

Exploiting prokaryotic nucleotide
excision repair as a novel antimicrobial
target for combinatorial therapies
in cancer patients



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I declare that no part of this thesis has been submitted for an application for another degree or other qualification at the University of Kent or any Institution of learning.

COVID-19 statement.

The global pandemic caused by COVID-19 has significantly impacted the progress of this PhD. The measures undertaken during the pandemic (such as lockdown) resulted in restricted access to the laboratory halting the experiments till the re-opening of the University. Furthermore, the return was staged with limited access to the laboratory, restricting the work for several months afterwards. Specifically, the University was closed for approximately three months, from the end of March to the beginning of July 2020. After re-opening, our group had to work in rotation or through a booking system to avoid exceeding the maximum number of people allowed in the lab. This situation was then repeated with similar restrictions at the beginning of 2021. Due to these events, some of the work reported in this thesis remained unfinished.

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Table of contents

LIST OF ABBREVIATIONS:	15
ABSTRACT	16
CHAPTER 1 – INTRODUCTION	17
1.1 Drug discovery.....	17
1.1.1 Computer-aided drug design	20
Ligand-based drug design	20
Structure-based drug design.....	21
1.1.2 Drug repurposing	23
1.2 An introduction to cancer	25
1.2.1 Overview of DNA damage.....	28
Strand breaks	29
Structural modifications.....	30
1.2.2 Anticancer chemotherapy and adverse effects	33
Principal pharmacological therapies.....	33
Most common toxicities in oncological treatments	36
Leukopenia and the risk of infection in cancer patients.....	37
1.3 Antibiotic chemotherapy and its stagnation.....	39
1.3.1 Antimicrobial resistance.....	41
AMR in cancer patients	43
1.4 DNA repair in bacteria	44
1.4.1 Direct reversal repair	44
1.4.2 Mismatch repair (MMR).....	44
1.4.3 Base excision repair (BER).....	45
1.4.4 Homologous recombination (HR) and non-homologous end-joining (NHEJ).....	45

1.4.5	Nucleotide excision repair in bacteria (NER)	46
	Damage location	48
	Damage verification	49
	Incisions and re-synthesis	51
1.5	Aims of this thesis	53
CHAPTER 2 – MATERIAL AND METHODS		54
2.1	Buffers and solutions	54
2.2	Plasmids and bacterial strains	55
2.3	Drugs and drug library	55
2.4	Phage lysate preparation and P1 Vir transduction	56
	Colony PCR	59
2.5	Protein purification	60
2.6	Survival assays	62
	Minimal inhibitory concentration assay	62
	Growth curves	63
	Checkerboard assay	63
	Complementation assay with recombinant UvrA	64
	FDA-approved drug screening	65
2.7	Solubility test for ATBC	66
2.8	NADH-coupled ATPase assay	67
2.9	Gel-based incision assay	69
2.10	Fluorescence-based incision	70
	HindIII Incision control in ABC	73
2.11	UV-damage repair assay	74

UV-damage repair assay control.....	76
2.12 In-silico molecular docking.....	77
Virtual screening	78
Speed evaluation depending on the parallelism level	80
 CHAPTER 3 – IDENTIFICATION OF THE TARGET AND MODE OF ACTION FOR THE PROKARYOTIC NUCLEOTIDE EXCISION REPAIR INHIBITOR ATBC.....	 81
3.1 Introduction	82
3.1.1 UvrA.....	82
The structure of UvrA.....	82
The zinc fingers.....	84
The ATP-binding pockets.....	85
DNA binding	86
Discrepancy on UvrA	87
3.1.2 NER as a target for antimicrobial therapy.....	89
3.1.3 Summary	90
3.2 Results	91
3.2.1 UvrA is necessary for efficient nucleotide excision repair.....	91
3.2.2 ATBC solubility in water	93
3.2.3 ATBC does not impair survivability on its own.	94
3.2.4 ATBC is effective against <i>E. coli</i> nucleotide excision repair	96
3.2.5 ATBC targets UvrA competing for its ATP binding sites.....	99
All surface exploration	99
Real-time NADH-coupled ATPase assay to validate the in-silico results	100
Directly visualizing ATBC effect on UvrA DNA-binding ability	102
3.2.6 Evaluating ATBC's ability to inhibit incision	104
3.3 Discussion	106
3.3.1 ATBC	106
3.3.2 UvrA as a target.....	108

3.4	Future works	110
3.4.1	ATBC in therapy.....	110
3.4.2	ATBC as a proof of concept.....	111
3.4.3	Why NER?.....	111
3.5	Conclusions	112
CHAPTER 4 – <i>COMBINING CANCER CHEMOTHERAPEUTICS WITH BACTERIAL</i>		
<i>DNA REPAIR INHIBITORS TO DEVELOP NOVEL ANTIMICROBIALS.</i>		113
4.1	Introduction	114
4.1.1	Cisplatin in therapy	114
4.1.2	Combinatorial therapies	116
4.1.3	Summary	118
4.2	Results	119
4.2.1	Cisplatin activity on different strains	119
4.2.2	Screening an FDA-approved drug library.....	120
4.2.3	Testing the compound <i>in vivo</i>	122
4.2.4	Confirming the activity against NER.....	124
4.2.5	UV-damage repair assay as further <i>in vivo</i> confirmation	126
4.2.6	Evaluating UvrA ATPase cassette as the possible molecular target	127
	NADH-coupled ATPase assay shows activity against UvrA	128
	Studying the possible binding modes on UvrA	129
4.2.7	Evaluation of the best hits on EC958 clinical isolate.....	131
4.3	Discussion	132
4.3.1	FDA-approved screening.....	132
4.3.2	Characterization of the 5 leads	133
4.4	Future work.....	135
4.5	Conclusions	137

CHAPTER 5 – SCREENING OUTLIERS: EXPLORING COMPOUNDS WITH
INTERESTING PROPERTIES 138

5.1 Summary..... 139

5.2 Bleomycin 140

5.2.1 Introduction140

5.2.2 Bleomycin shows an opposite effect against NER142

5.2.3 Bleomycin is strongly synergistic with cisplatin in clinical isolates.....144

5.2.4 UV-damage repair assay confirms its anti-NER activity.....146

5.2.5 Discussion and conclusions on bleomycin147

5.3 Aprotinin..... 150

5.3.1 Introduction150

5.3.2 Aprotinin impairs NER while causing a tremendous increase in DNA binding..153

5.3.3 Discussion and conclusions on aprotinin155

CHAPTER 6 – CHARACTERIZATION OF BEMCENTINIB AS A NER INHIBITOR
ACTING AS AN ADJUVANT ANTIMICROBIAL AGAINST A CLINICAL ISOLATE
STRAIN..... 157

6.1 Introduction 158

6.2 Results 159

6.2.1 Screening time improvements with higher levels of parallelization159

6.2.2 Virtual screening highlights a possible new hit.....161

6.2.3 Bemcentinib effectively inhibits UvrA *in vitro*164

UvrA DNA binding ability is compromised when in the presence of bemcentinib ...166

6.2.4 Bemcentinib inhibits DNA incision *in vitro*.....167

6.2.5 Bemcentinib significantly impairs bacterial survivability.....168

6.3 Discussion 170

6.3.1 In-silico study identifies bemcentinib170

6.3.2 Bemcentinib is effective against different strains of *E. coli*.....173

6.3.3	Bemcentinib/cisplatin for infections during cancer chemotherapy	177
6.4	Future work.....	179
6.5	Conclusions	181
CHAPTER 7 – LAST REMARKS AND CONCLUSIONS.....		182
7.1	General discussion.....	183
7.2	Conclusions	189
APPENDIX		190
A – Minimal inhibitory concentration controls		190
I - Resazurin colour changes when used at different volumes		190
II - DMSO impact on growth.....		191
III - MIC values of the interesting compounds in EC958.....		192
B – Growth curves comparing MG1655, MG1655 Δ tolC, MG1655 Δ tolC Δ uvrA		193
C – Additional compounds tested.....		194
D – Different intercalators show that DNA intercalation alone does not affect the incision assay.....		196
E - Apigenin, Hydroxyprogesterone and Meisoindigo exhibit synergism with cisplatin		197
F – Unfinished work on different hits analogues.....		199
I - Pirarubicin analogues show different degrees of activity		199
II - Pizxantrone, a non-active Mitoxantrone analogue		201
G – Hits` binding energies		202
H – ATP interactions with the distal and proximal sites		203
I – Lolitrem B		204

L – Code for virtual screening	205
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REFERENCES	207
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List of figures

Figure 1: Drug Discovery pipeline.	18
Figure 2: Share of population with cancer in 2017.	26
Figure 3: Common types of DNA structural damage.	29
Figure 4: Major DNA damages after exposure to UV radiation.	31
Figure 5: Schematic representation of Nucleotide excision repair in bacteria.	47
Figure 6: UvrA ₂ B ₂ complex.	50
Figure 7: Schematic representation of a P1 Vir transduction.	58
Figure 8: Agarose gel showing the chromosomal deletions.	59
Figure 9: Chromatography in HisTrap using HisTrap resins.	61
Figure 10: Stylized diagram of a survival assay for minimal inhibitory concentrations.	63
Figure 11: Stylized diagram of a checkerboard.	64
Figure 12: Example plate showing the screening setup.	65
Figure 13: NADH-coupled ATPase assay schematic.	67
Figure 14: UV irradiation titration.	69
Figure 15: Schematic representation of the fluorescence-based incision.	72
Figure 16: Incision control in ABC buffer.	73
Figure 17: Schematic representation of the UV-damage repair assay.	75
Figure 18: UV-damage repair assay drugs direct effect control.	76
Figure 19: AlphaFold UvrA showing the search space for the virtual screening.	79
Figure 20: UvrA homodimer (PDB:3PIH) crystal structure.	83
Figure 21: Survivability assay for UV radiation and UvrA complementation.	92
Figure 22: ATBC solubility in water and DMSO.	93
Figure 23: Colorimetric minimal inhibitory concentration.	95
Figure 24: ATBC effects on growth when combined with DNA-damaging agents.	98
Figure 25: In-silico docking defines a possible binding site for ATP.	99
Figure 26: NADH-coupled assay validates the in-silico findings.	101
Figure 27: ATBC significantly reduces UvrA DNA binding.	103
Figure 28: Gel-based incision assay.	105
Figure 29: Intra- and inter-links formed by cisplatin and DNA.	115

Figure 30: Survivability assay for cisplatin against different strains.....	120
Figure 31: Screening strategy.....	121
Figure 32: Checkerboard assay to test for the combinatorial effect of the selected compounds.....	123
Figure 33: Incision assay to test for NER inhibiting activity in vitro.....	125
Figure 34: UV-damage repair assay evaluates the inhibiting activity in vivo against NER.	127
Figure 35: NADH-coupled ATPase assay on recombinant UvrA.	128
Figure 36: Studying possible binding sites on AlphaFold UvrA.....	130
Figure 37: Three final hits are effective against the clinical isolate strain EC958.	131
Figure 38: Bleomycin A ₂ structure.	140
Figure 39: Bleomycin acts directly on the DNA.....	143
Figure 40: Survivability assay for Bleomycin and combinatorial effects with cisplatin.....	145
Figure 41: UV-damage repair assay with Bleomycin in EC958.	146
Figure 42: Summary of aprotinin's explored applications.....	151
Figure 43: Aprotinin can impair NER.....	154
Figure 44: Evaluating the efficiency of multiple jobs submission against the available computing power.....	160
Figure 45: In-silico screening shows a possible hit of ~120000 candidates.	163
Figure 46: Real-time NADH-coupled assay to confirm the molecular docking.	165
Figure 47: Real-time investigation of UvrA binding ability on suspended DNA.	166
Figure 48: Incision assay shows the activity of the compounds on NER.	167
Figure 49: In vivo characterisation of bemcentinib.	169
Figure 50: Representation of bemcentinib cooperative inhibition.	174
Figure 51: L-thyroxine and dienestrol docking in the allosteric sites.	187
Figure 52: Different volumes of resazurin do not affect the result.	190
Figure 53: DMSO's MIC in MG1655 Δ tolC.....	191
Figure 54: MICs experiments in EC958.	192
Figure 55: MG1655, MG1655 Δ tolC and MG1655 Δ tolC Δ uvrA growth curves.....	193
Figure 56: Intercalating agents do not affect the incision mediated by NER.	196
Figure 57: Survival assay with three interesting compounds.....	198
Figure 58: anti-NER activity of the rubicin family.	199

Figure 59: Doxorubicin inhibits UvrA similarly to Pirarubicin.....	200
Figure 60: The analogue Pixantrone doesn't maintain anti-NER activity.....	201
Figure 61: UvrA-ATP docking interactions.	203
Figure 62: Molecular docking of Lolitrem B on UvrA.....	204

List of Tables

Table 1: Screening result and antimicrobial activity of the 5 major hits.	122
Table 2: Summary of the additional compounds tested while working on Chapter 4.....	195
Table 3: Screening results for the three compounds.....	197
Table 4: Hits` binding energy.	202

List of abbreviations:

4-NQO	4-nitroquinoline 1-oxide
6-4 PPs	6-4 pyrimidine-pyrimidone photoproducts
Amp ^R	Ampicillin resistance
AMR	antimicrobial resistance
ATBC	2-(5-Amino-1,3,4-thiadiazol-2-yl) benzo(f)chromen-3-one
ATP	Adenosine triphosphate
BRS	Buffered resazurin solution
CADD	Computer-aided drug design
Cm ^R	Chloramphenicol resistance
CLSI	Clinical and Laboratory Standards Institute
CPDs	Cyclobutane pyrimidine dimers
Km ^R	Kanamycin resistance
MIC	Minimum inhibitory concentration
NER	Nucleotide excision repair
PEP	Phosphoenol pyruvate
PMSF	Phenylmethylsulfonyl fluoride
ROS	Reactive oxygen species
TCR	Transcription-coupled repair
UV	Ultraviolet

Abstract

Infections are a group of pathologies which are increasing in severity due to the lack of new therapeutic approaches and the concomitant rise of antimicrobial resistance. This issue is particularly concerning in cancer patients who are often immunocompromised as a side effect of chemotherapy.

Most anticancer treatments target rapidly dividing cancerous cells but also hit immune system cells and bacteria. Bacteria, however, possess efficient DNA repair systems that allow them to escape otherwise lethal damage. In this thesis, we evaluated strategies to extend the cytotoxic activity of DNA-damaging agents to bacteria by inhibiting nucleotide excision repair (NER), a central prokaryotic DNA repair system responsible for recognising various damages.

We began our study with ATBC, the first effective NER inhibitor in *mycobacteria*, to understand its mechanism of action and molecular target. We confirmed its activity on *Escherichia Coli* and used *in silico* tools to predict its binding to UvrA, the first responder in the NER pathway. We then confirmed this activity *in vitro* by directly measuring UvrA's ATP turnover and its compromised DNA binding ability. Furthermore, we evaluated the compound's ability to inhibit the activity of the entire UvrABC complex through an incision assay. This data provided the rationale for moving forward in our study.

We designed two screening approaches to develop novel NER inhibitors to be used alongside the widely used anticancer drug cisplatin. We screened an FDA-approved drug library composed of ~3000 compounds in combination with cisplatin as well as virtually screening ~120000 compounds directly against UvrA ATP-binding pockets. The hits that emerged from this approach were subjected to a wide range of *in vivo*, biochemical, *in silico* and single molecule studies to confirm NER as a molecular target and to elucidate their mechanism of action.

We highlighted 8 principal candidates, among which 5 showed increased antibacterial activity against a clinically relevant *E. coli* strain responsible for multidrug-resistant infections. In doing so, we have taken the first step in developing a novel antimicrobial approach that could represent a valuable tool in fighting co-infections in cancer patients.

Chapter 1 – Introduction

1.1 Drug discovery

The discovery of new drugs is a highly complex and expensive process requiring decades of research with a high possibility of failure (1). The entire process spans from the identification of a molecular target to the large-scale production of an effective drug-like molecule active *in vivo* and achieving success requires a multidisciplinary approach in various stages of research.

The drug discovery pipeline can be arbitrarily divided into 4 steps, spanning from conceptualization to clinical application. The first stage is the “early discovery” and usually involves the evaluation of a target and the screening of drug-like libraries. Its main goal is to validate the target and to isolate a selected group of active compounds (hits) from the vast chemical space. These hits will then be further tested and often chemically optimised, looking for a reduced handful of working candidates (hit-to-lead) progressed for more detailed investigations. In the next phase, the “pre-clinical development”, the lead(s) is extensively studied for its essential properties, such as toxicities and ADME (absorption, distribution, metabolism and excretion). If the candidate matches the expectations, it progresses to the last phase, the “clinical evaluation”, for *in vivo* testing on selected patients. Once all necessary data has been collected, the compound can be submitted for approval and eventually used in therapy (1, 2).

Drug discovery was historically linked to natural products (3, 4). Biological sources like bacteria, fungi, plants and even animals can produce, through their secondary metabolism, a vast number of compounds with pharmacological effects. These compounds and their derivatives are still playing central roles in therapy. The antibiotic penicillin (5), the antiarrhythmic digoxin (6), the antihypertensive captopril (7), the analgesic capsaicin (8) and the antitumoral paclitaxel (9) represent a few meaningful examples.

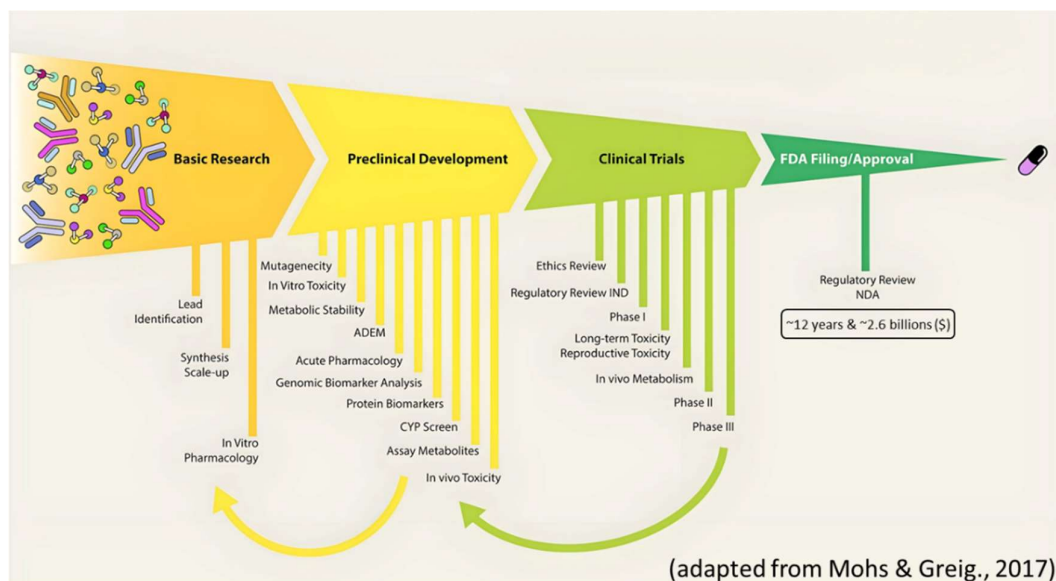


Figure 1: Drug Discovery pipeline. The adapted image (1) represents a novel chemical's long and expensive journey through the several steps leading to clinical use. The backwards arrows underline that a compound progressing the pipeline can be brought back for optimization or repositioning upon failing to meet criteria and expectations.

More recently, however, the drug discovery pipeline shifted its focus to higher throughput screening techniques (HTS), allowing for more automation and the rapid evaluation of thousands of compounds. A typical screen involves serial testing of drug-like molecule libraries against a biological target to identify several promising compounds. This approach has now become the standard method for drug discovery (3).

HTS can be considered a relatively young approach in light of the length of a drug discovery study. Nonetheless, numerous successful examples of FDA-approved drugs are available, including the hypertension drug ambrisentan, the anti-diabetic drug sitagliptin, the anti-hyponatraemic tolvaptan, and more (10).

More recently, fragment-based drug discovery (FBDD) has gone under the spotlight by combining both *in vitro* screening and *in silico* tools to facilitate drug design (11–13). FBDD is now complementary to the more traditional HTS and has led to numerous successes, with several compounds being approved by the FDA (11, 12). Compared to HTS, FBDD requires fewer compounds for the screening and usually leads to weak hits taken as the template for additional studies and optimizations (11).

Conventionally, a small molecule has to respect the rule of 3 to be defined as a “fragment” despite the possible limitation of such artificial restriction (14). According to these rules, the molecule has to have a low molecular weight (≤ 300 Da), ≤ 3 hydrogen bond donor/acceptors and a ≤ 3 computed logarithm of the partition or distribution coefficient (cLogP/cLogD). However, these rules can be violated, and successful hits can be identified by extending the definition of “fragment” to include more molecules (14).

FBDD is closely correlated with structure-based drug design (SBDD) (which will be discussed in the next section) since they both require structural knowledge of the target protein (11). In FBDD, a selection of fragments is tested against a target protein with one among the numerous biophysical techniques available (e.g., differential scanning fluorimeter, NMR, x-ray crystallography, Surface Plasmon Resonance) (13, 15) and the binding information are used to alongside the computational analysis for the design of a potent analogue able to bind the targeted site efficiently (11, 13, 15).

A screening should be as efficient as possible, therefore, when choosing among the numerous screening techniques (4), priority should be given to developing an efficient and reproducible pipeline that allows for automation. Furthermore, two additional strategies can increase the probability of success. As discussed in the following sections, using bioinformatics tools and repurposing already-approved drugs can significantly shorten the discovery process and minimize its costs, fast-tracking the basic research stage to preclinical development.

1.1.1 Computer-aided drug design

Computer-aided drug design (CADD) aims to accelerate the drug discovery process by providing shortcuts that enable a more rational approach compared to the traditional empirical method. With the use of computational tools, CADD provides shortcuts for the identification, optimization and understanding of drug candidates increasing the probability of success.

The fast improvement in technology and its accessibility played a crucial role in developing new informatic tools aiding drug discovery in recent years. Examples range from the use of artificial intelligence to generate unavailable 3D protein structures (16), to the creation of free ultra-large chemical library repositories (17, 18) and the availability of numerous accessible tools for bioinformatics (19–25). These software packages enable the user to perform fast and inexpensive molecular docking/modelling, binding site prediction, virtual screenings, dynamics simulations and evaluation of structure-activity relationship (26).

The discovery of the anti-HIV drug Maraviroc demonstrates the usefulness of the contribution of CADD. The compound was developed by combining similarity modelling and medicinal chemistry on a hit identified in a high-throughput screening (27). An additional example is Glivec, an anticancer drug that affects two or more targets, which was also rationally developed utilizing bioinformatics tools after screening results (28). Many more successful examples can be found in the literature underlying the use of these tools in drug discovery (29, 30).

CADD can be principally divided into two main approaches, ligand-based drug design (LBDD) and structure-based drug design (SBDD) (26, 31).

Ligand-based drug design

LBDD is based on the foundation concept that similar compounds have similar properties and functions (32, 33). Therefore, it can be used to sort ultra-large chemical libraries (up to billions of compounds) by their similarity to known ligands that exert the wanted effect. The process starts with the calculation of chemical descriptors and assigning a numerical identifier based on the chosen molecular property. Common descriptors can be 1-dimensional (e.g. molecular weight), 2-dimensional (dependent on the connection

between atoms) or 3-dimensional (dependent on the internal/external coordinates of the atoms) (32, 34). These descriptors can then be statistically combined with experimental data (e.g. machine learning) to model the quantitative structure-activity relationships (QSARs) of a ligand (32, 34). In addition, these molecules, sorted by their molecular descriptors and grouped in libraries, can be searched for compounds with the desired properties, from a simple molecular weight to their 3D-electroshapes (20, 32, 34).

LBDD represents a fast and accessible tool since it does not require any knowledge of the receptor and allows for high-speed search due to the relatively simple calculations required from the algorithm. For example, the SwissSimilarity Tool (20) is a web portal for remote LBDD that allows the user to input its ligand structure and perform ultra-large screening (up to ~16,000,000 molecules). The web page catalogues the compounds using various 2D and 3D descriptors, allowing for a quick search, outputting the first few hundred relevant molecules (in order of similarity) in a few minutes.

On the other hand, SBDD is considerably more complex, requiring the 3D structure of the receptor and longer times of execution.

Structure-based drug design

SBDD is the base for molecular docking. The required structural knowledge of the targeted protein can be retrieved either experimentally through x-ray crystallography, NMR, cryo-electron microscopy or through homology modelling and, more recently, with the aid of artificial intelligence (35–37). The structure can then be fed to various docking software (e.g., AutoDock Vina, GOLD, HDOCK, Dock3.7, Glide etc.) along with a 3D representation of a ligand to generate a snapshot of the best theoretical interactions. This process is based on Fisher's observation during the late 1890s that an enzyme and a ligand interact similarly to a “lock and its key” (38). This oversimplification that permits molecular docking also harbours the intrinsic limit of any computational approach which relies on shortcuts in favour of speed. Some approximations, such as ligand/protein flexibility, presence of the solvent, specific transition states or ionic charges, can be addressed to improve the accuracy of CADD but at the expensive cost of additional computational power. Structural data on the interaction of previous ligands can guide the docking and validate the process. However, this information is not always available. Without a known and defined binding

site, the entirety of the protein can still be explored (39) at the expense of the accuracy of the results. Nonetheless, allowing the docking software to explore all the possible interactions across the entirety of the protein can be beneficial and help discover allosteric sites, which can indirectly modulate the protein activity.

To provide an example of molecular docking, we describe the software used in this thesis, AutoDock Vina, developed for fast and accessible docking (19). This open-access program can calculate a ligand affinity for a protein, also outputting the best-bound conformations in only minutes (depending on the exhaustiveness of the search). Once the search space boundaries have been established, the algorithm performs several independent “runs” (decided by the user through the exhaustiveness value) from random ligand conformations. Each run includes numerous “steps”, starting with a random molecule perturbation based on the ligand torsional freedom. These perturbations are then optimised, evaluated, stored, or rejected according to Vina’s scoring function. Depending on the ligand flexibility, the number of these “steps” iterations is then heuristically decided. Each run can produce several independent results that are ultimately merged and sorted, generating the final output (19, 40). Importantly the docking score given by the software is an approximation of the interacting energies being calculated in the vacuum of the docking and not absolute empirical values (41). Furthermore, these tools can be used to virtually screen (VS) libraries of compounds for their computed affinity to a protein by sorting the outputs by their predicted docking score, heavily shrinking the number of candidates for biochemical testing.

SBDD has the advantage over LBDD of creating a more specific output displaying the interactions between the ligand and the protein. Furthermore, the interaction dynamics can be studied by introducing factors like temperature, pressure, solvent, and ions (26) with an exponential increase in computational power.

LBDD and SBDD are not mutually exclusive and can be combined in the screening process (34). For example, when a new molecule is found active in structure-based simulations, a ligand-based approach could be used to probe the chemical space in search of similar compounds possibly exhibiting stronger activity or better chemical characteristics.

1.1.2 Drug repurposing

As shown in Figure 1, developing a new drug-like molecule, from conceptualization to its approval, is challenging and incredibly costly. After years of basic research, the candidate can enter the pre-clinical phase (~3-4 years) and, if successful, proceed to clinical trials (~5 years) without insurance of success (42). During this process, the investigator has to work with national agencies such as the Food and Drug Administration (FDA) and provide the effectiveness and safety information required for approval (42). Examples of these requirements include animal testing results, manufacturing process, pharmacology and toxicology data (42). For a novel chemical, this process is uncertain, with a limited likelihood of approval (LOA). An analysis of the FDA approval rate on 7300 drugs developed by 835 independent developers (between 2003 and 2011) found the LOA to be only ~10% (43). Therefore, the possibility of re-evaluating years of research spent on developing a pharmaceutical entity to increase its therapeutic value is attractive. Furthermore, a practical strategy to reduce many drug development uncertainties can benefit both the researcher and the patients. For these reasons, drug repurposing represents an integral part of drug development.

Drug repurposing (or repositioning) is the evaluation of existing drugs (approved or not) for applications beyond the initial indication (44). This strategy aims to breathe new life into drugs that have lost their effectiveness, failed to match the expectations during development, or just to enhance their therapeutic profile.

Repurposing already developed drugs allows investigators to save time on various stages of the discovery process, from design and manufacturing to animal testing (44). In many cases, the repositioned drug has already undergone chemical optimization, scale-up or even clinical tests; therefore, the approval pathway shortens from 10/17 years for a novel chemical to 3/12 years for a repositioned drug (44). Furthermore, repositioning an already approved drug can provide shortcuts in the administrative side of the process, too (45). Taking the FDA as an example, the approval of a new chemical entity requires filing an investigational new drug (IND) application to begin the clinical evaluation (45). In contrast, an IND might be unnecessary for a repurposed drug unless the new application significantly increases patient risks (45).

Thanks to the increased accessibility and money-saving prospects, pharmaceutical companies and academic institutions have exploited this approach, successfully repurposing several drugs, among which the anti-angina sildenafil now used for erectile dysfunction or the infamous thalidomide now approved for myeloma (46). A particularly relevant example is the case of Aspirin. The already-approved and commonly used analgesic was later discovered to have other activities, such as a blood thinning effect. This new therapeutic property made Aspirin a drug now commonly used to treat cardiovascular diseases (47).

1.2 An introduction to cancer

Cancer is a disease that has always accompanied humanity throughout history. The first report of the condition can be found in the “Edwin Smith Papyrus”, written ~5000 years ago, where the author described a breast tumour causing an untreatable disease. Successive ancient records depict different types of cancer and primitive remedies going from surgery to herbal treatments (48).

Commonly a cancerous cell has lost its function and differentiation while growing uncontrollably without appropriate signalling. Also, a cancerous cell usually has the ability to infiltrate other tissues and escape programmed death (49, 50). A tumoral cell can grow and divide without proper signalling bypassing apoptotic requests almost anywhere in the body. It can infiltrate adjacent areas and hide from the immune system to avoid cell death with grim outcomes. Most importantly, it carries an unstable genotype that can readily undergo changes and mutations, progressively becoming even more unstable and dangerous (49–51).

More than 100 kinds of tumours can be classified according to different characteristics (49). Usually, these diseases are named either after the original organ containing the tumoral formation (e.g. lung cancer, breast cancer, etc.), the original cell types (e.g. carcinomas for epithelial cells, sarcomas for bones and soft tissues, etc.) or the liquid content (solid or blood cancers) (49). However, possibly the most crucial classification is between benign (not cancerous) and malignant (cancerous) tumours (49, 52). The main difference is that a benign tumour cannot spread to other tissues, grows slowly and has defined borders. Furthermore, once removed, it does not usually grow back; therefore, these groups of formations are primarily associated with good prognosis (49, 50, 52). On the other hand, a malignant tumour is able to disseminate around the body in a process called metastasis, resulting in a much more complex and severe pathology often accompanied by a poor prognosis (49, 50, 52).

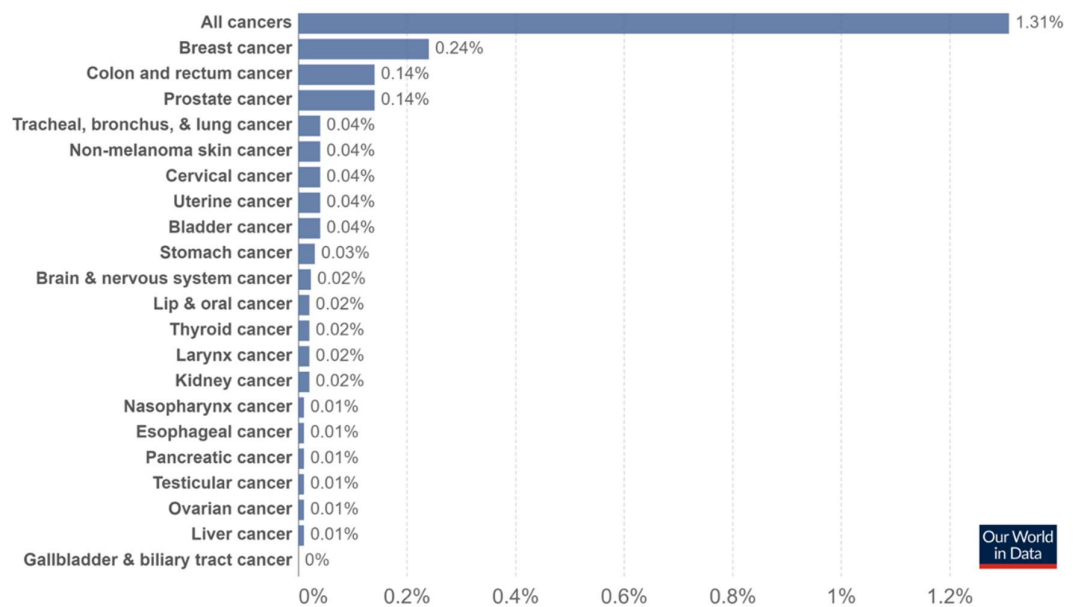


Figure 2: Share of population with cancer in 2017. Global population with cancer in 2017. The figure retrieved from OurWorldInData.org (53) shows the population's cancer percentage in 2017. The Institute for Health Metrics and Evaluation (IHME) (54) collected the data presented.

As shown in Figure 2 (53, 54)), cancer is a widespread disease, often resulting in death, representing one of the most significant health problems globally, with ~100 million cases and 9.53 million deaths worldwide (2017) (55–57). It has been estimated that 39.5% of all men and women will be diagnosed with cancer during their lifetime (58). In the US alone, 1,806,590 new cancer cases were expected, with 606,520 people perishing from the disease in 2020 (58).

To become a cancerous cell, a typical cell must acquire specific mutations, often due to unrepaired DNA damage that allows for uncontrolled and abnormal growth and survival (50, 59–61). It is well established that all the tumoral cells in cancer derive from a single progenitor that started reproducing improperly, accumulating genetic errors. Mutations in specific genes are defined as drivers for cancer (49, 51). These genes are divided among proto-oncogenes, tumour suppressors and DNA repair genes. Proto-oncogenes encode for proteins involved in cell division, differentiation and cell death, and their disruption usually leads to an abnormal division with inhibition of programmed cell death (62, 63). Tumour suppressor genes regulate cellular growth inhibiting cell proliferation and tumour development (64). Ultimately, DNA repair genes are needed for the biosynthesis of the

proteins required to maintain the integrity of the genome. Their malfunctions lead to the accumulation of mutations that eventually can cause cancer development (65).

These mutations occur spontaneously in the cells due to mistakes during DNA replication or damage (discussed in more detail in the next chapter). These errors have a chance of being maintained and propagated, causing the development of the disease due to failures in several DNA repair systems monitoring the integrity of the genome.

1.2.1 Overview of DNA damage

As with every biological structure, DNA spontaneously undergoes decay and damage that must be repaired for cell survival and to guarantee the correct maintenance of genomic information (61, 66, 67). Mutations can play a crucial role in evolution and therefore are not always deleterious. Instances of beneficial mutations can be found in both microorganisms and humans. One such example is the development of antimicrobial resistance (AMR) in bacteria conferring enhanced environmental adaptability (68). Another example in humans is the mutation of the *SLC30A8* gene, which decreases the probability of developing type 2 diabetes (69). However, detrimental mutations are far more prevalent and can cause catastrophic consequences (70, 71).

Some estimations suggest that spontaneous mistakes get incorporated into DNA every ~10,000 – 100,000 nucleotide polymerized for 100,000 – 1,000,000 polymerase errors every mammalian cell cycle (72). Furthermore, at significant rates, DNA's integrity is further compromised by physiological damage due to oxidations, hydrolysis and nonenzymatic methylation reactions, with oxidations alone producing ~10'000 damage sites per cell per day in humans (66, 73). Lastly, both chemical (e.g., intercalating and crosslinking compounds) and physical agents (e.g., UV radiation) can contribute to DNA damage by directly interacting with the DNA molecule causing mutations or strand breakage. If not repaired, these "errors" would be catastrophic for the organism causing ageing, cell death or mutations leading to several diseases, including cancer and neurological pathologies (59, 60, 74, 75).

Figure 3 shows a simple overview of typical DNA damage divided between strand breaks and structural modifications most pertinent to the work undertaken in this thesis.

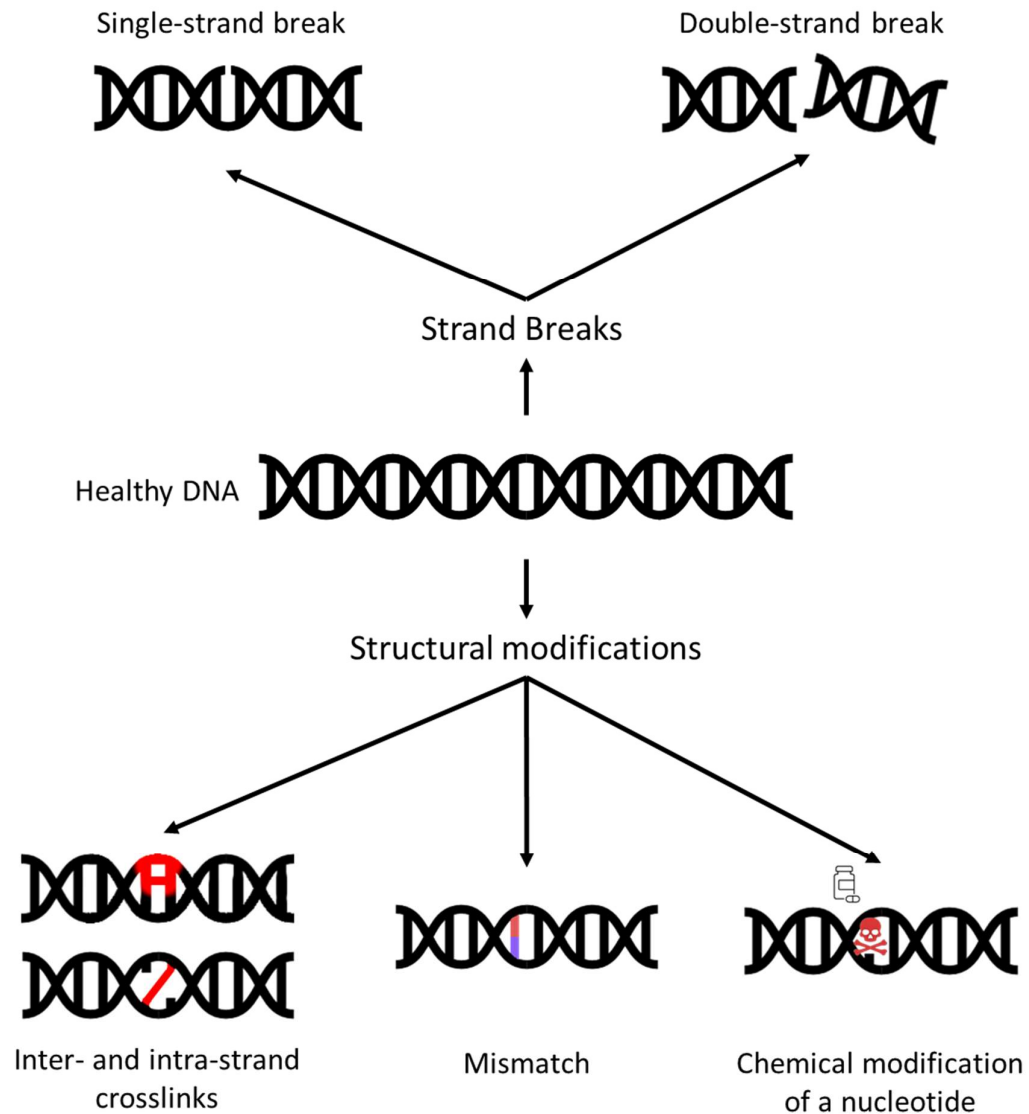


Figure 3: Common types of DNA structural damage. Representation of the most common types of DNA damage that can occur and must be repaired to ensure cell survival.

Strand breaks

Strand breaks are the most severe form of DNA damage in a cell. Single-strand breaks (the phosphodiester bond between two nucleotides in the same strand is broken) can cause several pathologies in humans and can also turn into double-strand breaks (complete break in the DNA) if not promptly repaired, causing an even increased toxicity for the cell due to the loss of genetic information around the break (76–78). Common causes of these severe types of DNA damage are reactive oxygen species, along with ionizing radiation, the

collapse of the replication fork and aggressive chemicals producing damage that must be repaired for cell survival (79–82).

Structural modifications

Structural modifications in the DNA can also happen without breakage but can still be critical for the host's survival. Different types of structural modifications require the intervention of the DNA repair system.

DNA polymerase is not infallible and can incorporate mistakes during replication, inserting the wrong nucleotide (mismatch) or skipping/adding multiple nucleotides altogether (insertion/deletion), with consequences that can even lead to cancerous formations if the mismatch involves a coding region of the genome (83, 84). Misincorporation can also be chemically induced by compounds mimicking endogenous molecules called antimetabolites. One example is 5-fluorouracil, used in anticancer therapy due to its ability to be misincorporated into the DNA and RNA, causing transcriptional problems (85).

The most common DNA modifications happen on a single base level comporting infidelity in replication or the stalling of DNA transcription (66, 86). Reactive molecules (e.g., free oxygen radicals) can attack the DNA molecule, leading to base modifications and mispairing. Well-known examples can be found by looking at the oxidation of bases due to the reaction with reactive oxygen species (ROS). This species can be formed in multiple ways, such as exposure to toxic chemicals or radiation or even as a byproduct during cell metabolisms (87). These highly reactive species interact with the DNA and can lead to base modification, mispairing or abasic (AP) sites contributing to several pathologies if unrepaired (87, 88). For example, modifications such as 8-oxoguanine (8-oxoG) cause guanine-to-thymine misreading leading to base misincorporation and a change to the genetic code, which can result in the development of diseases, such as cancer or premature ageing (87, 89).

Ultraviolet (UV) radiation represents another important source of DNA damage able to lead to cell death or mutations (90, 91). UV radiation is divided among UVA (320 to 400 nm), UVB (280 to 320 nm) and UVC (100 to 280 nm) depending on its wavelength (90). The ozone and the air strongly absorb high-energy wavelengths such as UVC. However, part of UVB

and UVA make it to the ground and, despite being considerably less dangerous to UVC, are still able to induce damage with mutagenic effects, even leading to cancer (90, 92–94). The major distortions produced by DNA irradiation are cyclobutane pyrimidine dimers (CPDs) in which two pyrimidines are covalently bonded through a cyclic butane, and 6-4 pyrimidine-primidone photoproducts ((6-4) photoproducts) in which a covalent bond is formed between the atoms 6 and 4 of two adjacent pyrimidines (95) (shown in Figure 4). CPDs represent most of the photoproducts produced (~2 to 1 ratio), but PPs have been reported to be more mutagenic, although the argument is debated (96).

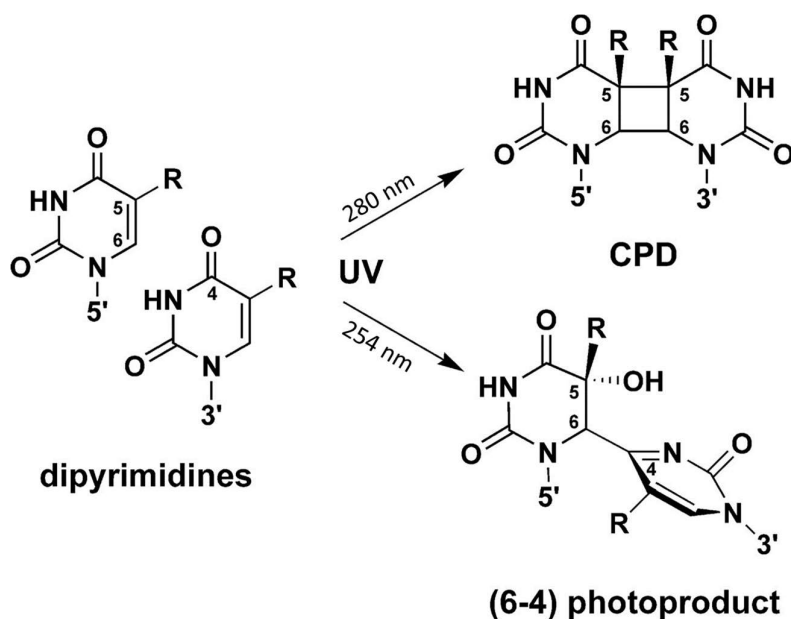


Figure 4: Major DNA damages after exposure to UV radiation. The adapted figure (95, 97) show the distortion caused by UV-radiation.

Furthermore, the effect caused by the exposure to highly reactive compounds able to directly interact with the DNA represent another relevant type of damage. These chemicals can produce alkylation and cross-linkage within the DNA molecule with devastating effects if left unrepaired. These alkylating agents are molecules that can covalently bind to electron-rich groups through nucleophilic substitutions. The binding of these compounds greatly interferes with transcription and replication. The transfer of an alkyl group onto a base can induce enzyme-mediated DNA fragmentation in the attempt of fixing the lesions. Furthermore, if the agent is bifunctional, the interaction might cause crosslinks leading to

severe DNA damage and ultimately cell death. (98). These molecules can be generated endogenously or found in toxic compounds, some of which are used in cancer therapy (61, 98–100).

Among these compounds, bifunctional alkylating agents show potent cytotoxicity and represent some of the most used anticancer agents (101, 102). These chemicals (e.g. cisplatin, mitomycin C, cyclophosphamide, etc.) (103–105) can cause the formation of covalent bonds between DNA - proteins or DNA - DNA either in the same strand (intra-strand crosslinks) or on opposite strands (inter-strand crosslinks). If not repaired, these new bonds can lock the DNA blocking access to other proteins, preventing the DNA-unwinding necessary for cell division and DNA transcription and causing base mispairing (98, 101, 102).

A summary of the bacterial DNA repair systems, used to overcome DNA damage is reported in the next chapter, primarily focusing on nucleotide excision repair.

1.2.2 Anticancer chemotherapy and adverse effects

Principal pharmacological therapies

Antitumoral drugs are cytotoxic, which conventionally is achieved by directly damaging DNA or interfering with cellular processes that regulate cell survival and replication (106).

Unfortunately, cancerous cells are still highly similar to non-cancerous cells. Therefore, developing a targeted therapy that does not affect healthy cells is challenging. By aiming at the processes regulating cell replication, fast-replicating cells (such as cancer cells and bacteria) are more susceptible to the antitumoral compounds' killing effects than physiological cells. However, these therapies still possess a narrow therapeutic index (doses in which the drug exerts its effects without unacceptable toxicities) with severe adverse effects (106) which will be discussed later.

Prior to the 1900s, the most viable option to treat cancer was surgical removal, resulting in high death rates (106). The aim of this treatment is the mechanical asportation of the cancer (when solid) from the body, and even to this day, surgery has remained an effective approach both alone and in combination with other therapies (107). Among those, radiation treatments are frequently employed alongside surgery to prevent relapse, mainly due to the incomplete removal of all the cancer cells (107, 108). Radiation therapy can physically destroy the tumoral cells, directly targeting their DNA and has been reported to be responsible for 40% of cancer cures while being significantly cheaper than other anticancer treatments (107).

New pharmacological treatments for cancer patients have been heavily investigated since the early 1900s, and while sorting anticancer treatments is arbitrary, they can easily be categorized into 6 principal classes based on their mode of action: (98, 106, 109, 110):

- Alkylating agents (e.g., nitrogen mustards, aziridines, platinum compounds)
- Topoisomerase poisons (e.g., anthracyclines, anthracenediones)
- Antimetabolites (e.g., analogues of the folic acid, purines, or pyrimidines)
- Antimitotic agents (e.g., taxanes and vinca alkaloids)
- Signal transducer inhibitors (e.g., tyrosine kinase inhibitors)
- Immunotherapy (e.g., interferon- α , interleukin-2, monoclonal antibodies)

- Immune checkpoint inhibitors (e.g., PD-1 and CTLA-4 inhibitors)

As already stated, these agents target cell survival and replication, acting in different stages of the cell cycle and through different molecular interactions. However, discussing the details of these therapies goes beyond the scope of this thesis. Nonetheless, the alkylating agents, poisons of the topoisomerases and signal transducer inhibitors are worth mentioning, as they will be encountered in chapters 4 and 6.

In a major turning point during the First World War, a poison developed for chemical warfare (mustard gas) was found able to produce tumoral regression in animals (111). This discovery led to the development of numerous compounds giving effective weapons against cancer for the first time. These drugs were called “alkylating agents”, electrophile compounds with a high reactivity towards electron-rich atoms (nucleophiles) in the DNA where they exert their therapeutic effect (102). Purines (guanine and adenine) possess the most reactive centres in the DNA towards these compounds, with the N7 atom in guanine as the preferential attacked target (followed by N3 and N1 adenine) (98).

Some of the most known examples are the nitrogen mustards (e.g. cyclophosphamide), the aziridines and epoxides (e.g. mitomycin C and dianhydrogalactitol), the alkyl sulfonates (e.g. busulfan), the nitrosoureas (e.g. carmustine), the platinum compounds (e.g. cisplatin) and compounds that decompose into active alkylating agents (e.g. temozolomide) (102). These agents are either monofunctional or bifunctional, covalently bonding with one or two sites to produce strong crosslinks on DNA that compromise the target function (101). These crosslinks lead to catastrophic consequences for the cell through three principal mechanisms. Once firmly crosslinked, the DNA cannot separate, preventing transcription and replication. Additionally, these alkylations can induce nucleotide mispairing, causing undesirable mutations. Furthermore, in an attempt to fix the DNA, the repair enzymes can cause DNA fragmentation and breakage (101), one of the most severe forms of DNA damage. All these effects disrupt essential cellular processes, causing mutations or cell death.

Drugs able to poison the topoisomerases were discovered in the 1950s by studying a *Streptomyces* strain and quickly entered the anticancer arsenal. Briefly, the topoisomerases (type I and II) modulate the DNA topology through highly reversible DNA cleavage to

control for the torsional stress permitting the cellular processes that require DNA unwinding (112). The impairment of the topoisomerases and their essential activity results in a dysregulation of the controlled DNA breaks, which leads to catastrophic damage to the cells (113). These drugs mainly act by inhibiting the enzyme's ability to re-join cleaved DNA or by promoting the topoisomerase DNA cleavage (113).

The main members of this family are anthracyclines (e.g., doxorubicin and pirarubicin) and anthracenediones (e.g., mitoxantrone and pixantrone), which exert their mechanism of action through an impairment of the topoisomerase mediated by DNA intercalation (98). Taking the anthracyclines as an example, their planar structure combined with a positively charged sugar motif enables them to intercalate the DNA between two adjacent bases and form a stable DNA-anthracycline-Topoisomerase complex preventing DNA resealing (98, 114). Furthermore, they produce toxic radical compounds during enzymatic metabolism, further contributing to DNA damage (98).

More recently, signal transducer inhibitors have been under the spotlight. Glivec (imatinib mesylate) and its rapid success stand out as an example, spotlighting this cancer treatment approach (115, 116). These drugs target critical pathways involved in tumorigenesis. They interfere with the activation and downstream effects of growth factors, cytokines and other signalling molecules that regulate cell proliferation, survival, invasion, angiogenesis and apoptosis (106). Some examples are rituximab, a monoclonal antibody that targets CD20 antigen on B-cell malignancies, and imatinib (as already mentioned), a tyrosine kinase inhibitor that inhibits BCR-ABL fusion protein in chronic myeloid leukaemia (106, 115, 117).

Ultimately, the discovery of immune checkpoint proteins (e.g., PD-1/PDL-1 and CTLA-4) represented another significant advancement in the fight against cancer (110). Cancer cells can hijack the repressors of programmed cell death, such as (PD-1) and cytotoxic T lymphocyte antigen 4 (CTLA-4), acquiring tolerance to death signals and the ability to escape the immune response. However, anti-PD-1 (e.g., nivolumab) or anti-CTLA-4 (e.g., ipilimumab) can target these inhibitory receptors by acting directly on the T-lymphocytes, causing the reactivation of the immune system against the cancer cells (110). These new agents were only recently approved for use by the FDA for different types of cancer and represent an exciting and novel anticancer approach (118).

Most common toxicities in oncological treatments

The sentence “the dose makes the poison”, credited to Paracelsus, emphasizes the importance of dosage in drug toxicology. Ideally, treatments should have a beneficial effect without causing unwanted side effects; however, this is not possible, especially regarding antitumoral therapies. As previously mentioned, cancer cells are normal cells which acquire abnormalities in growth and survival rates. Therefore, they share their targets with the rest of the organism. Furthermore, cancer is often associated with relatively fast progression and poor prognosis, making the prompt eradication of the disease an absolute priority. Out of these necessities, the cancer arsenal is composed of highly reactive compounds used at the maximum tolerated doses causing many side effects. The most common acute toxicities are nausea and vomiting, hair loss, mucositis and bone marrow toxicities. Other adverse effects that occur less frequently include cardio-, neuro-, lung-, renal toxicities and extravasations (119). Unfortunately, the adverse effects do not end here; hepatic toxicity, embolism, fatigue, diarrhoea, inflammation etc., can be caused by different anticancer drugs (120).

Chemotherapy agents can induce nausea and vomiting by stimulating the chemoreceptor trigger zone in the medulla oblongata or by irritating the gastrointestinal mucosa. Hair loss and mucositis are a consequence of the cytotoxic effects of the oncologic compounds on rapidly proliferating cells, leading to tissue damage. Especially mucositis can cause severe pain, ulceration, and infections. Another off-target victim of chemotherapy is the bone marrow. This anti-proliferating action causes particularly severe complications resulting in anaemia, leukopenia and thrombocytopenia (119, 121). A comprehensive list of specific toxicities linked to different anticancer treatments has been recently reviewed (120), but its discussion goes beyond the scope of this thesis. However, the bone marrow adverse effects and particularly leukopenia, are of particular relevance for the work undertaken in this project.

Leukopenia and the risk of infection in cancer patients

Leukopenia is a medical condition characterized by a decrease in the total number of white blood cells below the normal range (less than $0.5-1 \times 10^9$ /litre and “falling” (122)). While the term is sometimes used interchangeably with neutropenia, which refers specifically to a reduction in neutrophils, leukopenia encompasses all white blood cells, including monocytes and lymphocytes (123). Neutrophils or granulocytes are produced, inactive, in the bone marrow, and they constitute up to 60-70% of the total leukocyte count. After an injury or an infection, signals are released to attract neutrophils to the damaged site, activating other immune system components and contributing to the fight against the intruder via phagocytosis and degranulation. The journey of neutrophils from the bone marrow takes ~6 days, and their circulating lifespan ranges from a few hours to a few days (122, 123). By targeting highly proliferating cells, chemotherapeutic agents cause significant loss in the white cell count.

In the 5-7 days following an oncological treatment, the neutrophils count drops and require 2-4 weeks to recover, leaving the patient extremely vulnerable to infections and sepsis (122). Infections in cancer patients are especially problematic; they can cause antitumoral treatment delays and harm already compromised tissues and organs. It has been reported that approximately half of cancer patients' deaths are caused or associated with infections, making it the second leading cause of oncological mortality (124, 125). Unfortunately, neutropenia alone is not the only cancer-related factor responsible for acquired infections in cancer patients. The disruption of anatomic barriers caused by hospital procedures and devices (e.g., catheters, shunts, endoscopies, surgical interventions) or other treatment side effects (e.g., mucositis) also contribute to an increased risk of healthcare-associated (nosocomial) infections. Furthermore, hospitalization exposes patients to multidrug-resistant bacteria, raising the risk associated with the infection. Additionally, obstructions resulting from metastatic tumours (e.g., bowel obstruction, necrosis perforation) can further increase the probability of acquiring the additional disease. Even psychological distress and poor nutritional status can heighten the risk of infection (124–126).

Two main strategies are advised to prevent neutropenic sepsis, the use of prophylactic antibiotics and growth factors (such as the granulocyte colony-stimulating factor) to

replenish the white blood count (122, 127). However, the empirical use of broad-spectrum antibiotics carries problems such as the fast rise of multidrug-resistant (MDR) bacteria, rapidly lowering the effectiveness of the antimicrobial arsenal and leading to poorer prognosis (125, 128).

1.3 Antibiotic chemotherapy and its stagnation

Humanity has been fighting against pathogenic microbes since long before their discovery. When an invading microorganism enters its host, the immune system's response leads to the release of mediators of inflammation, creating an inflammatory status. This response aims to quickly contain and eliminate the invading pathogen (129). Looking back in time, we can find reports describing infectious diseases like smallpox and poliomyelitis that go far back as ~3000 years ago in Egypt (130) and even 4000 years ago if we consider the herbal treatment for symptoms associated with infection (fever, inflammation, cough, etc.) described in the Chinese Traditional Medicine (TCMs) (131).

The term "antibiotic" was initially defined as a natural metabolite synthesized by an organism for its survival, acting through the destruction of competitive microorganisms in its environmental niches (132). Nowadays, the definition has extended to all those molecules chemically modified or de-novo synthesized with an antimicrobial purpose. Antiviral, antiparasitic and antifungal compounds also belong in this category (133) but would not be discussed as not relevant to this thesis.

Antibiotic treatments are another form of chemotherapy in which the drug aims to quickly and selectively eliminate the agents responsible for the infections. Instances of overlapping between antibiotics and antitumoral are evident in the use of anthracyclines, also called "anticancer antibiotics" (134). Furthermore, antibacterial/anticancer drugs share chemotherapeutic strategies, such as disrupting metabolic pathways or interacting with DNA to poison topoisomerases (98, 135). However, antibacterial chemotherapy carries considerably fewer toxicities than its oncologic counterpart, thanks to the substantial differences between the eukaryotic and the prokaryotic cells (136). Meaningful examples are the bacterial cell wall targeted by β -lactams and the 30s ribosomal subunit targeted by the aminoglycosides (137, 138). These differences allow for more selective treatments that minimize the drugs' off-target activities, permitting safer therapeutic profiles with reduced side effects for most antibiotics.

The division of different antibiotics is arbitrary and depends on the common characteristics chosen to group them. A straightforward classification divides the compounds between

bacteriostatic (prevents growth) and bactericidal (causes cell death). However, this distinction seems unclear in clinical settings (139). A more precise division can be obtained by sorting the compounds by their chemical similarities and mode of action (135, 136). The main categories are:

- Drugs targeting cell wall synthesis (e.g., penicillins, cephalosporins)
- Drugs affecting the cell membrane (e.g., lipopeptides, polymyxins)
- Drugs impairing protein synthesis (e.g., chloramphenicol, tetracyclines, aminoglycosides)
- Drugs acting on nucleic acids (e.g., quinolones, rifamycins)
- Drugs disrupting essential metabolic pathways (e.g., sulfamidics, diaminopyrimidines)

The discovery of each one of these categories consisted of a significant step forward in antimicrobial therapy, and their development represented one of the most significant achievements in drug discovery, saving countless lives, raising life expectancy and removing infections as one of the leading causes of death in industrialized countries (140, 141). However, antibacterials cannot be used indefinitely, similarly to other drugs, due to the bacteria's ability to adapt and mutate, quickly acquiring resistance to toxic substances (68, 142). This natural phenomenon is an essential drawback in pharmaceutical treatment leading to the rise of antimicrobial resistance (AMR) with a loss of therapeutic options (the impacts of AMR will be discussed in Chapter 1.3.1). Furthermore, no new major additions have been introduced for clinical use in ~35 years (143–147). However, this does not mean that researchers have abandoned antibiotic research. Several new drugs have been approved for use in recent years with lefamulin (a pleuromutilin antibiotic approved in 2019) (148, 149), the pretomanid (a nitroimidazole approved in 2019) (150, 151), the eravacycline (a tetracycline-derived approved in 2018) (152, 153) and plazomycine (an aminoglycoside approved in 2018) (154, 155) representing some of the examples. However, these new compounds are based on the same long-known antibiotic structures and classes listed before (156–159).

The lack of new antimicrobial class development has been underlined in a recent report (2021) on the antibiotic clinical and preclinical pipeline by the World Health Organization

(WHO) (160). They report 292 antibacterial agents in preclinical evaluation, among which 101 non-traditional products, including bacteriophages, virulence inhibitors and drug potentiators. However, only a fraction of these compounds make it to the market. The report ultimately states that the approved antibiotics are insufficient to overcome the danger of antimicrobial resistance. Despite this ever-increasing need for new antimicrobials, big pharmaceutical companies seem to have left the burden of developing new antibiotics mainly on the small biotech companies' shoulders (146, 161). Approximately 46% of the institutions progressing the antibacterial in pre-clinical studies are micro-entities with less than 10 employees (160). The reasons for this exodus are mainly economic (146), with big companies unwilling to take the costly bet. As already discussed, drug discovery is a long, expensive and complicated process requiring up to billions of dollars and years of development without assurance of success (1, 162). GlaxoSmithKline (GSK) led 70 high-throughput screening (HTS) studies over 7 years, spending ~1 million (\$) per project to find only 5 hits, none of which reached clinical use (162).

Antibacterial development faces additional obstacles compared to the challenges commonly faced during drug discovery. For example, traditional antibiotics do not remain effective indefinitely, and their use is discouraged even at their peak to prevent the rise in antimicrobial resistance. Also, antimicrobial therapies do not require prolonged administrations as other treatments do, further reducing their financial appeal to pharmaceutical companies (145). Consequently, new antibacterial strategies are not entering the market fast enough, causing more difficult-to-treat infections and predicted disastrous healthcare outcomes in the near future (160, 161).

1.3.1 Antimicrobial resistance

Antimicrobial drug resistance (AMR) is a natural phenomenon that leads bacteria to acquire resistance to a toxic substance present in their environment. They can achieve it mainly by restricting access to the antibiotic (by efflux, reduced influx or by binding to other proteins), destroying the compound enzymatically, modifying the target or bypassing the metabolic pathway targeted by the drug (163–165). After Alexander Fleming discovered penicillin returning from a holiday (5), the Oxford Team purified it and allowed its use on a large scale

during the Second World War. This novel drug saved countless lives that otherwise would have been lost. However, already in 1940, evidence of a microorganism able to produce enzymes that could inactivate penicillin had already been found (166). Furthermore, *Staphylococcus aureus* strains isolated from hospitalized patients showed resistance to penicillin as soon as 1942 (167). These facts quickly sparked concerns about the decline of the antimicrobial development pipeline becoming gradually ineffective (168). Twenty years later, reports indicate that more than 90% of every *Staphylococcal* isolate was able to produce penicillinase enzyme, rendering them resistant to β -lactam antimicrobial agents (169).

In 2011, a survey conducted through the Emerging Infections Network (EIN) highlighted that 63% of specialists had encountered at least one patient suffering from an infection resistant to all available antimicrobials, and 56% stated that the ratio of the untreatable infections was increasing (170). Being naturally occurring, AMR cannot be stopped, but the overuse and often misuse of the available antibiotics exacerbates its spread and the consequent impacts on healthcare (164). Recent estimates indicate ~700,000 infections caused by antibiotic-resistant bacteria, with 33,000 deaths in the EU in 2015. Furthermore, infants and people over 65 appeared to be the most affected, with more cases registered in Italy and Greece (171). Among the bacteria with the stronger impact, they reported: III gen cephalosporin-resistant *E. coli*, Carbapenem-resistant *P. aeruginosa*, III gen cephalosporin-resistant, *K. pneumoniae* and Methicillin-resistant *S. aureus* (MRSA) to be of particular concern (171).

In 2018 more than half of the *E. coli* isolates in the EU, and more than a third of *K. pneumoniae* isolates were resistant to at least one antimicrobial group; despite the numerous warnings from public health institutions, it is clear that the efforts made to improve the antimicrobial-resistant issue are still insufficient (172). Recent data estimated that unless an effective strategy is soon implemented, antimicrobial resistance could cost Europe around 1.1 billion (€) per year from 2015 to 2050 in terms of longer recovery (e.g., more than 569 million extra hospitalization days per year by 2050) and greater risk for complications (173). In addition, it is estimated that since 2007 the overall burden on global health due to antimicrobial resistance has been increasing, and last-line antibiotics are becoming increasingly ineffective (172).

Antimicrobial agents and possibly new strategies to treat infections are needed to tackle these issues effectively.

AMR in cancer patients

It appears clear that AMR is particularly problematic in cancer patients with a compromised immune system and susceptible to hospital-acquired infections.

A 2019/2020 survey of 100 UK oncologists (174) reported that 95% were concerned about the threat AMR poses on cancer treatments, with 46% believing that some chemotherapeutic options will soon become unavailable. Moreover, 41% of the oncologists reported a rise in drug-resistant infections in the past 12 months.

As we discussed before, it is already accepted that one of the major causes for the rapid insurgence of multi-drug resistant (MDR) bacteria is the misuse of antibiotics; however, choosing the proper antibiotic treatment is not easy. In the case of infections, tests to characterise the bacteria can take up to 24/72 h to provide results; therefore, the initial treatment is empirical and dependent on the physician. One side of this double edge sword is that delays in antimicrobial treatments increase the mortality rates (175), but also inadequate therapies for clinically ill patients are often associated with poor prognosis (133). It has been reported that at least 27% of the pathogenic bacteria responsible for cancer-associated infections are resistant to the antibiotic prophylaxis treatments used to counterbalance the loss of immune defences (128). Furthermore, prolonged exposure to antibiotics has side effects and can even reduce the efficacy of cancer chemotherapy or be the cause of the insurgence of MDR infections (125). However, when using combinatorial therapy, the probability of developing resistance to the effect of two drugs is much lower than of one alone if the resistance mechanism is not the same (133). Furthermore, minimizing the infection rate provides the opportunity to reduce the use of antibiotics.

With these premises, we set out to define a strategy to bypass the need for an antibiotic to minimize the insurgence of infections in cancer patients by “activating” the antimicrobial effects of the antitumoral agent itself. To achieve our goal, we looked at one of the DNA repair mechanisms allowing bacterial survival after exposure to an anticancer agent.

1.4 DNA repair in bacteria

DNA replication needs to maintain exceptional accuracy to avoid accumulating mutations. This fidelity has been estimated to be of the order of one misincorporation per 10^8 to 10^{10} nucleotide polymerized. To achieve these rates, the polymerase itself can promptly repair mistakes occurring during replication through the process of “proofreading” (176).

However, this system is not always enough, requiring additional processes to accurately restore the DNA's integrity, using its inherently redundant information stored in two strands. After DNA damage, the cell cycle is delayed, allowing the DNA damage response to deal with the lesions. Specific DNA repair pathways are then activated depending on the different nature of the detected damage (177–179).

1.4.1 Direct reversal repair

The simplest system to overcome damage is the direct reversal repair. These systems are limited, but they represent a fast way to fix simple errors in the DNA. Photoreactivation is the most known example. In this pathway, photolyase uses the energy from near-visible light (300-400 nm) to reverse UV damage breaking the pyrimidine dimers' unwanted bonds (180).

1.4.2 Mismatch repair (MMR)

The mismatch repair system (MMR) is highly conserved (in eukaryotes and prokaryotes) and can rectify mistakes escaping proofreading, improving the replication fidelity by ~20-400 fold (181). In *E. coli*, MutS binds to mismatched bases (up to 3 misincorporated nucleotides) and distinguishes the newly synthesized strand from the template by its undermethylation. With the help of MutL and MutH, the mismatch in the unmethylated strand is excised from the DNA, allowing for the gap to be correctly re-synthesized (182, 183).

1.4.3 Base excision repair (BER)

Base excision repair represents another DNA repair system available to both eukaryotes and prokaryotes. It recognises commonly modified bases due to DNA decay or damaging agents. (66, 184, 185). In *E. coli*, BER was reconstituted *in vitro* in the 90s using purified proteins (185). The authors were able to confirm that a glycosylase initiates the repair process by excising the damaged base and leaving an abasic site (AP site). The repair is then followed by the action of an AP endonuclease and a phosphodiesterase to remove the AP site, followed by the DNA polymerase I and ligase to complete the repair. They also describe a minor alternative pathway allowing slightly longer repair patches (2-5 nucleotides), with the DNA polymerase I taking over the lesion immediately after the AP endonuclease.

1.4.4 Homologous recombination (HR) and non-homologous end-joining (NHEJ)

As discussed before, DNA strand breaks are among the most toxic forms of damage. In bacteria, there are two primary mechanisms to repair these damages: homologous recombination (HR) and non-homologous end-joining (NHEJ) (186).

During HR, the DNA is repaired using an analogue DNA strand allowing for higher fidelity. The overall pathway is a multistep reaction initiated by the proteins RecBCD binding to the damaged DNA and preparing a single-strand region for RecA. RecA is then responsible for the search for the homologous strand used by the DNA polymerase I as a template for re-synthesis (186, 187). Furthermore, the RecBCD proteins can also repair single-strand breaks in bacteria via HR (188).

NHEJ represent an additional and simpler repair system available to some bacterial species. This pathway has the advantage of not requiring a sister DNA strand as a template and the disadvantage of having a lower level of fidelity. The prokaryotic protein Ku binds to the DNA ends as a homodimer forming weak synapsis (pairing the DNA strands together) and protecting the ends from degradation. The ligase LigD can then join the lesion, stabilizing the synapsis and sealing the ends together (189).

1.4.5 Nucleotide excision repair in bacteria (NER)

Nucleotide excision repair (NER) was discovered as a consequence of multiple observations on UV-damaged DNA in the 1960s (190–192). These findings led to the isolation and characterization of the UvrABC system (193, 194).

Even before NER's formal discovery, evidence of a promiscuous repair system able to respond to unrelated damages producing distortions in the DNA had already been published (195). This characteristic renders NER a more versatile pathway that can tackle an increased spectrum of DNA damage compared to other repair systems (e.g. photoreactivation or MMR) (196). NER is essential for effectively removing UV-induced damage (193, 197). Among the two major UV-induced distortions (Figure 4), the UvrABC system has been found to repair PPs much more efficiently than CPDs (with a difference of up to 9-fold) (96, 198). Interestingly, a complementary effect is seen in *E. coli* photolyase which efficiently repairs only CPDs (199). Other damages repaired by NER include single base adducts and DNA backbone modifications (e.g. cholesterol, fluorescein and 4-nitroquinoline-1-oxide) and crosslinking agents (e.g. cisplatin, mitomycin C) (196).

The pathway has been recently reviewed (200). Briefly, the three main proteins involved in the NER pathway are UvrA, UvrB and UvrC, followed by UvrD, DNA polymerase I and DNA ligase. During global genome repair (GGR), UvrA is responsible for the damage location as a homodimer (UvrA₂) or in complex with UvrB (UvrA₂B₂) and represents the first protein to contact the damaged DNA. UvrB is the second molecule of the system whose role it is to verify the damage prior to UvrC recruitment. After the damage is verified, UvrB ejects UvrA forming the UvrB-DNA pre-incision complex. Then the dual endonuclease UvrC can finally join the lesion to generate two nicks on the same DNA strand both 5' and 3' of the lesion. The helicase UvrD removes the damaged oligos, and the DNA polymerase I re-synthesizes the fragment using the undamaged strand as a template. The ligase can ultimately seal the gap restoring the DNA. A simplified summary of the process is reported in Figure 5.

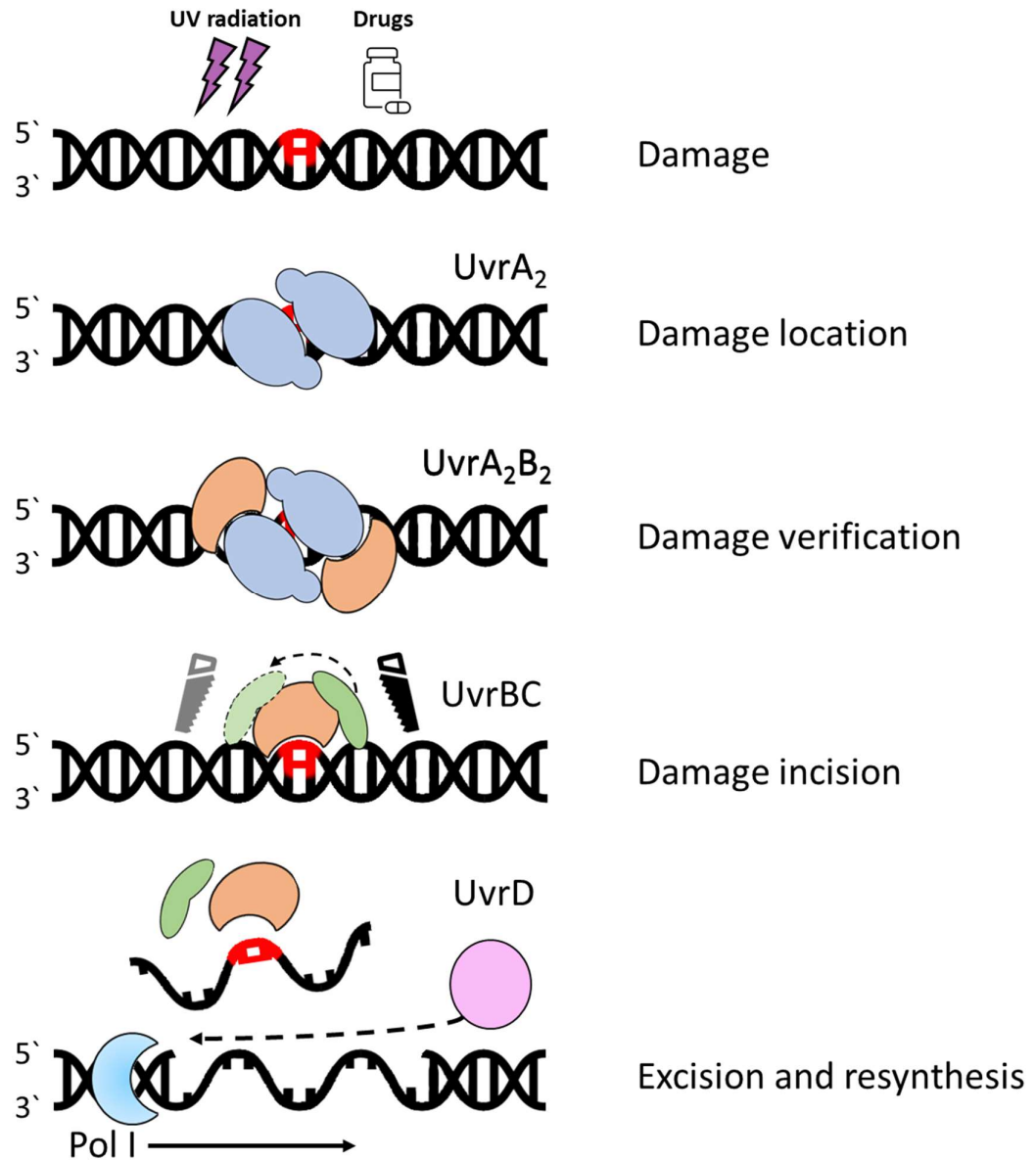


Figure 5: Schematic representation of Nucleotide excision repair in bacteria. Simplified representation of the nucleotide excision repair pathway from the formation of DNA damage to DNA resynthesis.

Damage location

For NER to initiate repair, the damage has to be detected and verified to prevent spurious incisions in the genome. The precise mechanism for recruiting NER onto various lesions is still under investigation. Three main processes have been described culminating with the handoff of the damaged site from UvrA to UvrB, global genome repair (GGR), transcription-coupled repair Mfd dependent (TCR-mfd) and a newly proposed TCR UvrD-dependent repair (TCR-UvrD).

The observation that the actively transcribed DNA strand is repaired more efficiently led to the discovery of a transcription-coupled mechanism linked to NER (201). A lesion can stall the RNA polymerase (RNAP) on the damage site and has to be removed for the repair systems to fix the distortion (202, 203). The protein Mfd can then displace the stalled RNAP and recruit UvrA through its UvrB-homology domain (exposed during the interaction with the lesion), initiating NER (204). More recent findings led to the proposal of an additional TCR pathway mediated by UvrD, not Mfd. Observations showed that in the absence of UvrD (6-4) PPs were still repaired, while CPDs repair through TCR was compromised (205). Furthermore, it has been reported that UvrD can pull the RNAP away from the lesion, making the damage accessible to repair systems (206). The authors proposed this alternative pathway in which UvrD will mediate the backtracking of the RNAP, permitting the recruitment of the UvrAB complex on the lesion to facilitate repair without the need for Mfd. These mechanisms of transcription coupling are hotly debated in the field, with strong arguments both for and against. These will not be discussed further, but the latest arguments can be found here (207, 208).

NER also locates the damage without the aid of additional proteins. During global genome repair, the system relies on UvrA for its damage search. The homodimer UvrA₂ uses a 3-dimensional search to scan the genome jumping from one strand to another (within ~1 µm) (209) with increased affinity for damaged and single-stranded DNA (210, 211). In its search, UvrA₂ briefly contacts the DNA (~130 ms) and switches to longer dwell times (using ATP) only when damage is suspected. This dual-contact mode is believed to allow for rapid scanning enabling UvrA to search the entire genome each replication cycle (212).

The complex UvrA₂B₂ was also believed to be responsible for the damaged locations. UvrA₂B₂ can slide onto the DNA backbone, looking for damage 1-dimensionally in contrast with the faster 3D search performed by UvrA₂ alone (209). However, an *in vivo* study implied that this complex could not be a primary search mechanism since UvrB rarely binds UvrA in solution (213).

The specific mechanism that allows UvrA to discriminate damaged DNA is not clear, but suggestions from structural data indicate that upon binding, UvrA detects deformations of the double helix by switching between an open and closed conformation (214, 215). This mechanism allows the protein to sense various lesions but requires another member to locate the damaged strand. More on UvrA will be discussed the chapter 3.1.1.

Therefore, UvrB (the system's second protein) must verify the lesion for the pathway to proceed.

Damage verification

UvrB has been crystallized both bound to DNA (PDB: 2FDC) (216), as UvrA₂B₂, the damaging sensing complex (PDB: 3UWX) (215) shown in Figure 6 and recently trapped on the lesion (PDB: 6O8F) (217). The structural data shows the complex UvrA₂B₂ forming a UvrB-UvrA-UvrA-UvrB “sandwich” (215), later confirmed with electron microscope data (218). The complex exhibits a DNA binding region that can accommodate ~45 bp, among which ~32 within the UvrA₂ dimer (215). This conformation places UvrB ~80 Å away from the lesion (215). It has long been known that the pre-incision complex is formed only by UvrB-DNA, which requires the addition of UvrC to effectuate the dual incision (219). Therefore, one molecule of UvrB would have to translocate onto the damaged nucleotide, and UvrA₂ must be ejected.

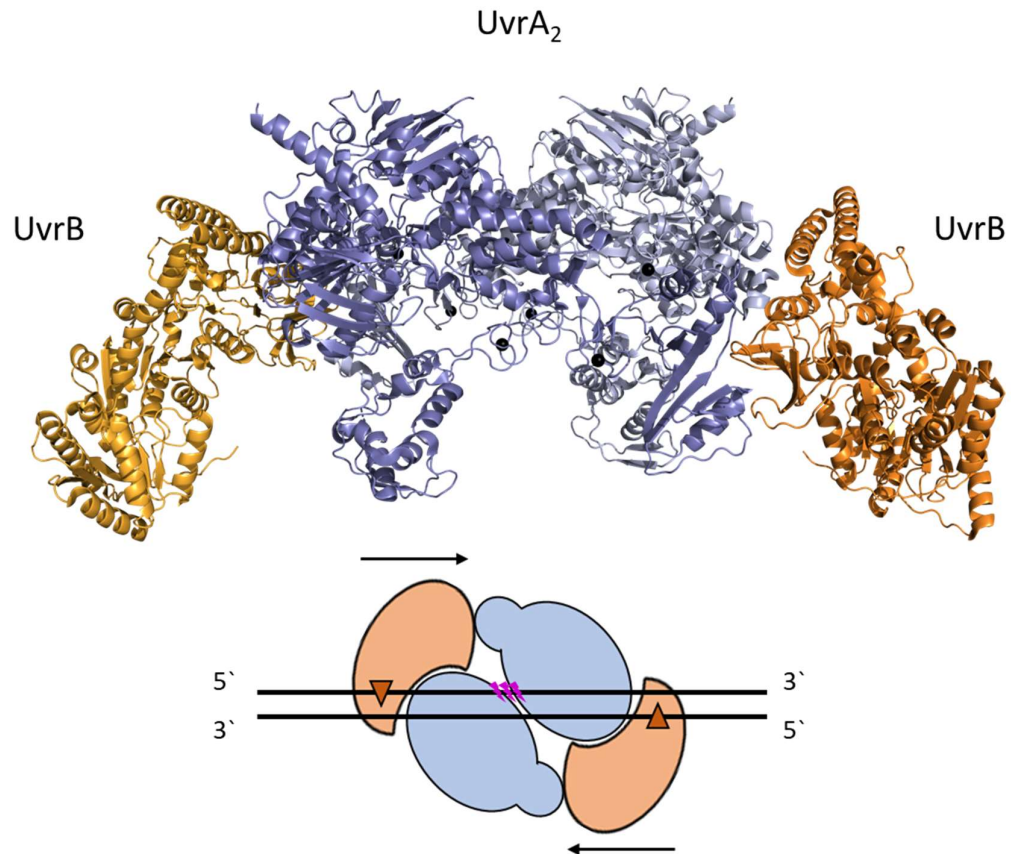


Figure 6: UvrA₂B₂ complex. On top, the figure generated in PyMOL (3UWX) shows the UvrABBA complex. (UvrA is coloured in purple and UvrB in orange). The bottom represents the complex bound to UV-damaged DNA (violet rays). The two molecules of UvrB clamp the DNA strands through their β -hairpin (shown as a brown triangle). The arrows indicate the 5' $\rightarrow$ 3' direction followed by UvrB when it translocates onto the DNA to approach the damaged nucleotides.

The damage discrimination process is still being investigated. However, recent findings have shed light on its mechanism.

UvrB belongs to the helicase family, also exhibiting an ATP-dependent translocase activity. Mutants lacking ATPase activity (K45A) remain trapped bound to UvrA onto the DNA, unable to form the pre-incision complex (220). The UvrA-stimulated helicase activity enables minor local unwinding and allows UvrB to translocate in direction 5' $\rightarrow$ 3' (221, 222). Notably, this activity allows UvrB to move towards the damaged nucleotides (223) and seems to be halted by DNA lesions (221, 222). A central structural motif seems essential for UvrB activity, the β -hairpin. It allows for the formation of the pre-incision complex and damage discrimination. It has been shown in mutational studies (lacking residues 97 – 112

of the β -hairpin) that UvrB is rendered unable to create a stable pre-incision complex abolishing the incision mediated by UvrABC (224). Furthermore, biochemical and structural data have shown that UvrB uses its β -hairpin to clamp the two different strands (one per UvrB) ~18 bp upstream from the damage (215, 218). The authors also proposed that the bulky adducts recognised by NER cannot be accommodated in the interface between UvrB and its hairpin, being the cause for UvrB stalling. Ultimately, recent structural data (217) showed UvrB being trapped in the pre-incision complex with its β -hairpin inserted in a loop between the two DNA strands and clamping only one against itself. Based on this evidence, a mechanism for damage discrimination was recently proposed (217, 218). UvrA₂B₂ binds the DNA with UvrB offset from the lesion clamping the two strands with its β -hairpin (Figure 6). The complex UvrAB locally unwinds the DNA and allows UvrB to translocate towards the lesion (5' $\rightarrow$ 3') in an ATP-dependent manner. The UvrB molecule holding the damaged strands stalls at the lesion, which cannot be accommodated in the space beneath the β -hairpin. In the meantime, the other UvrB proceeds in its translocation and disassociates along with UvrA₂. Now that the pre-incision complex is formed, UvrC can join to progress the pathway in its final stage.

Incisions and re-synthesis

The dual endonuclease UvrC performs in order a 3' and 5' incision in the same DNA strand, creating a ~12 oligonucleotides nicked fragment isolating the damage for removal (193, 225, 226). UvrC has been less investigated than the other NER members, and it is the system's only protein not yet successfully crystallised. As for the damage location and verification, the precise mechanism leading to the specific incisions is still unclear.

It has been shown that UvrC's N-terminal domain (Arg⁴²) is responsible for the 3' incision while the 5' incision is achieved in the C-terminal domain (Asp^{399, 438, 466} and His⁵³⁸) (225, 226). Furthermore, incision experiments in the early 1980s on NER showed that the nicks are not in random locations but situated at the 4th/5th phosphodiester bond 3' and the 8th bond 5' of the lesion (193, 227). Once the DNA has been nicked, the DNA fragment and proteins must be removed to allow re-synthesis. UvrD (Helicase II) and DNA polymerase I can free the region (at a higher rate when combined) and use the undamaged strands as a

template to replace the appropriate nucleotides. Ultimately, DNA ligase seals the remaining nick restoring the DNA (228, 229).

1.5 Aims of this thesis

Antitumoral agents (specifically cisplatin) significantly affect cell growth, but bacteria can overcome the damage mainly thanks to the activity of NER (230, 231). By interfering with the UvrABC proteins, we would remove the primary natural defence against cisplatin, preventing proliferation without the need for additional antibiotics.

Nucleotide excision repair is only functionally conserved between eukaryotes and prokaryotes. The two systems do not share any biochemical similarity, representing an example of convergent evolution (232). Eukaryotic NER involves significantly more proteins and steps than the bacterial counterpart (233), making the UvrABC system an ideal selectively druggable target.

Therefore, the work undertaken for this thesis aims to explore NER as a new possible antimicrobial target in combinatorial therapy. Following the premises described so far, we have defined strategies for repurposing different drugs to be used as adjuvants in combination with antitumoral agents to fight co-infection with cancer. In doing so, we were not only looking for a solution to be immediately applied, but we wanted to define a new approach and new compounds as a starting point for further explorations. To achieve this goal, we started by understanding how a drug-like molecule reported as an inhibitor (ATBC) worked to impair NER in *mycobacteria*. We followed it up using both a traditional screening of thousands of compounds and an in-silico screening of hundreds of thousands of drug-like molecules to identify new possible hits. In doing so, we have established a pipeline of microbiological, biochemical, computational and single-molecule assays to identify their possible mechanism of action and confirm their intended activity.

Ultimately, we aim to be able to help in the fight against cancer by preventing or even treating the frequent bacterial infections acquired during hospitalization for cancer chemotherapy.

Chapter 2 – Material and Methods

2.1 Buffers and solutions

ABC buffer

50 mM Tris (pH 7.5), 50 mM KCl, 10 mM MgCl₂

Standard Lysis Buffer

50 mM NaPO₄ (pH 7.5), 20 mM imidazole, 500 mM NaCl

UvrA storage buffer

50 mM Tris (pH 7.5), 500 mM KCl, 0.1 mM EDTA, 2.5 mM DTT, 50% v/v Glycerol

UvrB storage buffer

50 mM Tris (pH 7.5), 100 mM KCl, 0.1 mM EDTA, 2.5 mM DTT, 50% v/v Glycerol

UvrC storage buffer

50 mM Tris (pH 8), 400 mM KCl, 0.1 mM EDTA, 5 mM DTT, 50% v/v Glycerol

Buffered resazurin solution (BRS)

0.3 mg/ml resazurin sodium salt, 400 mM MOPS, 40 mM Tricine (pH 7.4)

Bacteriophage buffer

10 mM CaCl₂, 20 mM MgSO₄

2.2 Plasmids and bacterial strains

The pJB vectors (IPTG inducible) containing the genes for the proteins UvrA, B and C were assembled by Dr Alexandra Moores with a Gibson assembly kit (New England Biolabs (NEB)). The vectors containing the desired genes were engineered to have a flexible C-terminal linker connecting the protein to an AviTag, TEV protease site and a hexa-histidine (His) purification tag. The fluorescent proteins were cloned similarly, but a fluorophore (mNeonGreen) was inserted between the linker and the his-tag. The strains chosen for the experiments were *E. coli* K-12 MG1655, MG1655 $\Delta tolC$, MG1655 $\Delta tolC \Delta uvrA$ and *E. coli* H4-ST131 EC958, a multidrug-resistant clinical isolate (234–236) (Dr Mark Shepherd gently provided the wild type and the MDR strains). The strain BL21-DE3 cells (NEB) $\Delta uvrA \Delta uvrB$ was used for protein purification. The mutants were prepared through P1 virus transduction, as described in the next paragraph.

2.3 Drugs and drug library

The respective suppliers provided the storage conditions, solubility profiles and purity information of the compounds used in this thesis.

- (2-(5-amino-1,3,4-thiadiazol-2-yl)benzo(f)chromen-3-one) ATBC: Green Pharma, Ambinter (purity $\geq 90\%$).
- 4-Nitroquinoline N-oxide (4-NQO): Merck (purity $\geq 98\%$).
- Bleomycin (sulfate): Cayman chemicals (purity $\geq 98\%$).
- cis-Diamineplatinum(II) dichloride (cisplatin): Merck (purity $\geq 99.9\%$)
- Dienestrol: Merck (purity $\geq 95\%$)
- Pirarubicin, aprotinin, bemcentinib, mitoxantrone, l-thyroxine, 9-aminoacridine: MedChemExpress: (purity $\geq 98\%$)

2.4 Phage lysate preparation and P1 Vir transduction

The mutants were generated through P1 Vir transduction, a well-established technique that exploits the sloppy packaging mechanism of the P1 virus (able to pack ~100 kb of DNA) to move a mutation into a clean *E. coli* strain (237). The procedure was based on the knockout strains obtained from the excellent Keio collection (238).

For the creation of multiple knockout strains, the transductions were repeated sequentially. The first transduction swaps the gene of interest with a Km^R cassette that can be cured using the thermosensitive vector pCP20, as previously described (239), allowing for sequential transductions. For the phage lysate preparation, a donor knockout strain (e.g., *ΔtolC* Km^R Keio mutant) was grown overnight in LB broth (Miller) in the presence of 25 µg/ml kanamycin at 37°C with aeration. After approximately 16 h of incubation, 200 µl were inoculated in two prewarmed flasks containing 10 ml LB supplemented with 0.2 % glucose and further re-incubated for 30 minutes at 37°C in a shaking incubator at 250 RPM. Then, 200 µl of a premade phage lysate containing a different antibiotic resistance (e.g., Tet^R) was added to only one of the flasks and further incubated as previously described while checking the solution every 30 minutes for clearance, indicating cell lysis. After adding 200 µl of chloroform, the phage solution was transferred to a 50 ml falcon tube and vortexed. The tube was centrifuged at 4000 RPM for 10 minutes at 4°C. 5 ml of the supernatant was then transferred into a 15 ml falcon tube with 100 µl of chloroform and stored at 4°C overnight. The transduction was done the next day. 200 µl from an overnight culture of a recipient strain (e.g., MG1655) were doubled with 200 µl of bacteriophage buffer (10 mM CaCl₂, 20 mM MgSO₄) and then divided into three different tubes. 10 and 50 µl of the phage lysate prepared previously were added to two tubes, keeping the last one as a control. An additional control was made containing only 50 µl of the phage but no overnight culture. The samples were mixed gently and incubated at 28°C for 30 minutes to allow the incorporation into the cell. 1 ml of LB supplemented with sterile 0.1 M sodium citrate (with the function of chelating the calcium fundamental for P1 to minimize secondary infections) was added to the tubes and further incubated for 2-3 hours at 37°C and 180 RPM. Ultimately, 100 µl of the cultures were spread onto L-agar plates containing the appropriate antibiotic selection (e.g., Kan) and grown overnight at 37°C. Obtained colonies were re-streaked and re-incubated for an additional overnight to ensure the

Page | 56

complete loss of residual P1 virus. Finally, they were inoculated in LB in the presence of the appropriate selection and checked for successful mutation through colony PCR, as shown in Figure 8 (a simplified representation of the process is available in Figure 7).

The curing required the transformation of the plasmid pCP20 containing the FLP gene required for removing the Km^R cassette, which proved challenging in MG1655 ΔtoI . The curing was successful after the cells were transformed with pCP20 and incubated at 28°C overnight instead of the suggested 30°C (239). After successful transformation, the cells were grown without selection at 43°C and then tested for loss of antibiotic resistance.

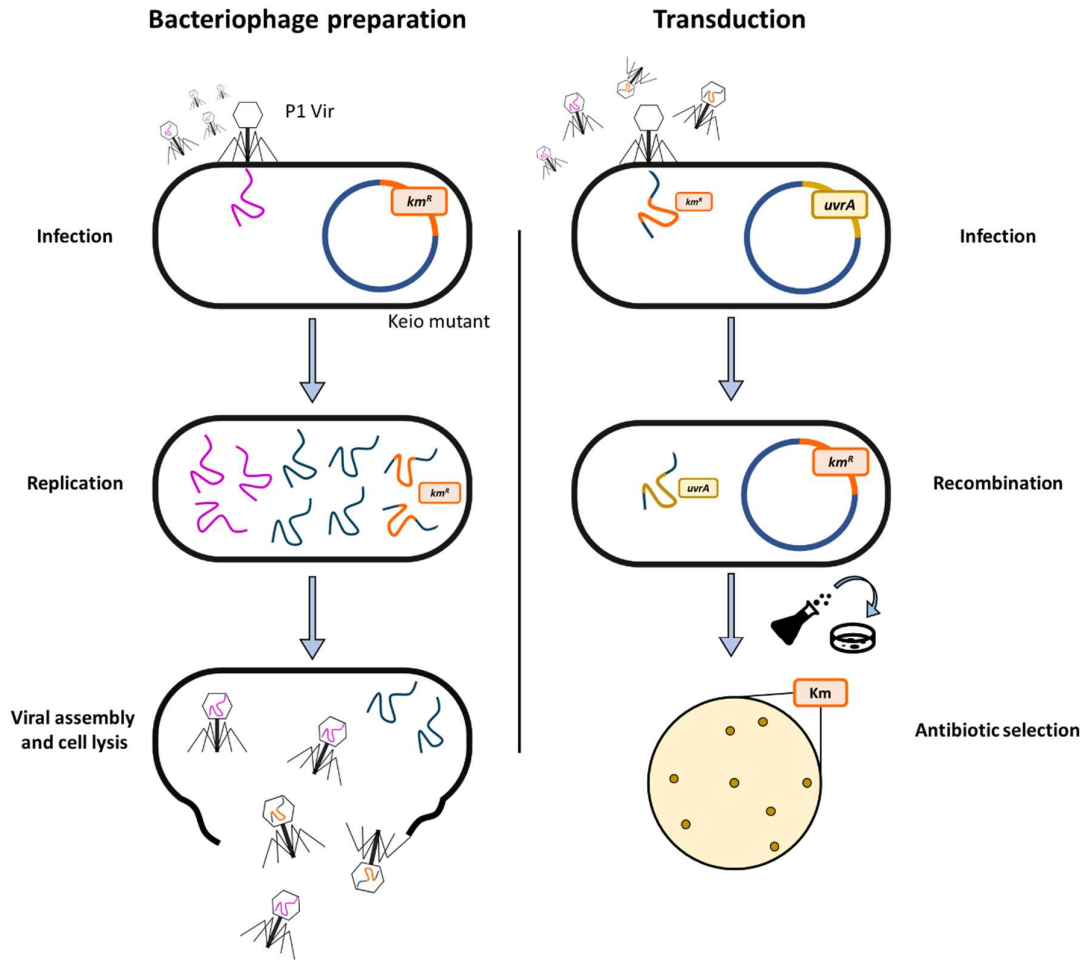


Figure 7: Schematic representation of a P1 Vir transduction. The figure visualizes the main steps in the bacteriophage lysate preparation (left) and the viral transduction (right) used to swap the *uvrA* gene in *E. coli* MG1655 with a Km^R cassette. **(Infection)** A P1 virus (capable of packing ~100 kb DNA) infects a Keio mutant cell already containing the desired mutation (in this case, a kanamycin cassette replacing the *uvrA* gene). **(Replication)** The infected cell undergoes DNA replication, creating copies of the genetic material. **(Viral assembly and cell lysis)** The virus re-assembles and, while packing DNA, accidentally produces molecules containing the desired gene fragment. **(Infection)** The new bacteriophage solution is used to infect a wild-type cell, transferring the DNA fragment to a clean host. **(Recombination)** The new fragment (Km^R cassette) may exchange with the gene of interest through homologous recombination. **(Antibiotic selection).** The infection is then stopped, and the culture is plated in selective LB agar (in the presence of kanamycin). Only the cells that successfully express the resistance promoted by the Km cassette will be able to grow after incubation.

Colony PCR

The colony PCR was made following NEB guidelines. Internal primers of the deleted gene were chosen to test for successful transduction. A master PCR mix was prepared to contain 200 nM forward and reverse primer, 1x Taq Master mix (purchased from NEB), and ddH₂O to a total volume of 25 μ l. One colony was then picked from an overnight selective LB-agar plate with a sterile 10 μ l tip, mixed with the master mix, and then streaked in a fresh selective LB-agar plate. The primers were chosen to produce an amplicon of 553bp and 624bp, respectively, for *tolC* and *uvrA* both internal to the gene. The samples were then placed in a thermocycler (Alpha Cyclor 1 PCR max) for amplification. The thermocycler settings were chosen according to NEB guidelines, and the PCR products were then run on a 1% agarose gel pre-stained with Ethidium bromide. Successful deletion would appear as a lack of the amplicon of the regions for the mutant colonies but not for the positive control carrying the gene of interest (Figure 8).

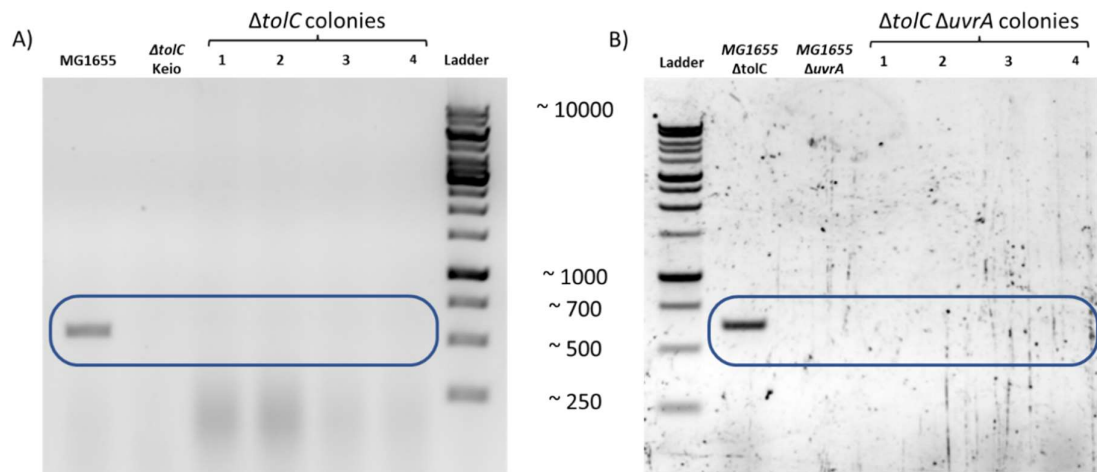


Figure 8: Agarose gel showing the chromosomal deletions. Colony PCR samples were run in 1% agarose gels stained with ethidium bromide. **A)** The gel shows the successful deletion of $\Delta tolC$ from MG1655. The original strain MG1655 was used as a positive control, and MG1655 $\Delta tolC$ Keio mutant as a negative control to check for the absence of chromosomal *tolC*. The band's presence in the positive control (MG1655) and the band's absence in the $\Delta tolC$ colonies show the success of the deletion. **B)** The gel shows the successful deletion of chromosomal *uvrA* from MG1655 $\Delta tolC$.

2.5 Protein purification

The proteins used in this project were purified after numerous optimizations adapting a previously described protocol (240). Initially, we started with the ÄKTA™ (chromatography systems from Cytiva), which allows more granular control over the fractionation step. Then we moved to a faster purification method using a simple spin column with Nickel resin to increase the output.

Freshly transformed cultures of BL21-DE3 cells (NEB) $\Delta uvrA \Delta uvrB$ were grown to an OD₆₀₀ of 0.4 - 0.6 in selective LB (Miller) at 37°C while shaking at 180 RPM in baffled flasks. When ready, the cells were induced with 0.5 mM IPTG. The flasks were then incubated at 18°C overnight after the re-addition of appropriate antibiotics to maintain the selection. In the morning, the cultures were pelleted in a JA10 rotor (Beckman) at 6000 RPM at 4°C for 20 minutes. The cells were then resuspended in standard lysis buffer (50 mM NaPO₄ (pH 7.5), 20 mM imidazole, 500 mM NaCl) supplemented with protease inhibitor cocktail ((no EDTA) Thermo Fisher Scientific)), and 1 mM phenylmethylsulfonyl fluoride (PMSF). After resuspension, 100 µg/ml lysozyme was added on ice to aid cell lyses before sonication (30 seconds on, 59 off, ~65% amp, for 7.5 minutes of 'on'). The tubes were submerged in an ice slurry during the sonication step to prevent heat accumulation. After lysis, 50 µg/ml DNase I (Roche) were added to the solution and incubated on ice for 30 minutes. The solution was centrifuged at 20k RPM for 30 mins at 4°C in a JA25.50 rotor (Beckman). The pellet was discarded, and the supernatant was transferred into a Proteus '1-step batch' midi plus spin column (Protein Ark column) with Ni-NTA resin (Thermo Scientific™ HisPur™) pre-equilibrated with standard lysis buffer.

The proteins were then purified with increasing concentrations of imidazole according to the manufacturer's protocol. The sample was loaded and incubated while gently spinning in a tube roller at 4°C for 2 h before being washed twice with 15 ml of standard lysis buffer. The imidazole concentrations used were 90 mM, 130 mM, and 160 mM for optimal elution, with a final wash at 500 mM imidazole. The protein was eluted with 10 ml of standard lysis buffer + imidazole. The protein purity and content in the fractions were visualized by a 10% SDS-PAGE gel with Coomassie staining. The best fractions were pulled together, and buffer exchanged using a Viva-spin filter with a cut-off at least 50 % smaller than the size of the protein. 2x of the appropriate storage buffer was used for the exchange (repeated at least

3 times). The last exchange was used to concentrate the protein in solution to a volume of approximately 500 μ l, then made 50% v/v with glycerol and kept at -20°C for immediate use or at -80°C for more extended storage. The concentration was estimated with a spectrophotometer reading the absorbance at 280 nm through the Beer-lambert law. *E. coli* Uvr proteins are notoriously challenging to work with (240), particularly UvrA and UvrC. Therefore, we were forced to repurify periodically for loss of activity. An example of an SDS-PAGE gel showing the purity of the protein used in this research can be seen in Figure 9.

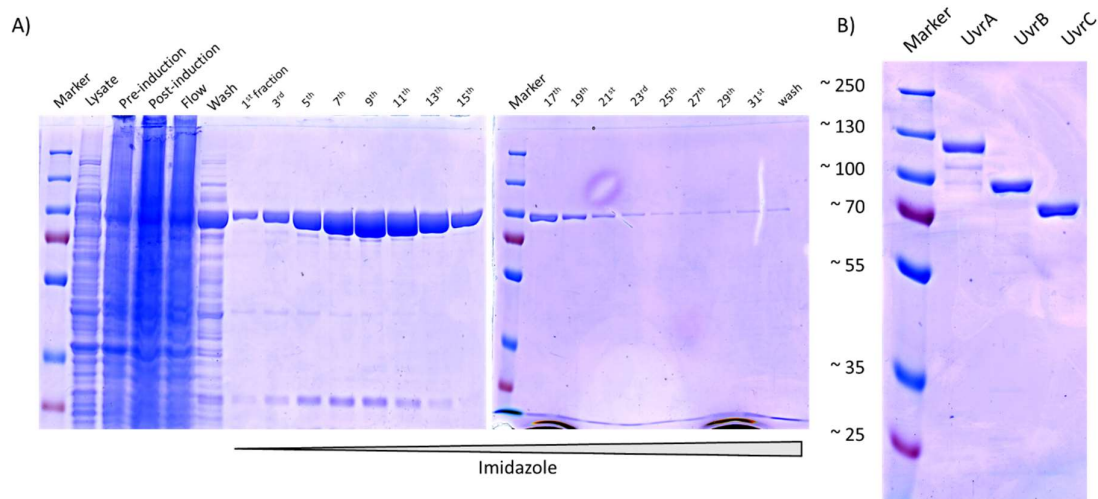


Figure 9: Chromatography in HisTrap using HisTrap resins. A) Representative 10% SDS-PAGE gel stained with Coomassie showing the purification of UvrB using the ÄKTA™ system. Overexpression of UvrB can be seen as soon as the first wash of the unbound protein. The gel shows a gradient elution due to increasing imidazole concentrations up to a peak around the 9th fraction. In this example, fractions 5 to 16 were pulled together, buffer exchanged, spin concentrated and stored for use. **B)** Representative 10% SDS-PAGE gel stained with Coomassie showing the final purity of the stored UvrABC for usage.

2.6 Survival assays

We based our survival assay on adapting the Clinical and Laboratory Standards Institute (CLSI) guidelines (241). A single colony grown in selective LB-agar for 16 h at 37°C was inoculated into fresh sterile LB with the appropriate selective antibiotic. The new culture was incubated for 16 h at 37°C with aeration. In the morning, the bacterial suspension was aliquoted with 25% sterile glycerol and kept at - 80°C for long-term storage. The day prior to the experiment, the bacteria were inoculated in fresh LB and grown overnight at 37°C while shaking. The next day prior to the experiment, the culture was re-inoculated in fresh sterile MOPS Minimal Medium (242) supplemented with 0.4% glucose to a final OD₆₂₅ of 0.001 to be used for the experiments. Most of the compounds used in this research are dissolved in DMSO. Therefore, we measured a MIC value for the solvent (Appendix A, Figure 53), and we used 2.5% v/v as the optimal concentration for DMSO to not interfere with growth.

The chemicals were added to the empty microplate before adding bacteria to avoid cross-contamination and minimize plastic usage. When UV irradiation was to be tested, the bacteria were irradiated with UVC (254 nm) in a 90 mm petri dish using a UV cross-linker (UVP/Analytik Jena UV Crosslinker CX-2000). Then an appropriate volume of culture solution (to a final volume of 100 µl) was plated for incubation. Different steps of preparation were then required depending on the assay.

Minimal inhibitory concentration assay

For the higher throughput colorimetric assay, we used resazurin, a blue dye that gets metabolized irreversibly to the pink compound resorufin (243). After the plate preparation, as previously described, the microplate was sealed with parafilm and covered in tinfoil for protection against light. Then the bacteria were incubated for 16 h at 37°C shaking. At the end of the incubation, 50 µl of buffered resazurin solution (BRS from now on) was added. The cells were further incubated at 37°C (in a static incubator) in the dark for 4 h before scanning the plate. The picture and plate were inspected, and the MIC was recorded based on the color change on the best representative plate among the three independent replicates, as shown in Figure 10.

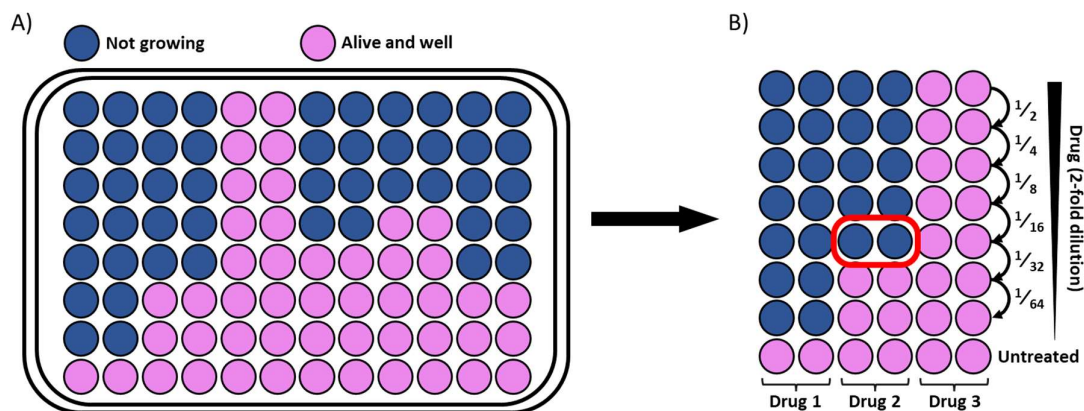


Figure 10: Stylized diagram of a survival assay for minimal inhibitory concentrations.

Representation of a microplate with different MICs at their endpoint (16 h incubation + 4 h incubation with resazurin). **A)** The plate represents a standard set-up for a colourimetric MIC evaluation of multiple compounds. **B)** Instructions on how to read the results (left half of plate A). The first two wells in blue colour indicate the minimal amount of compound required to inhibit growth, shown with a red circle in drug 2. The MIC cannot be extrapolated for drug 1 and drug 3 due to their high and low concentrations, respectively.

Growth curves

For the less efficient but more informative and precise assessment of bacterial growth, we incubated the plate in a plate reader (id5 Spectramax, Molecular Devices) in the dark at 37°C while shaking (double orbital). Readings at OD₆₀₀ were acquired every 30 minutes for 16-24 h, depending on the experiment. All the assays were repeated multiple times with independent measurements.

Checkerboard assay

The checkerboard assays are 2-dimensional MICs and therefore prepared as described previously. Briefly, 2.5 µl of two different compounds are plated to create a checkerboard with different concentrations. The plate will then contain the MIC for the two compounds on different axes, and the bacteria will be untreated at the intersection. Since the survivability assays can display fluctuations around their MIC values, the drug was plated at a starting concentration to ensure inhibition of growth to enable the estimation of the shift when combined with the other compound. The results were reported measuring the

fold increase in inhibitory activity in the two axes based on the colour change of the best representative plate among the three independent replicates, as shown in Figure 11.

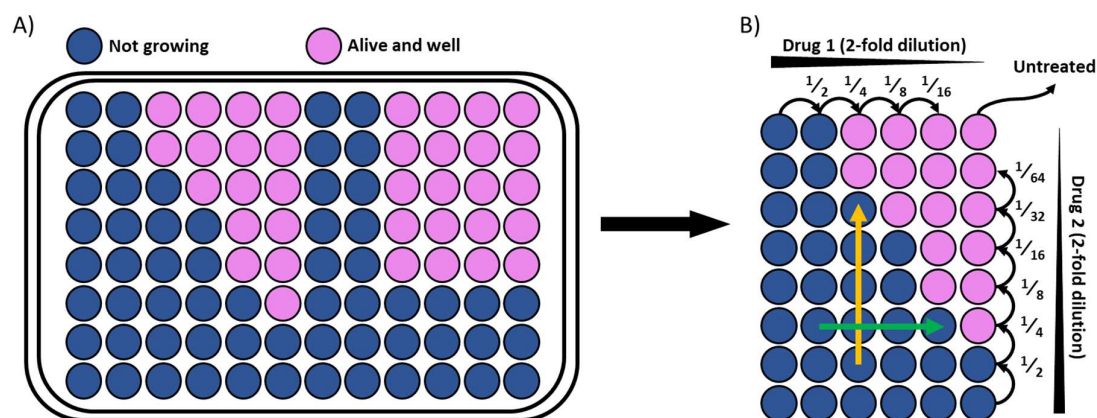


Figure 11: Stylized diagram of a checkerboard. Representation of a microplate with two different checkerboards at their endpoint (16 h incubation + 4 h incubation with resazurin). **A)** The left half of the plate shows strong cooperativity between the two drugs when combined, while the half-right experiment shows no cooperation between the two compounds, and only the respective MICs can be seen. **B)** Instructions on how to read the checkerboard (left half of plate A). The shift in MICs (green arrow for drug 1 and yellow for drug 2) indicates cooperativity between the two compounds. For simplicity, only the major shift was reported.

Complementation assay with recombinant UvrA

We performed a complementation assay to verify if the recombinant UvrA we purified could restore NER activity and UV survivability. MG1655 $\Delta toI/C \Delta uvrA$ was made competent following the NEB protocol and then transformed with the pJB plasmid used to purify UvrA. A survivability assay with UV was performed as described before titrating the intensity of the irradiation to test for the cell's ability to overcome UV damage. The cultures were not supplemented with IPTG since the pJB plasmid DNA shows leaky expression in MG1655 thanks to its T5 promoter. The experiments were repeated multiple times with technical replicates.

FDA-approved drug screening

An FDA Approved & Pharmacopeial Drug Library purchased from MedChemExpress containing 2731 compounds were screened against *E. coli* MG1655 $\Delta tolC$ in the presence and absence of 4 $\mu\text{g/mL}$ cisplatin in MOPS minimal medium (242) supplemented with 2% glucose, as shown in Figure 12. The drugs and cisplatin were added to the microplate at a concentration of 20 μM and 4 $\mu\text{g/mL}$, respectively and later the bacteria were plated in the wells at a final volume of 100 μL and OD₆₂₅ 0.001 (241). The microplate was then incubated in the dark at 37°C while shaking for 16 h before adding BRS and further incubation for another 4 h at 37°C. Scans were then acquired, and the results were recorded. The screen for the shortlisted compounds was then repeated using UV radiation instead of cisplatin as a DNA-damaging agent. The cell cultures were irradiated with UV at 75 J/m² at 254 nm using a UV cross-linker (UVP/Analytik Jena UV Crosslinker CX-2000). Similarly, the compounds that showed activity when combined with UV radiation were shortlisted for further investigations.

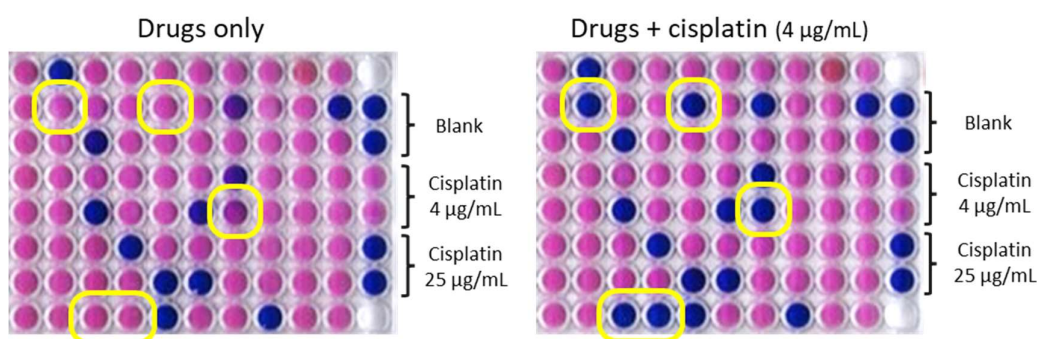


Figure 12: Example plate showing the screening setup. The example represents two screening plates containing several drugs incubated in the presence and absence of cisplatin. The yellow squares indicate compounds that were listed for further evaluation due to their activity only in the presence of cisplatin. Blue = not growing, pink = alive and well.

2.7 Solubility test for ATBC

ATBC purchased from Green Pharma (Ambinter), was solubilized in 100% DMSO at a concentration of 5 mM and kept frozen at -20°C. A two-fold dilution series was prepared in DMSO and water as solvents. The solutions were then intensively mixed by vortex, and then the solutions were centrifuged at 13000 rpm for 30 minutes in a microcentrifuge (Micro Star 12) to precipitate the eventual insolubilized crystal. 100 µl from the solutions was then taken, not disturbing the eventual precipitate, and then added to a microplate. The OD₄₁₂ was recorded with a plate reader (id5 Spectramax, Molecular Devices). The experiment was repeated 3 times.

2.8 NADH-coupled ATPase assay

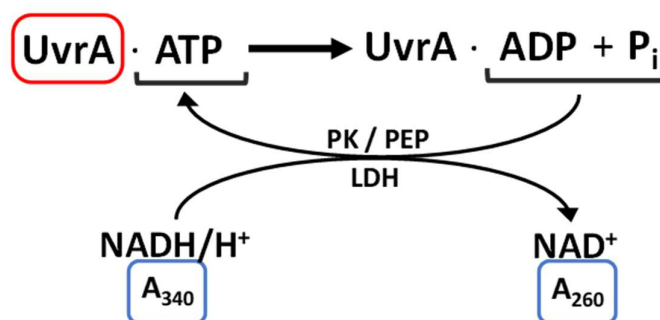


Figure 13: NADH-coupled ATPase assay schematic. A representation of how the ATP turnover is coupled to NADH oxidation and loss of absorbance at 340 nm.

The assay is schematically summarized in Figure 11. Briefly, the ADP and P_i produced by UvrA ATPase are recycled back to ATP with the generation of pyruvate from phosphoenolpyruvate (PEP) thanks to the activity of pyruvate kinase (PK). The pyruvate is then converted into lactate by the enzyme lactate dehydrogenase (LDH) with consequent oxidation of $NADH/H^+$ to NAD^+ ($NADH$, $6220 \text{ M}^{-1} \text{ cm}^{-1}$ at 340 nm). This reaction comports a decrease in absorbance at 340 nm, which is directly proportional to the ATP consumption by UvrA. To increase the output of this assay and test multiple inhibitors, we adapted the method to be carried in a microtiter plate (244). Prior to the experiment, 2 % v/v pyruvate kinase (600–1000 U/ml) and lactate dehydrogenase (900–1400 U/ml, premixed stock from Merck) were added to a master mix tube of ABC ATPase buffer (50 mM Tris (pH 7.5), 50 mM KCl, 10 mM $MgCl_2$, 0.5 mM PEP, 1 mM DTT, 210 μM NADH) supplemented with a concentration of 50 nM UvrA and kept on ice. The master mix was then divided in the microplate, and 20 μM of the drugs were added to the appropriate wells. One well was always kept as UvrA untreated control containing DMSO. The reactions were incubated for 6 minutes at ambient temperature, and then 1 mM ATP was added to start the reaction. The loss in absorbance at OD_{340} was then measured with a plate reader (id5 Spectramax, Molecular Devices) with readings every 10 seconds for 6 minutes at ambient temperature. Ultimately, 0.1 ng/ μl of pUC18 DNA was added to stimulate UvrA ATPase activity, and the OD_{340} was measured for additional 6 minutes at ambient temperature. The results were fitted linearly. The slope of the linear decrease in absorbance was calculated using the

“slope” function in Excel and used to measure the ATP conversion rate with the following formula:

$$ATPase\ activity\ (M\ sec^{-1}) = \frac{Rate\ of\ OD_{340}\ signal\ loss}{path\ length * \epsilon_{NADH}\ (6220\ M^{-1}cm^{-1})}$$

The ATPase activity is then divided by the protein concentration to get the catalytic constant (k_{cat}) value.

The data was then normalized by the activity of the untreated sample used as a control in each plate. The results were expressed in the percentage of inhibition. The assay was repeated 3 times, and the error was reported as the standard error of the mean (n=3). ADP does not accumulate in this assay.

The fits and IC_{50} were calculated based on the Hill equation (245, 246) using Excel data solver and Excel SolvStat through the equation:

$$Effect = E_{no\ drug} \left(1 - \frac{[C]^n}{[IC_{50}]^n + [C]^n} \right)$$

$E_{no\ drug}$ represents the maximum catalytic activity in the absence of the inhibitor, C is the ligand concentration, IC_{50} is the concentration causing a 50% inhibition, and n is the Hill constant.

2.9 Gel-based incision assay

A solution containing UvrA 100 nM, UvrB 200 nM and UvrC 100 nM was prepared in ABC buffer supplemented with 5 mM DTT and 1 mM ATP on ice. A final concentration of 2,8 nM of pUC18 plasmid DNA was irradiated at 200 J/m² (254 nm) and added to the solution. The reaction mix was then incubated for 15 or 30 minutes at 37°C in the presence or absence of different compounds. At the end of the incubation, the tubes containing the solutions were placed in a heat block at 65°C for 10 minutes to kill the reaction. The DNA was then examined on a 0.8% agarose gel stained with Ethidium Bromide (Et-Br). As a control, damaged and undamaged DNA was run in the same gel as well as a sample treated with T4 endonuclease V (New England Biolabs) instead of the UvrABC mixture. T4 endonuclease V is an enzyme that cleaves the DNA in the presence of CPDs (247) and can be used to visualise a full incision, as shown in Figure 14.

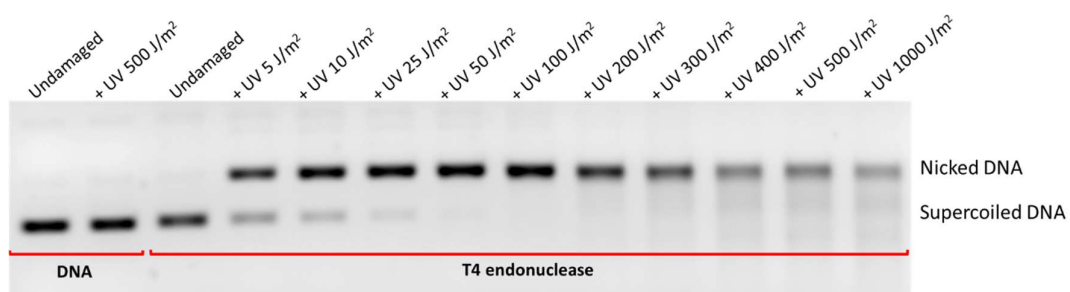


Figure 14: UV irradiation titration. 0.8% agarose gel shows the level of incision of a pUC18 plasmid by T4 endonuclease when irradiated with different intensities of UV light (254 nm). Supercoiled DNA completely disappears from 100 J/m². The experiment was repeated twice.

2.10 Fluorescence-based incision

It has been shown that UvrAB loading onto a lesion is an asymmetric process starting at the 5' side of the damage (223). After successful binding and UvrC recruitment, the two incisions are performed sequentially. The first incision occurs at 3' relative to the lesion at the 5th or 4th phosphodiester bond and is followed by a less specific 5' incision at the 8th phosphodiester bond (193, 225, 226)). With this in mind, we designed two complementary oligonucleotides (oligo) with fluorescein-modified thymine as a damage site for NER. Furthermore, the oligos were designed to have in 3' and 5', respectively, a fluorophore (Cy5, Ex: 633, Em: 647) and a quencher (Iowa black hole quencher), as shown in the schematic below (Figure 15). The modified thymine with fluorescein was placed 14 bases away from the 3' end (linked to Cy5). This would have ensured the production of a 10-base long oligo upon successful incision. The experiment was carried out in a 384-microplate (black with clear bottoms) incubated at 37°C. Without an incision, the fluorescence produced by Cy5 would be quenched with minimal readings in the emission spectrum. Upon successful completion of the NER pathway, the experiment temperature (37°C) would surpass the melting temperature of the incise oligo (~16°C), producing a separation of the two strands and therefore dequenching with an increase in fluorescence. To ensure the functionality of the designed oligos, a HindIII cutting site was placed at the 11th phosphodiester bond from the 3' end to generate an 11-base long oligo with a melting temperature of ~18°C (Figure 16B).

Prior to the experiment, the oligo containing the modification was resuspended in Tris buffer (10 mM Tris-Cl, pH 8.5) and then annealed at 95°C for 5 minutes in the dark. To ensure optimal annealing, the solution was slowly cooled to ambient temperature and then stored at 4°C repaired from light. The reaction mix (5 mM DTT, 200 nM UvrA, 400 nM UvrB, 200 nM UvrC, and 1 mM ATP in ABC buffer) was prepared on ice. After gentle mixing, the 2 volumes of the master mix were divided into 1.5 ml tubes, and 20 µM of the drugs (or the equivalent volume of DMSO as a control) were added to each reaction. To initiate the experiment, 100 nM of the annealed oligos were added to each tube as the last step. Finally, 50 µl were split in two wells in the microplate (25 µl each), and the plate was lidded and incubated at 37°C. The reactions were excited, and readings were taken from the

bottom after 0.5, 1.5, 2.5 and 16 h (Ex 633, Em 647). To control for the experiment's success, 2 wells were reserved for DNA alone to establish the level of base fluorescence, 2 wells contained untreated NER incubated with DMSO to control for successful incision, and 2 wells contained 10 U HindIII to assess for complete separation. The plate's first and last 2 wells, left and right, were filled with water to mitigate the vaporization at the extremes. The experiments for each compound were repeated at least twice with 2 technical replicates each. The error was reported as the standard error of the mean. The fluorescence data were visualized in a bar chart to calculate the percentage of inhibition. The basal fluorescence emitted by the untreated DNA was subtracted before normalizing the data to the untreated UvrABC kept as a baseline. A schematic representation of the assay is reported in Figure 15.

The sequences of the oligos were purchased from IDT (Integrated DNA Technologies, IDT, UK):

5` - /IowaBHQ/GT AAC TAA GCT TGA CGA TGG AGC CGT AAC AGT ACG TAG TCT G - 3`

5` - CAG ACT ACG TAC TGT TAC GGC TCC ATC /FluoresceinT/TC AAG CTT AGT TAC /Cy5/ - 3`

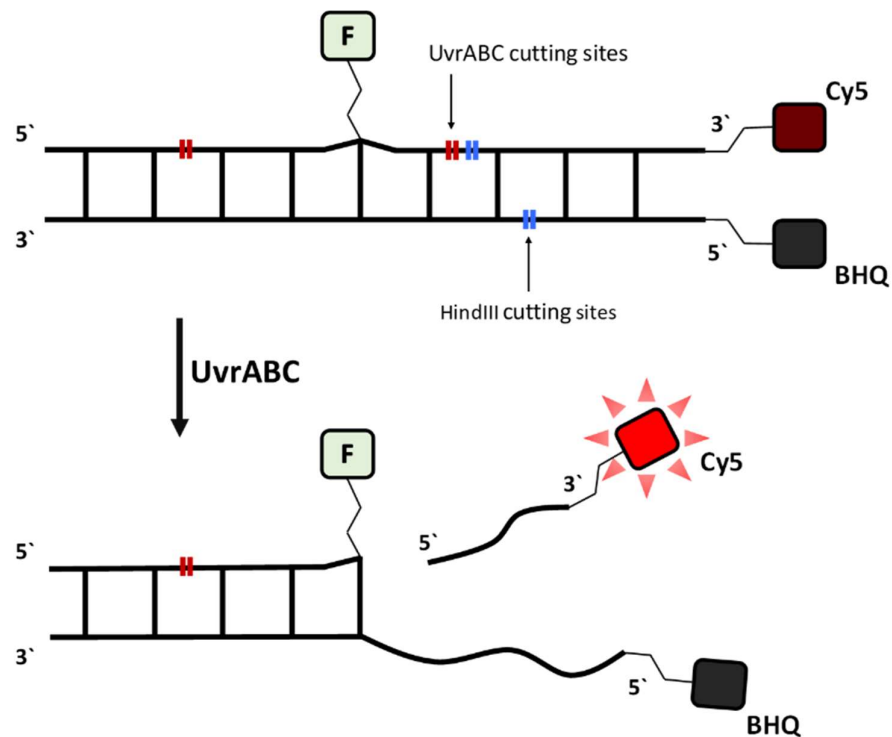


Figure 15: Schematic representation of the fluorescence-based incision. Simplified representation of the fluorescence-based incision assay. On the top, the annealed oligos show that the fluorophore (Cy5) can emit only minimal fluorescence due to the quenching effect of the Iowa black hole quencher (BHQ). On the bottom, the incision made by UvrABC at 37°C is shown to produce the release of the fluorophore (Cy5), causing dequenching and an increase in fluorescence.

HindIII Incision control in ABC

To assess if HindIII would have retained its activity in ABC buffer, necessary for the fluorescence-based incision assay, we performed a gel-base incision looking at 3-time points. 14 nM pUC18 containing 1 HindIII cutting site were incubated at 37°C in ABC buffer supplemented with 1 mM ATP, 1 mM DTT, and 10 units of HindIII. 5 µl were taken after 10, 20 and 30 minutes of incubation to be run on an agarose gel. The reactions were stopped by adding 1 µl loading dye and 1 µl EDTA 0.5 M and quickly placed at 4°C. The samples were then run in a 0.8 % agarose gel stained with ethidium bromide. As seen in Figure 16A a successful incision would result in an upwards shift of the band due to the change from supercoiled to linear. Figure 16B shows the HindIII activity in the plate assay compared with untreated quenched oligos (DNA) and free Cy5 to assess for maximum fluorescence.

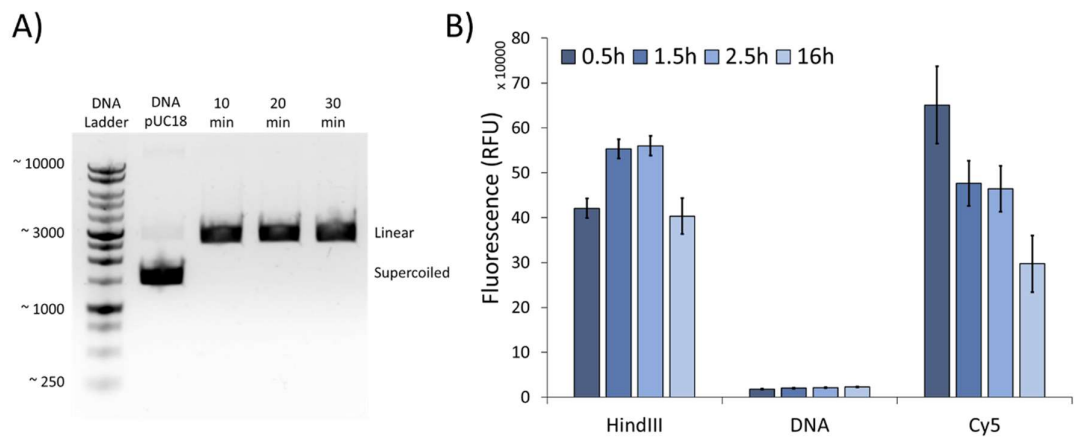


Figure 16: Incision control in ABC buffer. A) 0.8 % agarose gel showing HindIII activity in ABC buffer. pUC18 has 1 HindIII cutting site; therefore, upon incision, the DNA will change conformation from supercoiled to linear resulting in an upwards shift of the band. The gel shows a complete reaction after the first 10 minutes of incubation. **B)** Fluorescence incision control. The graph shows the maximum increase in fluorescence when the strands disassociate entirely due to the HindIII incision compared to the maximum fluorescence of Cy5 alone. The decrease in fluorescence allows an estimation of the photobleaching. The error bars represent the standard error of the mean (n≥5).

2.11 UV-damage repair assay

To make the cell competent, one colony from a fresh selective plate was inoculated in 5 ml LB containing the appropriate selection if required. After 16 h of incubation at 37°C in a shaking incubator, the culture was diluted 1 in 1000 into fresh selective LB media. The cells were grown as before to an OD₆₀₀ between 0.4 and 0.6. The cells were then pelleted at 4000 RPM (ALC PK 121R Centrifuge) for 15 minutes at 4°C and then gently resuspended in 100 mM CaCl₂. Once properly resuspended, the culture was pelleted again in the same conditions. The pellet was resuspended in 100 mM CaCl₂ and 15% v/v glycerol, then aliquoted and flash-frozen using liquid nitrogen.

Before the transformation, a pJB plasmid carrying a Cm^R (or a Amp^R) cassette was irradiated with 200 J/m² UV at 254 nm using a UV cross-linker (UVP/Analytik Jena UV Crosslinker CX-2000) to impede its expression. 100 ng of damaged plasmid DNA were added to 50 µl of competent cells. Immediately after adding DNA, the transformant cells were incubated on ice for 30 minutes, followed by rapid heat shock at 42°C (30 seconds) and re-incubation on ice (5 minutes) to allow the incorporation of the plasmid. After the incubation, 350 µl of LB containing 2xMIC of the appropriate compound (to account for the higher concentration of cells and the use of rich media) or an equivalent amount of DMSO was added to the bacteria and let them recover while shaking for 1 h. After the recovery, the bacteria were pelleted at 4000 RPM (5 minutes at room temperature) and resuspended in 350 µl fresh unselective LB before being quickly plated onto selective plates. The plates were then incubated at 37°C overnight before inspecting the plates. The experiment was repeated 3 times. A simplified representation of the assay is reported in Figure 17.

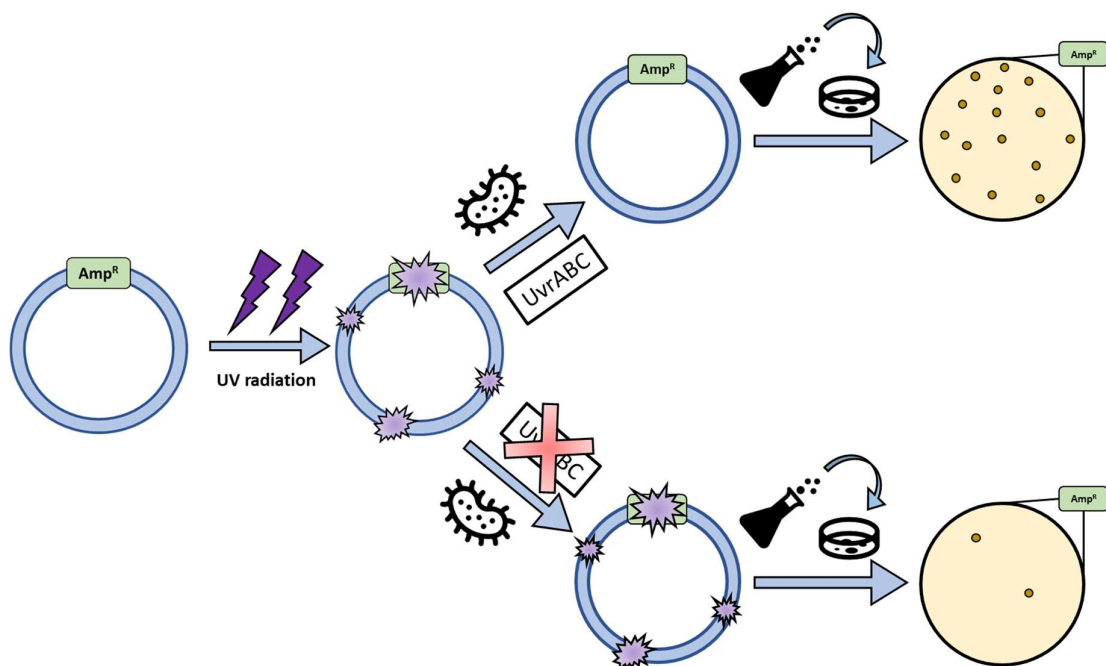


Figure 17: Schematic representation of the UV-damage repair assay. A simplified representation of the growth repair assay. A plasmid carrying an antibiotic resistance gets damaged with UV radiation (254 nm). The plasmid gets transformed into a cell in the presence and absence of the UvrABC inhibitor. The cells with functional UvrABC will then repair the damage allowing the cell to express antibiotic resistance and survive in the appropriate selective plate. The cell with an impaired NER will not be able to fix the plasmid and acquire resistance to the antibiotic, and therefore they will not survive when plated in a selective media.

UV-damage repair assay control

To assess the possible antibacterial effect of the compound, the cells were prepared at an equivalent OD₆₀₀ and growth state before being incubated for 1h with vigorous shaking in LB with 2xMIC of the respective compounds (an equivalent volume of DMSO). At the end of the incubation, the cells were pelleted and resuspended in 350 µl of fresh LB, and the absorbance at OD₆₀₀ was recorded. Furthermore, 5 µl of the cells were plated in unselective LB agar plates for overnight incubation at 37°C. The results showed that a 1 h-long exposure to 2xMIC in LB broth and with a high concentration of cells is not enough to be detrimental to bacterial growth, as shown in Figure 18.

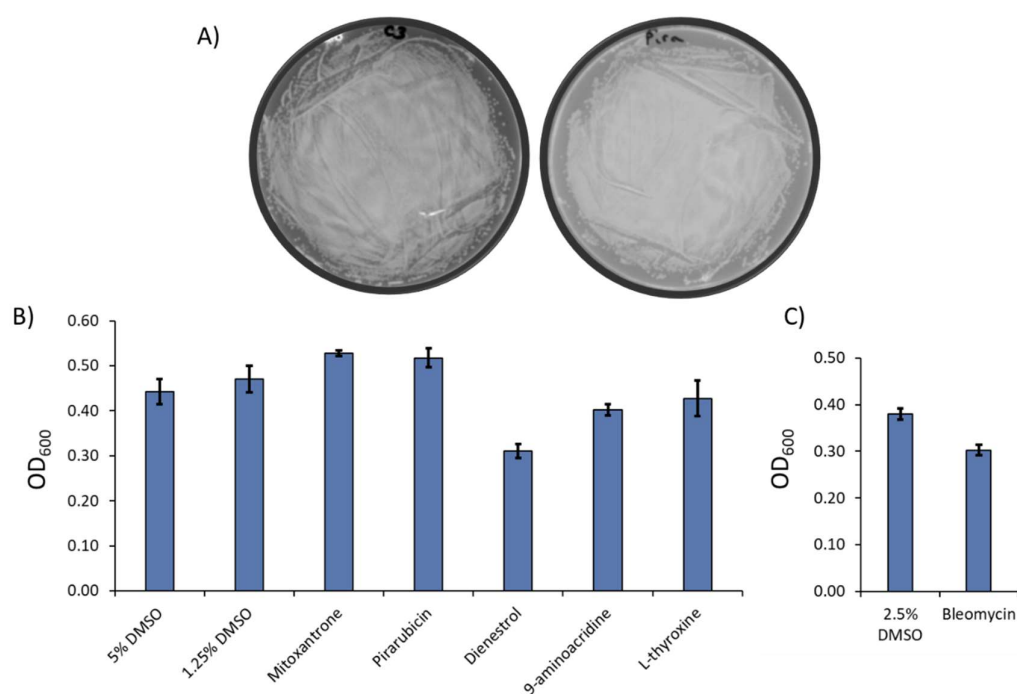


Figure 18: UV-damage repair assay drugs direct effect control. Growth control to estimate the direct effect of the compounds on MG1655 $\Delta tolC$ (A-B) and EC958 (C) incubated in the UV-damage repair conditions (2x MIC for 1h at 37°C in LB while vigorously shaking). **A)** Representative picture showing growth in the absence (left) and presence (right) of 2x MIC of Pirarubicin, only 5 µl of the suspension were plated in LB agar and further incubated overnight at 37°C. Complete coating of the plate can be observed in both plates. The experiment was repeated 3 times. **B)** Absorbance acquired at OD₆₀₀ of the same cultures allows to quantify the effect on the cells. Only marginal reduction can be seen. Dienestrol (~35%) appears as the most active compound in this assay, but it is still unable to produce enough effect to justify the complete absence of growth in the UV-damage repair assay. **C)** The same control was performed for Bleomycin using EC958. An approximate reduction of 20% can be measured. The data was acquired in three independent measurements with 2 technical replicates. The error bars represent the standard deviation.

2.12 In-silico molecular docking

The molecular docking was performed using the open-access and freely available algorithm AutoDock Vina (19, 248). Due to the lack of an *E. coli* 3D structure for UvrA, we decided to use the computationally generated structure from AlphaFold (16, 35), which utilised an AI (AlphaFold developed by DeepMind) to make accurate predictions of 3D conformations from just the amino acid sequences. Polar hydrogens were then made explicit, and the format was converted to .pdbqt using AutoDock Tools 1.5.7 (249). The 3D ligands were downloaded from ZINC or PubChem (respectively .mol2 and .sdf files), two of the largest freely available chemical databases (17, 18, 250). The molecules were then converted in the appropriate format accepted by AutoDock (.pdbqt) using OpenBabel, an open-access chemical toolbox which allows molecular translation in several formats while computing different parameters (e.g. different conformers at lower energy) (25). When energy minimization was relevant, we also recurred to OpenBabel to generate the structure using the MMFF94 force field that provides accuracy for drug-like molecules as suggested in the documentation (251, 252). The coordinates for the search space were calculated using AutoDock Tools 1.5.7 and adjusted depending on the need of the experiment. For every study done with computational tools, the protein was considered a rigid object, and the compounds could only rotate at their torsional point. To evaluate the potential binding site, we maximise the search space across the entirety of the protein to allow the algorithm to explore every possible binding site. When more precise docking was required, a search space was defined as suggested “As small as possible, but not smaller” (aiming for a box of ~25x25x25 Angstrom when possible) (40). The exhaustiveness was maintained at 8, the default setting unless specified. ATP, prepared as described before, was docked alongside the other ligands as a control. The results were then analysed and imaged using PyMOL.

Virtual screening

A collection of drugs reported active *in vitro* and available for sale was downloaded from ZINC as a .mol2 file (250). The library contained 173234 compounds, among which 117760 unique drugs. The ligands were then converted in bulk in .pdbqt using OpenBabel (25). The protein structure was prepared as described before, as well as the search space. For the virtual screening on UvrA, we included both the ATPase cassette and the channel connecting them, as shown in Figure 19. A Python code was written with the help of Dr Jake McGreig, Dr Robert Charman, and Antoine Paris for the bulk preparation of the ligands and the automatic execution of the virtual screening and data sorting (Appendix L – Code for virtual screening). The code was designed to have easy control of the parameters required by AutoDock Vina as well as the level of parallelism. The lowest binding energy conformation for each one of the ligands docked was then automatically printed in a .csv file and sorted for binding energy. The first unique 50 molecules were evaluated singularly, and the main hit was chosen for characterization.

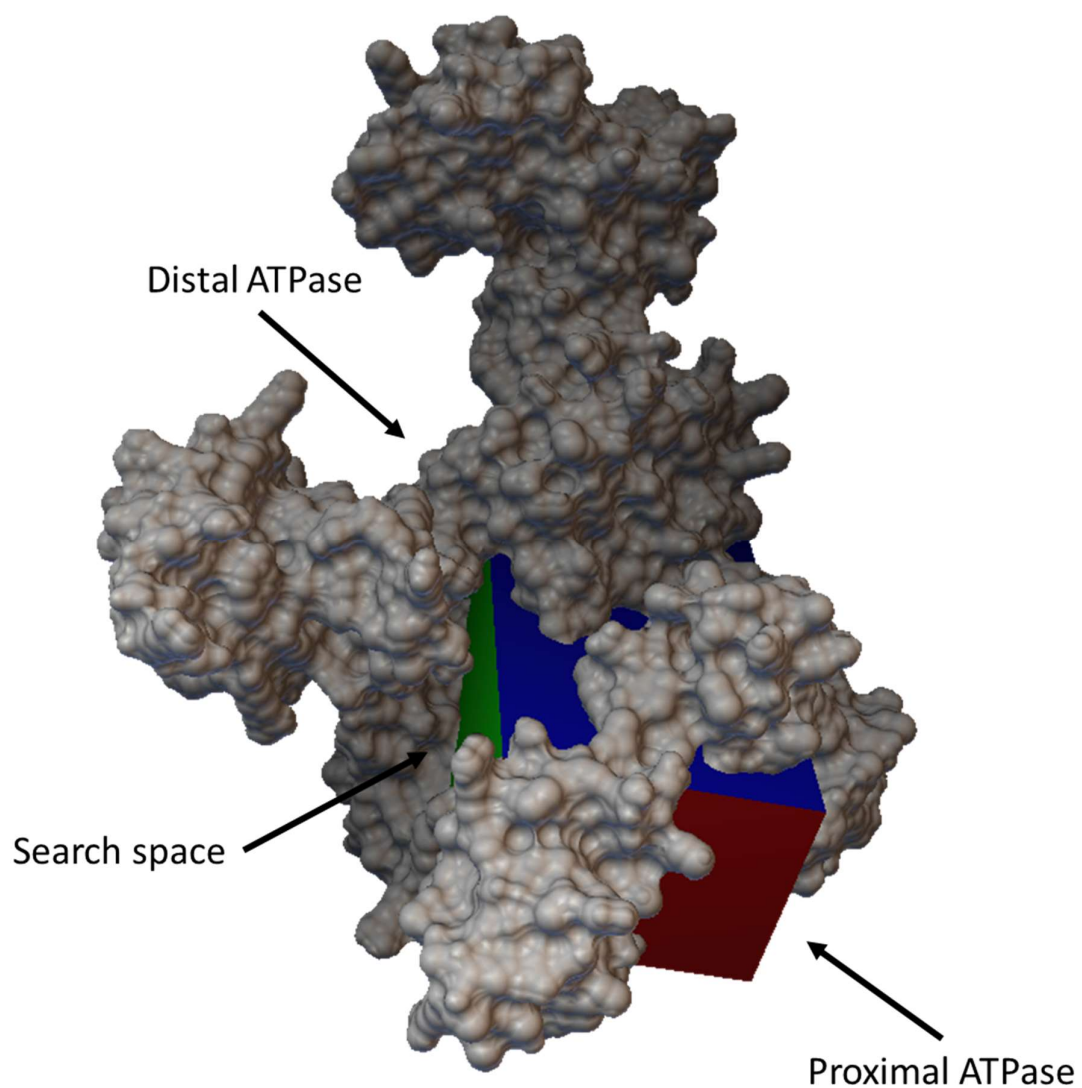


Figure 19: AlphaFold UvrA showing the search space for the virtual screening. Structural surface of *E. coli* UvrA (retrieved from AlphaFold and generated in MGL tools) shows the search space calculated using AutoDock Tools 1.5.7. The box includes the two ATPases and the channel that connects them.

Speed evaluation depending on the parallelism level

We screened a set of 500 molecules and a set of 10000 molecules while changing the level of parallelization to estimate the fastest conditions. We tested the maximum computational power available for one molecule at a time, and then we increased the parallelization to 1/32th of the available CPUs per job (with 32 jobs submitted simultaneously). The results were then plotted and analysed in Excel.

Chapter 3 – Identification of the target and mode of action for the prokaryotic nucleotide excision repair inhibitor ATBC.

Sections of this chapter are part of published data “Identification of the target and mode of action for the prokaryotic nucleotide excision repair inhibitor ATBC” (253) and include a small section with work done by Antoine Paris (data collection, analysis and methodology on the optical trap) for the visualization of UvrA on suspended DNA. External contributions were left in the chapter for clarity, and they are specified in the figure legend and text.

3.1 Introduction

3.1.1 UvrA

As previously described, nucleotide excision repair is a multistep sequential reaction initiated by UvrA (in its dimeric form UvrA₂), necessitating ATP for its proper function (212, 244, 254, 255).

The structure of UvrA

Eight UvrA crystal structures from 4 different bacterial species (*Geobacillus stearothermophilus*, *Deinococcus radiodurans*, *Thermotoga maritima*, *Mycobacterium tuberculosis*) are now available (PDB: 3UWX, 3PIH, 2R6F, 3ZQJ, 3UX8, 2VF7, 2VF8, 6N9L) (214, 215, 255–258) but unfortunately, none of them is derived from *E. coli*.

UvrA is composed of two halves separated by a linker sensitive to proteases (196). The protein belongs to the ABC superfamily (ATP-binding cassette), including two different ATP-binding domains (proximal or N-terminal (AA 31-36) and distal or C-terminal (AA 640-654)) likely originated through gene duplication (259, 260). Each ATPase contains a nucleotide-binding domain (NBD) with motifs characteristic of the ABC superfamily as the Walker A, Walker B and Q - D - H loops. However, UvrA also possesses specific structural elements not found in other members of the family and with unclear functions, such as the insert connecting the walker A to the ABC motif (256, 261). As mentioned in the introduction on NER, UvrA forms a homodimer in solution (UvrA₂), which is believed to be promoted by ATP (211, 256). This head-to-head dimer (UvrA₂) presents 4 ATPases (2 per monomer) positioned below the DNA-binding site, which extends in a groove running across all the available surfaces, as shown in Figure 20 (214). The two ATPases (distal and proximal) seem to be connected by a hollow region (tunnel) with an unclear structural function which has been hypothesized to allow an exchange of phosphate groups driving the cooperative nature of the two sites (244).

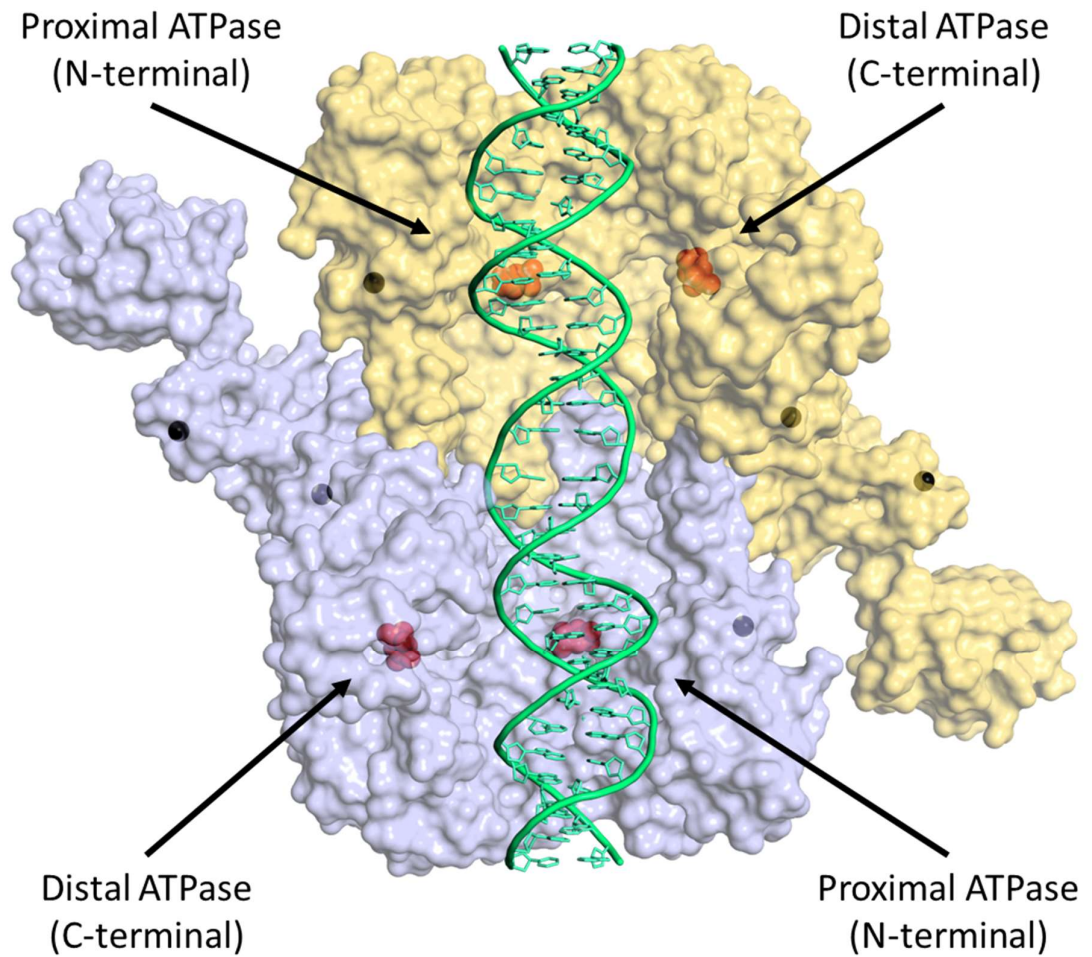


Figure 20: UvrA homodimer (PDB:3PIH) crystal structure. The dimeric structure of the protein (from *T. maritima*, generated in PyMOL) shows the two homodimers (violet and yellow) crystallized while bound to fluorescein-modified DNA (green). The phosphates groups bound to the protein are highlighted as red spheres to indicate the location of the ATPase cassettes (showed through 40% surface transparency). The zinc atoms bound to the proteins are shown as black spheres.

The zinc fingers

In addition to its ATPases, UvrA possesses another structural characteristic involved in its functions, 3 zinc fingers per monomer (214, 256, 262). Their meaning for the protein is not entirely understood, but mutational studies tried to describe their impact on UvrA's activity (263, 264). Visse was able to show by modifying the zinc-coordinating cysteine in serine or glycine (unable to coordinate Zn) that the N-Terminal zinc finger is not necessary for UvrA's activity while losing the C-terminal finger renders the bacteria susceptible to UV irradiation (264). Also, the N-terminal mutant still retained its ATPase functionality and DNA binding. Shortly after, Wang showed that the mutation of the cysteine 643 to a large amino acid impaired DNA binding and bacterial survival if exposed to UV (263). Interestingly a later study showed how the zinc finger domain does not seem directly responsible for DNA binding, but it appears to be involved in regulating damage-specific binding (265).

It was then proposed that the C-terminal finger carries a more structural role in helping the maintenance of a functional conformation, contributing to communicating the damage location to the ATPases for their activation (196, 262). Although the complete mechanism has not yet been understood, more recently, a mode of action has been highlighted (266), clarifying the role of the zinc finger and the effects of UvrA dimerization. The investigators showed that the damaged-dependent DNA binding was impaired by crosslinking the Zn3hp to impede its movement. Furthermore, UvrA had lost its capacity to hydrolyse ATP and load UvrB onto the DNA. Moreover, the results showed by using K37 and K643 mutants (with abolished ATPase activity in the proximal and distal sites, respectively) that only the distal mutation affected the movement of the Zn3hp. Therefore, the authors conclude that during UvrA's search for damage, its distal ATPase is conformationally inhibited by the zinc finger alpha helices (Zn3hp). Upon contacting the lesions, a structural re-arrangement is caused. These modifications propagate into the distal ATP-binding region, allowing for structural rearrangements with subsequent ATP hydrolysis and damage recognition (266).

The ATP-binding pockets

The role of the two ATPases is complex and has been investigated through the years since their purification in the 80s (267). It is now agreed that the two ATPases per monomer work in a cooperative manner and their activity highly affects DNA binding (212, 244, 268), however their unique functions are not yet fully understood. Most of the early knowledge on the ATPase comes from mutational studies that identified two residues (in the ATP binding pockets) to be essential, K37 (proximal or N-terminal site) and K646 (distal or C-terminal site) (91). Mutating the lysine to an alanine (K37A and K646A) seems to disrupt the ATPase activity, interrupting the cooperation between the two sites and causing a deficient DNA repair *in vitro* with a consequential increase in UV sensitivity *in vivo* (91, 213, 254, 269, 270). It is now generally accepted that the distal site is involved in damage discrimination while the proximal site is necessary for the interaction with DNA and UvrB (200, 268).

Recent studies have tried to determine the mechanistic roles of ATP hydrolysis in the two ATP binding pockets and their cooperation.

Specifically, a single-molecule study on UvrA showed how UvrA could bind to DNA in its ATP-bound state. Also, they noticed that the dimer could hydrolyse 2 molecules of ATP when in the presence of DNA, regardless of damage. In contrast, they measured the attached lifetime the affinity for ATP increased significantly. To reconcile these facts, the authors proposed a cooperative system in which only if the damage is detected, the hydrolysis of the first ATP molecule can activate the second ATPase through an exchange of a phosphate group and stimulate the consumption of an additional ATP in the opposite ATP-binding site (244).

Moreover, in 2019 a complex study monitored the phosphate release from the ATPases in UvrA mutants, trying to define the nature of the communication between the two sites (258). Briefly, the authors showed how the distal sites (higher ATP affinity) are responsible for fast hydrolysis and are affected by nucleotide binding in the proximal sites (lower affinity) but not vice versa. They also showed the mechanistic difference due to the presence of DNA, proposing that when the homodimer UvrA₂ is alone, both the nucleotide-binding pockets are filled, but only the distal site can turn over ATP. In contrast, when DNA

is in solution, both pockets come into play in a system where the proximal site permits ATP hydrolysis in the distal site and simultaneously, the distal site accelerates hydrolysis in the proximal. Ultimately, they conclude that if the damage is detected, the hydrolysis in the proximal site gets delayed maintaining the protein in a state with a higher affinity for UvrB.

The most recent investigation (220) examined the interaction between UvrA, UvrB and DNA with a sophisticated Forster energy transfer (FRET) system that allowed them to measure the different steps in DNA binding and UvrB loading. In summary, their findings showed that mutants lacking proximal ATPase activity (K37A) had an impaired ability to load UvrB onto DNA, forming the pre-incision complex. At the same time, the distal mutant (K643A) could still form a stable DNA complex but at a reduced rate. It has to be noted, though, that the distal mutant can still retain some of its ATPase activity (258). These studies also agree with previously proposed damage recognition and UvrB recruitment roles (213, 266).

DNA binding

UvrA has been crystallized both bound to DNA and in complex with UvrB (PDB id: 3PIH and 3UWX) (214, 215). However, the precise mechanism of DNA binding and damage recognition is still unclear and under investigation. It is known that UvrA has an increased (~4x) affinity for damaged DNA with a reported change in K_d from 45 nM in the absence of damage to 12 nM when damaged is present (258). Also, it is well established that UvrA possesses a non-specific binding site for DNA that allows for recognising a wide variety of structural damages, as reported in an extensive review from 2006 (196). Structural analysis of UvrA from *Bacillus stearothermophilus* found 3 highly conserved and positive charged regions forming a groove in the protein's ventral surface, able to accommodate ~30 DNA bp DNA (256). Timmins later confirmed these findings in its work with UvrA from *Deinococcus radiodurans* (255). As immediately clear from the crystal structure represented in Figure 20, published in 2011, the homodimer (UvrA₂) DNA binding region runs across a concavity immediately above the ATP binding pockets able to accommodate 30-35 bp on the opposite side of a flat dorsal surface (214). The study also shows how UvrA doesn't bind directly to the lesion but interacts only with the DNA backbone, binding on both sides of the damage, detecting distortions of the double helix. A later study done by crosslinking the protein to block rearrangements identified a β -hairpin within the 3rd Zinc-

binding finger (Zn3hp) to be involved in damaged discrimination and UvrB binding due to a conformational change promoted by ATP hydrolysis in the distal site (266). They observed that when the protein is crosslinked to prevent Zn3hp from moving, UvrA could still bind undamaged DNA, but the protein had a reduced affinity for damaged DNA and a greatly diminished ATPase rate.

Ultimately, a recent investigation on *E. coli* UvrA using iSCAT spectroscopy revealed two different binding modes used to search for damage for the first time. A previously known slow phase (~1.3 s) coupled with ATP consumption and a much faster binding mode (~130 ms) that doesn't require any nucleotides. The authors argue that UvrA *in vivo* binds to DNA without spending an ATP molecule and rapidly disassociates if the damage is not detected to quickly bind to a closer DNA strand until a lesion is found. This fast-searching mode would allow UvrA to probe the entire bacterial genome every replication cycle in search for damage (212). Critically, the authors noted that using ATP analogues (such as ADP and the non-hydrolysable ATPγS) dramatically reduces UvrA's ability to detect damage. According to the authors, UvrA performs a fast and high-efficiency search of the DNA until a lesion is sensed by the 3rd Zinc finger, locking UvrA on the site by ATP hydrolysis, allowing for UvrB recruitment and the progression of the pathway.

Discrepancy on UvrA

The information on UvrA reported in this chapter is based on structural work done in different bacterial species since no crystallographic information is available for *E. coli* UvrA, probably due to the protein's poor stability. These stability issues were also encountered in the making of this thesis. The differences in bacterial species (especially their growth temperatures), the protein's poor intrinsic stability in *E. coli* and differences in experimental approaches led to some discrepancies in the available data regarding UvrA despite the numerous efforts to clarify the mechanistic action of the protein. A relevant example for this work is the effect of DNA on the rate of ATP hydrolysis. It has been shown that adding DNA to UvrA doesn't stimulate the catalytic activity of the protein and can even cause a drop in the ATP turnover (221, 254, 270). However, the opposite effect has also been shown by other groups, including ours (244, 258, 271). In conclusion, additional work has to be done to clarify the distinct roles of the ATPases and how UvrA uses the cooperation

between the two sites to drive the damage verification, UvrB recruitment and disassociation from DNA.

3.1.2 NER as a target for antimicrobial therapy

NER represent an underexplored antimicrobial target with minimal efforts done over the years to evaluate its role in therapy. The excision repair pathway is well conserved among all kingdoms of life, but the mechanism and proteins responsible for the systems are unique (232). This diversity makes for an interesting target for a chemotherapeutic agent where the aim lies in maximising the concentration to quickly eliminate the infectant without affecting the host. Moreover, as already described in the introduction, NER is involved in the repair of a wide variety of DNA damages, including drugs such as cisplatin, physical agents such as UV radiation, and it has even been implicated in repairing the DNA against ROS, heavily produced during the immune host defence (196, 272–275). These facts combined could make prokaryotic NER a broad and selective target for antimicrobial applications in combinatorial therapy.

A review published in 2011 (276) proposed the investigation of bacterial DNA repair systems in antibiotic drug discovery, but to our knowledge, only one study was conducted on prokaryotic NER (275). While looking for a molecule that could help understand NER better, the group performed an extensive screening on 38000 drug-like molecules, searching for a NER inhibitor (275). For their screening, they used *Mycobacterium smegmatis* Δ uvrB complemented with *Mycobacterium tuberculosis* uvrB used as a faster-growing and non-pathogenic *M. tuberculosis* NER representative. Their study discovered the molecule 2-(5-amino-1,3,4-thiadiazol-2-yl)-3H-benzo[f]chromen-3-one (ATBC, PubChem CID = 399112) (structure shown in Figure 22 A) as a small, non-intercalating mycobacterial NER inhibitor. The group could not show evidence of its molecular target despite advancing the hypothesis of possible action against UvrA.

3.1.3 Summary

In this study, we set out to demonstrate the direct effect of ATBC against *E. coli* UvrA and how this impairs the protein's normal function and ability to bind effectively to DNA. We have shown how the effects previously demonstrated on *mycobacteria* (275) are maintained in *E. coli* NER. We showed *in silico* molecular docking of the molecule bound to the ATPase sites and validated the result with a real-time NADH-linked ATPase assay. Furthermore, we showed how this effect results in defective binding by observing UvrA on suspended optical beads translating in a reduction of NER incision activity with a gel-based incision assay. In doing so, we validate UvrA and, specifically, its ATPase pockets as a novel molecular target for an adjuvant antimicrobial acting alongside a DNA-damaging agent.

3.2 Results

3.2.1 UvrA is necessary for efficient nucleotide excision repair

UvrA is the system's first protein to enter in contact with the lesion; therefore, it represents the mediator that allows for damage verification and subsequent incision. Its importance in repairing various DNA damages has been long known, and complementation studies were already done (91). We verified the ability of our recombinant UvrA to complement our MG1665 $\Delta tolC \Delta uvrA$ strain effectively after exposure to UV radiation (254 nm). TolC is an outer membrane efflux protein part of the AcrAB-TolC complex (277). Specifically the protein is a periplasmic tunnel inserted in the outer membrane forming a channel open to the outside but closed at its periplasmic entrance (278). Its function has been extensively investigated in literature due to its role in the efflux of toxic compounds, including numerous antimicrobials (277–280). Specifically, the AcrAB-TolC pump has been shown to be involved in the mechanisms of resistance to different drugs (281–284). Therefore, to ensure that ATBC's effect was not masked by its efflux from the bacteria, we have prepared and tested an *E. coli* MG1655 $\Delta tolC$ strain (as described in the material and methods).

As shown in Figure 21 the control strain with a complete NER ($\Delta tolC$) is able to survive irradiation up to 80 J/m² UVC, while the knockout strain lacking UvrA ($\Delta tolC \Delta uvrA$) is unable to recover even when irradiated with only 5 J/m² UVC. The complemented strain (MG1665 $\Delta tolC \Delta uvrA + uvrA$) recovered its survivability, showing optimal growth till 40 J/m². Minimal growth at 80 J/m² can be observed by the small deviation in colour. Importantly, 2-fold deviations are not uncommon when measuring MICs. Furthermore, it is worth mentioning that the protein expression was not induced by the addition of IPTG in the complemented bacteria therefore, UvrA's production would not be upregulated by the SOS response as a consequence of DNA damage (285) possibly explaining the slight deviation. For this reason, we didn't set out to quantify the protein expression levels (e.g., through RT-qPCR or western blot analysis).

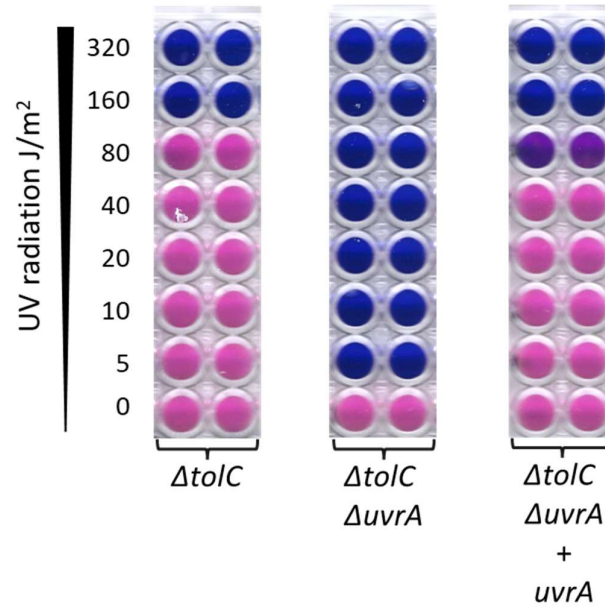


Figure 21: Survivability assay for UV radiation and UvrA complementation. Minimal inhibitory concentration for UV radiation in MG1655 $\Delta tolC$ incubated for 20 h (16 h + 4 h with resazurin) in the dark while shaking. An *E. coli* strain with an intact NER can overcome up to 80 J/m² of UVC radiation. On the contrary, a $\Delta uvrA$ strain cannot survive irradiation of 5 J/m². Ultimately, when the $\Delta uvrA$ strain is complemented back with plasmidic UvrA the bacteria recover its survivability up to 40-80 J/m². Representative plate with 3 technical replicates of 3 biological replicates.

3.2.2 ATBC solubility in water

ATBC's (Figure 22 A) first report as a NER inhibitor underlined solubility problems (275). We set out to quantify the solubility issue to account for eventual technical limitations. We found out that ATBC shows absorbance in the visible spectrum with a predominant peak at 412 nm (Figure 22 B). Therefore, we dissolved ATBC in DMSO and water at different concentrations to measure its eventual precipitation. As expected, ATBC shows optimal solubility in DMSO with concentrations directly proportional to the mass added. However, when in water, the trendline rapidly decays, showing a significant reduction in solubility (Figure 22 C). Furthermore, we noticed that while mixing the solution at higher concentrations, some precipitate could be immediately visible, even attached to the plastic tip of the pipette. This poor behaviour in an aqueous solution and its tendency to spontaneously form aggregates made it challenging to estimate the actual concentration for our experiments.

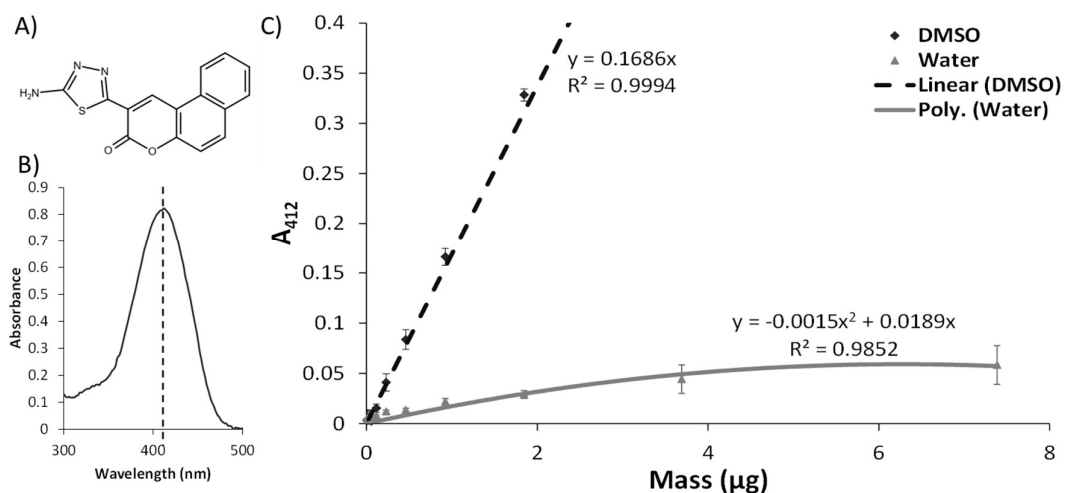


Figure 22: ATBC solubility in water and DMSO. **A)** 2D Molecular structure for ATBC (PubChem CID = 399112). **B)** Spectrum of absorbance for 100 μM ATBC in DMSO. ATBC shows a peak of absorbance at 412 nm. **C)** Solubility comparison between ATBC dissolved in water and DMSO. The difference between the linear part of the two trendlines shows a difference in solubility of approximately 10 times. The experiment was repeated 3 times, and the error bars represent the standard error of the mean.

3.2.3 ATBC does not impair survivability on its own.

To ensure that the effects of the drug in *E. coli* were not masked by the bacteria's capacity to remove it from the cytoplasm, we prepared and utilized an MG1655 $\Delta tolC$ strain to be used alongside the wild type (MG1655). To further confirm the success of the bacteriophage transduction in the deletion of TolC (in addition to the colony PCR results shown in Figure 8) we tested the mutants for their ability to survive ethidium bromide. We selected this compound among those previously reported to have an increased antimicrobial effect against *E. coli* $\Delta tolC$ (280). Figure 23 A shows how the knockout strain has a dramatically increased susceptibility to Ethidium Bromide with a ~64-fold difference (in both $\Delta tolC$ and $\Delta tolC \Delta uvrA$) phenotypically confirming the lack of a functional TolC. Having re-confirmed the presence of the mutation and due to the ethidium bromide inhibitory effects on NER reported in literature (196) we didn't utilize this compound for further investigations.

4-Nitroquinoline N-oxide (4-NQO) is a DNA-damaging agent reported to be a NER activator by forming adducts on DNA mimicking UV light (193, 286, 287). 4-NQO exhibited a MIC of 30 and 3.75 μ M, respectively in MG1655 $\Delta tolC$ and MG1655 $\Delta tolC \Delta uvrA$, showing a decreased survival if UvrA was knocked out (Figure 23 B). This expected result confirmed that 4-NQO's damages need to be repaired by NER, which is impaired by the lack of functioning UvrA. Ultimately, we tested ATBC's antimicrobials effect finding it to be non-toxic, with both strains able to grow in the presence of up to 125 μ M ATBC (Figure 23C). However, we noticed precipitations (as yellow spots) in the wells containing a higher drug concentration after the incubation period. ATBC's poor solubility in water makes the estimation of actual concentrations problematic.

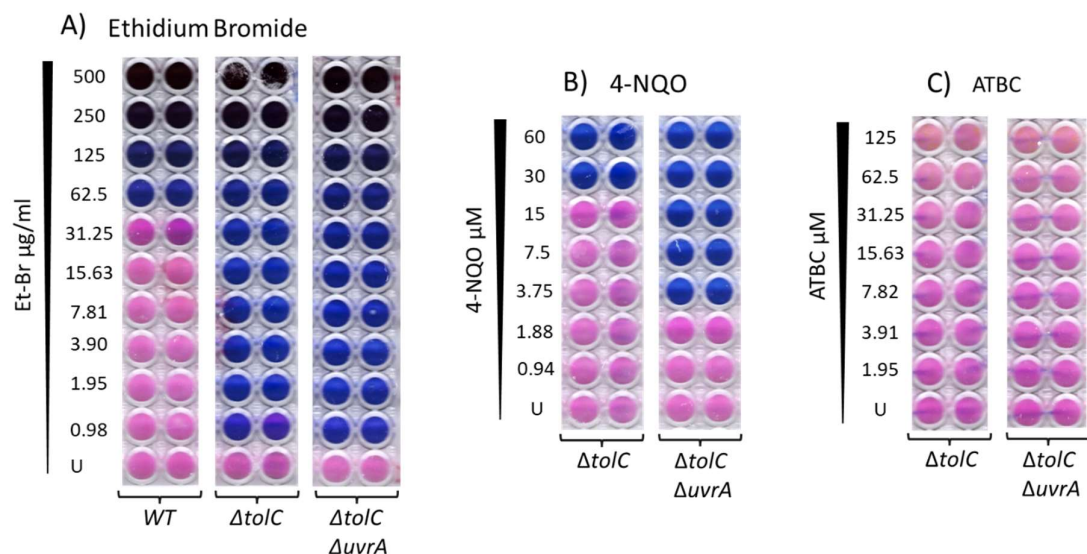


Figure 23: Colorimetric minimal inhibitory concentration. After 16 h of incubation at 37°C in the dark, while shaking, 50 μl of sterile BRS were added to the plate before further incubation at 37°C. **A)** Shows the phenotypical difference of the efflux pump mutants (ΔtolC and $\Delta\text{tolC } \Delta\text{uvrA}$) against the wild-type strain (WT). A ~64-fold shift in the MIC can be observed. **B)** 4-NQO displays a MIC of 30 μM in the ΔtolC strain and 3.75 μM in the double mutant $\Delta\text{tolC } \Delta\text{uvrA}$, confirming its nature as a NER activator. **C)** ATBC has no inhibitory concentration of up to 125 μM in either of the strains tested ($\Delta\text{tolC } \Delta\text{uvrA}$). The pictures are representative of three different independent experiments.

3.2.4 ATBC is effective against *E. coli* nucleotide excision repair

To test for a compound's ability to inhibit NER, DNA must be damaged first. We used two different DNA damaging agents (UV radiation and 4-NQO) known to cause damage repaired by NER in our assays (as shown in Figure 21 and Figure 23).

As shown in Figure 24 A&B irradiating the bacteria with a sub-MIC intensity of UV radiation (75 J/m^2) produces a $\sim 3\text{h}$ shift in mid-exponential (compare green to black lines in Figure 24 A&B). This shift is expected since the DNA cannot be replicated until the damages are completely repaired. On the other hand, when the bacteria are exposed to 5 or 10 μM ATBC, the growth profile does not deviate from the respective untreated sample (compare black to blue lines in Figure 24, A for MG1655, B&C&D for MG1655 ΔtolC). The results agree with the non-toxic nature of ATBC shown in Figure 23. However, when the bacteria are treated with UV radiation (75 J/m^2) alongside 5 μM ATBC, an additional $\sim 5\text{h}$ delay in growth appears in the ΔtolC strain (compare green to red lines in Figure 24 B). This additional delay is absent in the wild-type strain (compare green lines to red lines in Figure 24). This effect implies the multidrug efflux pump's ability to remove the intracellular ATBC, preventing it from exerting its effect in the wild-type strain. Having concluded that the drug is a TolC substrate, the subsequent experiments were conducted only in MG1655 ΔtolC .

When switching from a physical and discrete DNA damaging agent (UV irradiation) to the chemical 4-NQO, we could measure only a minor delay in growth when using a sub-lethal quantity of the drug (10 μM) (compare green to black lines in Figure 24 C&D). However, when combining 4-NQO with only 5 μM ATBC, a significant impairment of survivability appears (compare the red to the green line in Figure 24 C). If ATBC concentration is then further raised to 10 μM , the inhibition becomes a complete lack of growth (compare the red to green lines in Figure 24 D).

Considering that ATBC is non-toxic on its own (up to 125 μM Figure 23 C), these results suggest a synergistic action between ATBC and 4-NQO since the combined effect measured appears significantly greater than the action of the agents incubated alone. On the other hand, ATBC and UVC displayed a more additive effect with the NER inhibitor only able to double the effects caused by UV irradiation. The fractional inhibitory concentration (FIC) index or the isobole method could be used to distinguish between these possible effects

(288) after having measured the combined MIC while titrating the concentrations of the compounds. However, these additional investigations were not done due to the ATBC's poor solubility and the lack of direct applicability in clinical settings (as discussed later).

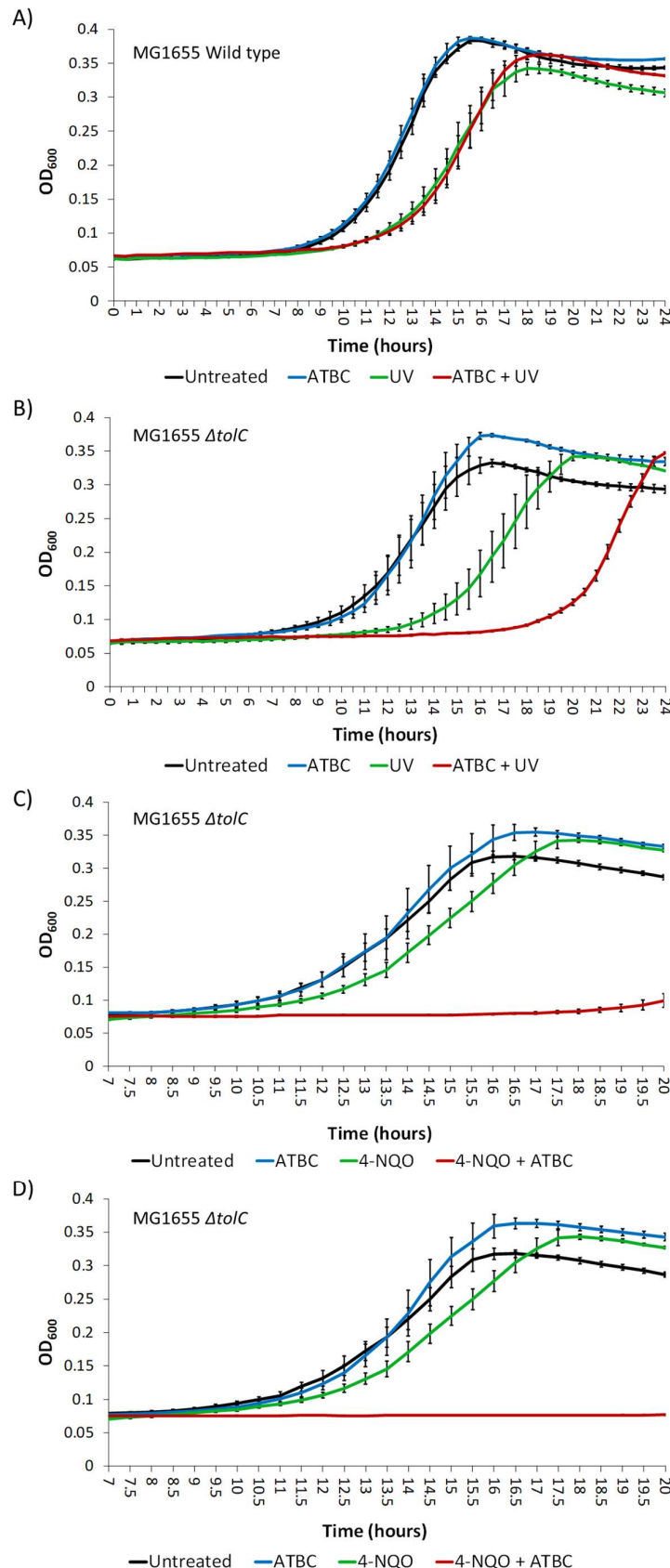


Figure 24: ATBC effects on growth when combined with DNA-damaging agents.

Evaluation of growth using a plate reader. **A)** Wilde-type strain (MG1655) growth in the presence and absence of 5 μ M ATBC (blue) and ± 75 J/m² of 254 nm UV radiation (green).

We can observe a delay in growth when the cells were treated with UV, but we cannot observe any effect caused by the presence of ATBC even when combined with UV radiation (red). **B)** The same shift in mid-exponential seen in figure A) can be observed for the mutant strain (MG1655 $\Delta tolC$) when the cell was exposed to UV radiation (green). An additional shift in mid-exponential appears only in the presence of 5 μ M ATBC due to their combined effects (red).

C) The $\Delta tolC$ was grown in the presence of 10 μ M 4-NQO (green) instead of UV. Significant growth inhibition can be seen with 5 μ M ATBC (red) and **(D)** complete inhibition with 10 μ M ATBC (red). The readings were acquired in independent measurements on different days, and the error bars represent the standard error of the means (n = 3)

3.2.5 ATBC targets UvrA competing for its ATP binding sites

All surface exploration

After confirming ATBC activity against NER in *E. coli*, we studied UvrA as its possible molecular target. We explored its affinity for the protein with a non-traditional approach. Instead of using the docking algorithm to identify the possible binding modes in a confined binding pocket, we performed a blind dock (39) to let it explore the entire protein surface, looking for possible binding sites. The lack of 3D structural information from crystalized *E. coli* proteins led us to the computationally generated model produced by AlphaFold, an artificial intelligence developed by DeepMind to reliably predict 3D structures from just their primary sequence (16, 35). For the docking algorithm, we choose AutoDock Vina, a fast and reliable open-access software (19). To verify that the increased search space was not too big for the program to locate an endogenous ligand, we docked ATP alongside ATBC. The algorithm successfully located the two ATP binding pockets when docking with the default exhaustiveness (=8). Moreover, ATBC was consistently docked alongside ATP (as shown in Figure 25) with comparable binding energies (ATP = -9.48 kcal/mol, SEM = 0.07, n = 6 and ATBC -9.53 = kcal/mol, SEM = 0.17, n = 6) for the highest scoring modes.

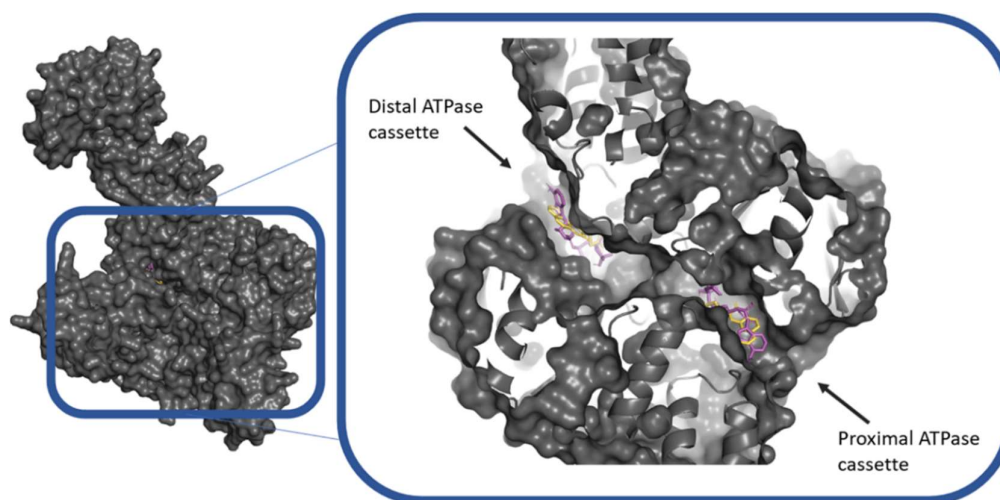


Figure 25: In-silico docking defines a possible binding site for ATP. UvrA 3D structure generated from α -fold is presented with ATP (magenta) and ATBC (yellow) binding to the proximal and distal ATPase cassette. The left image shows the integrity of the monomer of the protein. On the right, an enlargement of the channel and the ATPase binding site is made visible through slab transparency. The docking was repeated 6 times to evaluate the reproducibility.

Real-time NADH-coupled ATPase assay to validate the in-silico results

To verify the results obtained *in silico*, we looked at ATBC's ability to diminish the protein's catalytic activity for its ligand ATP. A real-time NADH-coupled ATPase assay can directly measure the loss rate in ATP through the diminishing absorbance at 340 nm due to the conversion of NADH/H⁺ to NAD⁺ (as shown previously in Figure 13). The ATP consumption is measured with and without adding pUC18 DNA which has been shown to further stimulate UvrA's activity by ~50% (244). Moreover, the assay quickly recycles ADP to ATP, preventing it from accumulating and inhibiting the reaction. The example reported in Figure 26 A visually shows the difference in ATP hydrolysis when in the presence or absence of 50 μ M ATBC (compare the slopes of the two reactions). Untreated UvrA exhibited a k_{cat} of $0.358 \pm 0.027 \text{ s}^{-1}$ in the absence of DNA, and as expected, a ~40% increased turnover to $0.493 \pm 0.015 \text{ s}^{-1}$ after adding pUC18 DNA (Figure 26 B). When the experiment was repeated in the presence of 50 μ M ATBC the k_{cat} was significantly reduced to $0.119 \pm 0.018 \text{ s}^{-1}$ and to $0.151 \pm 0.012 \text{ s}^{-1}$ when in the absence and presence of DNA. These findings indicate statistically significant reductions of UvrA's k_{cat} (~70%) both in the absence and presence of DNA when compared to the untreated control.

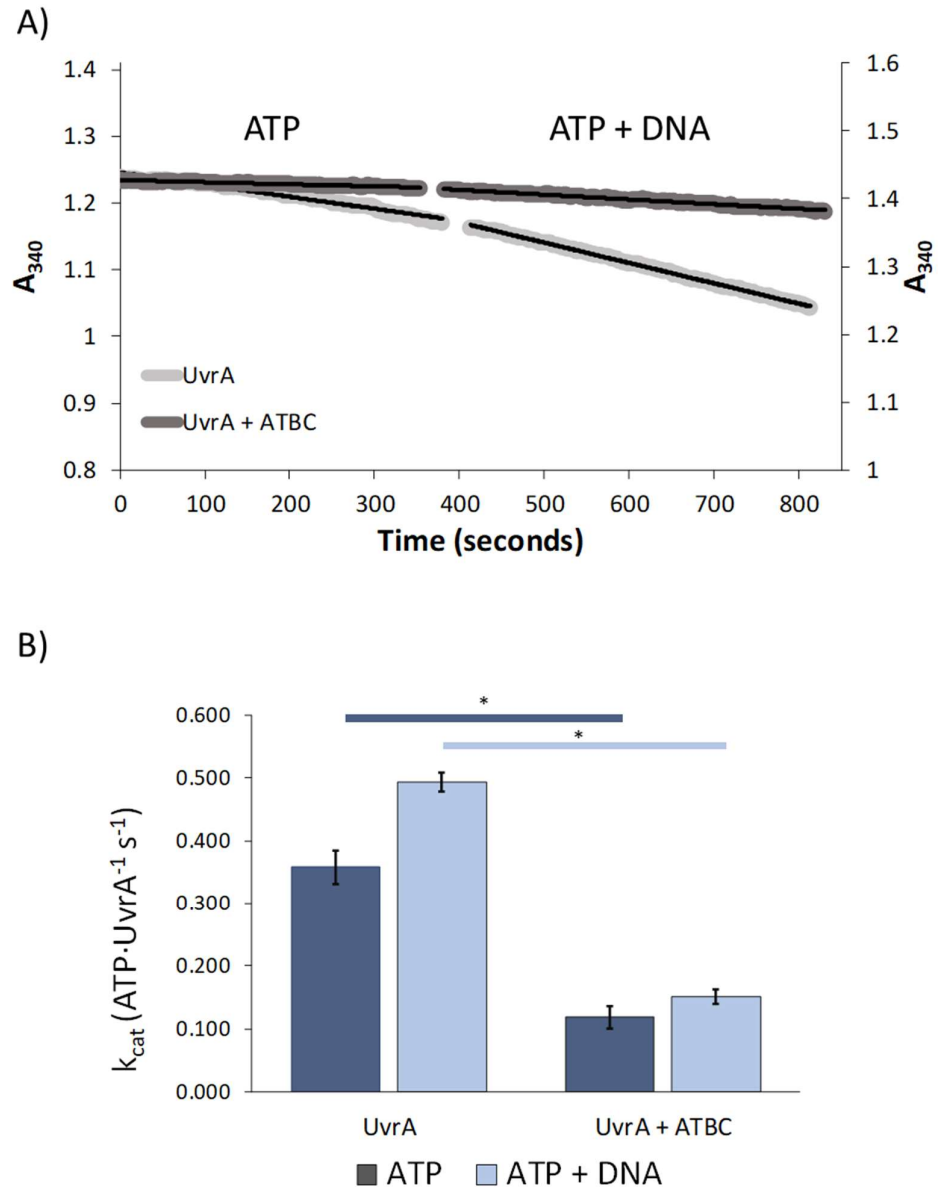


Figure 26: NADH-coupled assay validates the *in-silico* findings. An NADH-coupled ATPase assay with non-limiting ATP (1mM) was used to evaluate the inhibition of the ATPase activity. In this assay, the addition of ATP starts the reaction, which is further stimulated by a subsequential addition of pUC18 DNA (break in the line in A). **A)** Representative ATPase reaction visually shows the level of 50 μ M ATBC inhibition in ATP consumption. The slopes represent the V_{max} of the reaction. **B)** k_{cat} values (calculated from the concentration of UvrA) after adding ATP and DNA in presence and absence of 50 μ M ATBC. The graph shows significant inhibition of UvrA ATP usage up to ~70 %. The experiments were conducted at room temperature in ABC buffer. ADP is converted back to ATP. Hence it does not accumulate in the reaction mix. The error bars represent the standard error of the mean (UvrA $n = 26$, UvrA + ATBC $n = 6$). The results were considered significant if $p \leq 0.05$ (*) (T-test).

Directly visualizing ATBC effect on UvrA DNA-binding ability

This sub-chapter was discussed and planned in collaboration with Antoine Paris, who then defined the methodology, collected and analysed the data.

Our group showed that in the absence of ATP UvrA's ability to bind DNA is significantly affected (212, 244). Therefore, we tested the effects of ATBC on UvrA binding by directly visualising fluorescently labelled protein on suspended DNA. Lambda-phage DNA (48,502 bp) tagged with biotin was trapped with optical tweezers and suspended between 4.8 μm silica beads coated with streptavidin. Since, in this experiment, the DNA is unlabelled when the laser is turned on, only the binders are visible and in focus (as shown in the schematic in Figure 27 A). 5 nM UvrA mNeonGreen was flowed and incubated for 2 minutes in absence and presence of 50 μM ATBC before being imaged (Figure 27 B&C) revealing a dramatic decrease in binders when the drug was present in solution. To be able to quantify the binding and accommodate the unstable nature of ATBC, the workflow was adapted, and the single binders were counted over time. The results showed a ~ 7 -fold reduction in total binders when 50 μM ATBC were in solution.

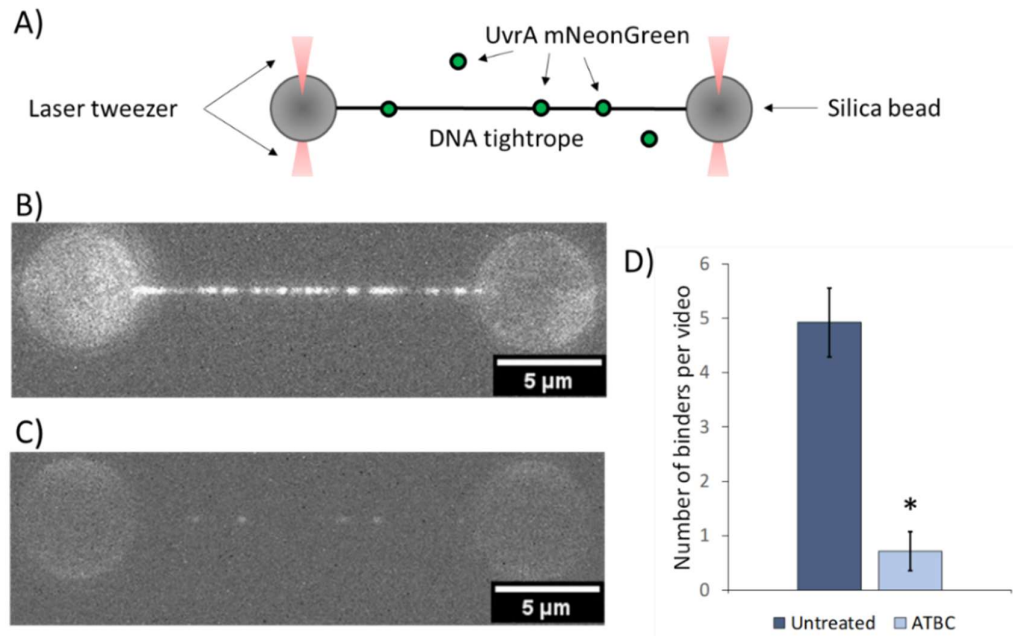


Figure 27: ATBC significantly reduces UvrA DNA binding. **A)** A simplified representation of the optical traps. Antoine Paris conducted and analysed the experiments on suspended DNA. **B)** 5 nM UvrA-mNeonGreen is shown to coat suspended DNA. **C)** A significant binding reduction can be seen in the presence of 50 μM ATBC. **D)** the assay was modified to enable the counting of binders. The graph shows the average number of binders in 200 frames (45.5 s), showing a ~7-fold decrease. The error bars represent the standard error of the mean, $n_{\text{untreated}} = 13$ (three strands). $n_{\text{ATBC}} = 7$ (five strands). The results were considered significant if $P \leq 0.05$ (*) (T-test). The assay was conducted in ABC buffer with 1 mM ATP and DTT.

3.2.6 Evaluating ATBC's ability to inhibit incision

At last, after having demonstrated a direct effect against UvrA, we tested ATBC's ability to interfere with the incision. We performed a gel-based incision assay (193) in which we utilized different DNA migration speeds when in a supercoiled state, unlike the nicked one. The intercalator ethidium bromide (Et-Br) was mixed in the gel matrix to enhance the supercoiling of the DNA (289) and to visualize the results. Supercoiled DNA runs quicker through the gel, but when nicked, it relaxes, passing through the gel matrix slower, resulting in a delayed band. 50 ng pUC18 DNA were used with or without damage caused by UV radiation (200 J/m^2 at 254 nm). T4 PDG V endonuclease can nick the DNA upon encountering CPDs and was used to verify the presence of the damage (247). Figure 28 shows the results of the assay. Only DNA is present in the first two lanes with and without damage. The following two lanes show the T4 endonuclease control with a complete upwards shift of the band with irradiated DNA. The UvrABC lanes show the untreated control, a modest but noticeable increase in concentration in the nicked state can be estimated when the DNA was subjected to UV damage. The ATBC lane shows the same experiment but in the presence of 100 μM ATBC. The nicked DNA band shows the same amount of DNA, indicating an inhibition of the incision. The experiment was repeated twice, and due to the difficulties in quantifying the result and the poor nature of the drug itself, the experiment was not repeated at lower concentrations of ATBC.

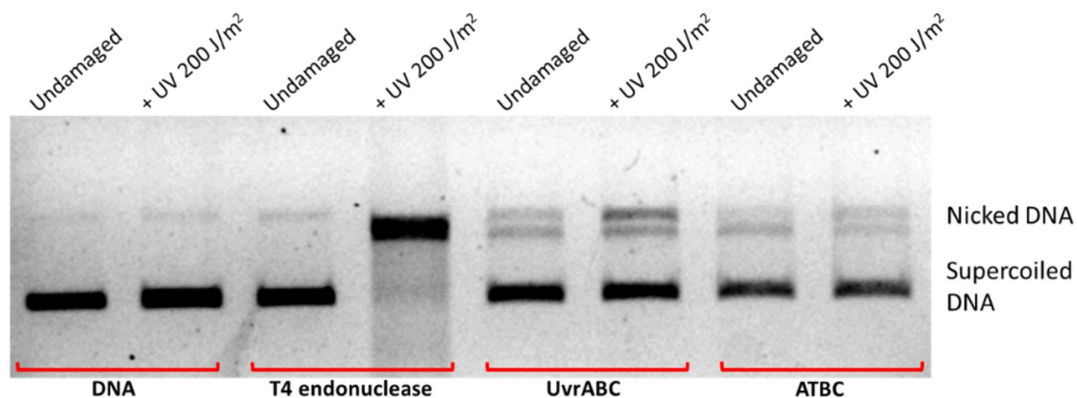


Figure 28: Gel-based incision assay. 0.8% agarose gel stained with Ethidium Bromide. The DNA lanes contain only pUC18 as a control. The T4 endonuclease lanes contain the plasmid and the T4 endonuclease, which can cut the DNA when CPD are present. The UvrABC lane contains the plasmid and the Uvr complex. Ultimately the ATBC lane contains the plasmid, the Uvr complex and 100 μ M ATBC. The DNA control shows that the DNA runs unchanged when exposed to UV, while the T4 control demonstrates the switch to nicked DNA when fully incised. When looking at the UvrABC, we can see a measurable shift to the nicked state, which completely disappears in the presence of 100 μ M ATBC. The gel is representative of 2 independent replicates. Due to the resolution of the gel, the results were only interpreted as qualitative and not quantified. Additional compounds similar to ATBC had already been shown to be inactive in inhibiting incision (275) and they were not re-tested for their anti-NER activity.

3.3 Discussion

3.3.1 ATBC

NER can discriminate a wide variety of damages (196); however, it is not required for bacterial survival if the DNA is intact. Therefore, a drug would not lead to cell death by only inhibiting the system. This fact, coupled with a still unclear mechanism of action of the entire process, rendered NER an overlooked target for a possible antimicrobial therapy. However, a fundamental difference at a molecular level with its human counterpart (232) and its promiscuous nature to identify DNA damages from different sources (196) make NER look like an ideal candidate for further exploration.

In this chapter, we started from the work done by the Nathan group (275) on *mycobacteria* and proposed the mechanism of action of the first drug-like molecule known to inhibit NER both *in vitro* and *in vivo*. Furthermore, we have validated its activity in *E. coli* and characterised UvrA as a new target for novel future inhibitors.

ATBC didn't display toxicity when used on its own, even at the high concentrations of 125 μM in neither strain tested (ΔtolC and $\Delta\text{tolC } \Delta\text{uvrA}$). However, when exposed to a DNA-damaging agent at a sub-MIC concentration, the compound could dramatically impair recovery from low concentrations (5 μM). We repeated the evaluation with two damaging agents, UV irradiation and 4-NQO, both long known to be strong NER activators (193, 287, 290). When the bacteria (MG1655 ΔtolC) were treated with UV radiation (254 nm), a ~5 h delay in growth could be observed, followed by a recovery despite the presence of ATBC. We reasoned that the phenomenon is due to the discrete nature of the physical damage. The 75 J/m^2 were irradiated to bacteria with a fully functional NER and exposed to ATBC only afterwards. Therefore, even a small number of cells able to repair the damage before entering into contact with ATBC would be able to grow due to the non-toxic nature of the compound. We decided not to have ATBC present in the solution with the bacteria to avoid possible chemical deformation due to the exposure to UV irradiation and to minimize eventual precipitation during exposure. For these reasons, we believe the combination ATBC + 4-NQO displayed a more drastic effect. The chemical was administered to the

bacteria alongside ATBC and never left the solution maintaining the damaging pressure during the entirety of the experiment.

This activity profile makes ATBC a compound applicable in adjuvant therapy to allow other chemicals to exert their antimicrobial activity without being rendered inefficient by NER. Having a non-toxic compound on its own but able to enhance the therapeutic power of other drugs is not a novelty, and there are multiple examples of combinatorial therapies in the pharmaceutical arsenal (291–295). As discussed later in this thesis, possible applications could range from topical administration (in gels and creams) followed by direct sunlight exposure for treating bacterial dermatitis to co-administration during cancer chemotherapy.

However, ATBC presents challenges that make the compound not a viable option for systemic usage as it is. Firstly, its chemo-physical property makes the drug challenging to work with, most likely limiting its bioavailability and rendering the compound difficult to administer. However, this problem is not insurmountable. An example of a compound with a poor chemical profile can be found in tioconazole, an antifungal agent that was only usable topically. The medicinal chemists modified the antimicrobial for improved characteristics to allow systemic use and created the better drug fluconazole (296). Ultimately, we found ATBC to be a substrate recognised by the multi-drug efflux pump TolC as shown by the difference in growth comparing MG1655 wild type to MG1655 $\Delta tolC$ (Figure 24 A&B). TolC is a membrane protein responsible for the efflux of a wide variety of compounds, and it has been involved in the arise of antimicrobial resistance (279, 280). Therefore, ATBC application in clinical isolate strains would most likely be diminished.

3.3.2 UvrA as a target

As extensively described before, UvrA is the first protein to contact the lesion and represent the initiator of the NER pathway (268). For its central role, coupled with the suggestion made during ATBC discovery (275), we explored UvrA as the molecular target.

Computational methods are largely used in drug discovery with numerous applications, from structure-activity relationships to virtual screening (297–299). In this study, we used the open-access software AutoDock Vina to perform a less traditional “blind docking” (39). Instead of looking for the best binding mode in a small and defined search space, we allow the software to probe the entirety of the surface available, looking for binding sites with the highest affinity for ATBC. In doing so, the algorithm cannot be expected to find the absolute best bound conformation, however, when ATP was used as a control, we found that the software could place the nucleotide in the right binding pockets correctly. When ATBC was docked in the protein, AutoDock Vina placed ATBC alongside ATP with comparable binding energies (ATP = - 9.48 kcal/mol and ATBC = - 9.53 kcal/mol). Due to the approximating nature of our approach with an unusually large search space in a computationally generated protein structure, we didn't focus on the precise interpretation of the *in silico* data, but we decided to directly test the finding against purified UvrA.

The *in vitro* assay confirmed the computational results showing a ~70 % reduction in the k_{cat} when ATBC was present in the solution. Unsurprisingly the ATPase activity wasn't completely killed, in agreement with the *in silico* finding (comparable binding energy with the endogenous ligand) and the *in vivo* experiments (recovery of damage when UV radiation was used). These results indicate a competitive nature with the endogenous molecule ATP. However, as discussed before, the unstable nature of ATBC prevented us from measuring a K_i and acquiring more specific data due to the unreliable concentrations of the drug in the solution.

Following the evidence indicating that ATP is required by UvrA to achieve proper DNA binding (212, 244), we evaluated ATBC's ability to hinder binding on suspended DNA. We showed how the drug could heavily reduce DNA binding in excellent agreement with the ATPase data. Noticeably, the observed reduction appears similar to the one caused by the endogenous competitor ADP published previously by our group (244).

Taken together, this data provides a strong indication that ATBC directly binds to UvrA and specifically to the ATP-binding pockets. Furthermore, ATBC was shown to be unable to intercalate to the DNA (275) confirmed by the lack of DNA abnormalities observed in the optical experiments. Moreover, the ATPase assay was performed in both presence and absence of pUC18 DNA showing, comparable rates of inhibitions for both (~70% when compared to the untreated sample). Therefore, we reasoned that the activity had to be due to an interaction with the protein. However, the nature of the interaction could be further assessed with more direct evidence. The clearest confirmation would require crystallizing UvrA while bound to ATBC to evaluate the nature of their interaction precisely. Moreover, additional techniques could be used to measure protein-ligand binding directly. An extensive review on hit validation recently published (300) describes several assays to gather direct evidence of a target-ligand complex in solution from NMR spectroscopy to simpler methods such as the thermal shift assay (TSA) which assesses for a change in the melting temperature of the protein when bound to a ligand.

Ultimately, we have shown how the drug's effect gets propagated to the overall repair system through a gel-based incision assay. Unfortunately, the resolution of the gel was only good enough to extract qualitative data but not quantitative. To overcome this problem, we set out to develop an alternative assay (fluorescence-based incision assay) to allow precise reading and scalability to be used during the screening performed in the next chapter. We much later discovered that the three proteins in the system (UvrABC) must be purified simultaneously and used fresh to obtain better resolution in a gel-based incision assay, avoiding loss of quality.

3.4 Future works

3.4.1 ATBC in therapy

ATBC does not represent a viable therapeutic option for systemic use at the moment for the reasons discussed in the chapter above. However, it represents an interesting starting point for a new class of therapeutic agents that could work as adjuvants to NER activators to treat infections by targeting UvrA. An immediate step forward could be retrieving the ATBC analogues that surfaced during the initial screening but were reported inactive (275). These molecules could be tested against UvrA to evaluate the source of the inactivity (lack of binding to the protein or even poorer stability in water). Furthermore, ATBC could be easily modified to increase its solubility in water, allowing for more reliable concentrations during the experiments. These steps, alongside the generation of conformers and their relative activity, would allow the determination of QSAR (quantitative structure-activity relationship), guiding the rational design of a more potent analogue with improved biochemical characteristics.

Alongside the direct experiments with ATBC additional investigations could be done computationally. Our analysis was limited and used to predict a possible binding site. Precise docking with higher exhaustiveness, reduced search space, and flexible residues could provide higher-resolution data on ATBC binding energy and, most importantly, the residues involved in the interactions with the protein. Also, UvrA undergoes structural changes while binding with UvrB and DNA. Docking analysis of these states would produce insight into the most susceptible protein conformation to ATBC. Furthermore, additional studies could be done to evaluate a possible effect of ATBC on UvrB or even UvrD, both possessing an ATP-binding pocket required for their function. We only conducted a preliminary docking on UvrB which showed a diminished affinity when compared with UvrA but didn't explore the combined effects on both proteins. Unfortunately, the lack of a crystal structure for *E. coli* UvrA limits the reliability of the structural data and the level of detail obtainable.

3.4.2 ATBC as a proof of concept

This study shows that inhibiting UvrA *in vitro* and *in vivo* by competing with ATP represents a new strategy to obtain an antimicrobial effect. ATBC could be just used as a template for a ligand-based search using freely available tools like SwissSimilarity (20) to look for other drug-like molecules possessing the same chemical footprint. Alternatively, UvrA's binding region could be used as a target for a rational structure-based drug design utilising the structural data available for the protein (for example, the importance of the residues K37 and K646). Ultimately, the ATPase cassette could be set as a target for a virtual screening of grand libraries of compounds searching for high-affinity binders to be tested *in vitro* and *in vivo* (as it was done for this thesis for chapter 6).

3.4.3 Why NER?

The ideal application for a NER inhibitor can be found when looking at cancer therapy. As discussed previously, antitumoral drugs are toxic compounds targeting fast-dividing cells. For that reason, their effect is also exerted on bacteria. The most relevant example for this thesis is cisplatin, initially discovered for its antimicrobial properties but later used for cancer therapy (230, 301). Bacteria are able to survive cisplatin damage thanks to NER activity, as discussed in the next chapter. Therefore, by inhibiting UvrA during cancer treatment, cisplatin could also exert its toxic action on the infectant.

As mentioned, more investigations are needed to explore possible topical applications of UvrA inhibitors. In summer, our skin is usually exposed to UV light therefore, arguably the inclusion of a NER inhibitor in topical pharmaceutical form could help in the treatment of different forms of skin infections without the need for systemic drugs.

Another question that remains to be answered pertains to the role NER plays when the host immune response is activated. Reports indicate NER involvement in repairing damages caused by free radicals but the extent of this contribution, especially in pathogen species, is not yet known. It would be interesting to evaluate the survival rate of a pathogen bacteria with an impaired NER tested directly in serum in the presence of components of the immune system.

3.5 Conclusions

In summary, we have shown how combining computational techniques and biochemical analysis can drive drug discovery. We have demonstrated how inhibiting the UvrA ATPases could be pursued as a possible novel antimicrobial target. We have fully characterized ATBC as a drug-like molecule likely acting as an ADP analogue against NER in *E. coli* and described its drawbacks as a poorly soluble and TolC substrate compound. Therefore, we set out to evaluate different strategies to define possible therapeutic options from the inhibition of NER and, specifically, UvrA. We concluded that, before a possible application, ATBC must undergo extensive improvements through chemical modifications.

Chapter 4 – Combining cancer chemotherapeutics with bacterial DNA repair inhibitors to develop novel antimicrobials.

The data in this chapter has been submitted for review as “Combining cancer chemotherapeutics with bacterial DNA repair inhibitors to develop novel antimicrobials” and is available in bioRxiv (302). For clarity, I have included sections with data collected by Dr Arya Gupta (data collection and analysis on some of the work with the wild-type strains, gel-based incision assay and part of the UV-damage repair assay). External contributions are specified in the figure legend and text.

4.1 Introduction

4.1.1 Cisplatin in therapy

During an investigation examining the effects of electric fields on bacterial growth during the 60s, the researchers noticed an inhibited cell division. Furthermore, compared to the untreated control, the bacteria appeared elongated up to 300 times more. Upon testing, they realised that the electric field wasn't responsible for the inhibition but was the platinum electrode to create the effect (301). After this discovery, the group synthesised and tested these compounds assessing their antibacterial properties. They evaluate both the cis- and trans- form concluding that the cis-isomer was responsible for the observed antimicrobial properties (230, 301). Since then, cisplatin antimicrobial activity has been shown to affect gram-negative and positive bacteria (303), even able to eradicate *E. Coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* persister cells (304). Unfortunately, its cytotoxicity on eukaryotic cells renders the drug inapplicable as an antibacterial. Nonetheless, the same toxicities make the platinum compounds an excellent antitumoral agent still in use today for various types of cancer (103, 305, 306).

Cisplatin's mechanism of action has been long investigated (230, 301, 307), and it is not surprising that the compound was the subject of many reviews during the years (103, 305, 308, 309).

Platinum compounds are highly reactive and can bind to multiple biological targets with disastrous consequences for the cell. Once cisplatin passively enters the cell, its chloride atoms are displaced by water. The hydrolysed cisplatin becomes a potent electrophile able to bind nucleophilic centres in proteins, GSH (and other oxygen scavengers), and, more importantly, DNA (103, 305). Cisplatin's DNA binding abilities are crucial, and it is now accepted that they are principally responsible for cisplatin's effects (309).

Cisplatin mainly forms bifunctional intra- and inter-strand crosslinks on DNA between two adjacent bases. The vast majority of the adducts produced in the interaction between cisplatin and DNA are intrastrand crosslinks between two guanines with a ~40-60° distortion of the DNA backbone (309). This evidence is supported by different structural studies, among which Takahara showed the x-ray structure of a DNA dodecamer containing

a cisplatin adduct for the first time. In her analysis, the investigators measured a significant distortion ($\sim 40\text{-}50^\circ$) towards the major groove caused by the formation of an interstrand GG crosslink between DNA and cisplatin (Figure 29 left) (310). Shortly after, Coste solved the crystal structure of a DNA decamer containing one single cisplatin crosslink (311). In his study, the author shows the interstrand link formed by cisplatin and two guanines and evaluates the DNA backbone distortion as $\sim 47^\circ$ (Figure 29 right). Figure 29 shows these crystal structures (310, 311) and the consequence of cisplatin-guanine adducts on the DNA topology.

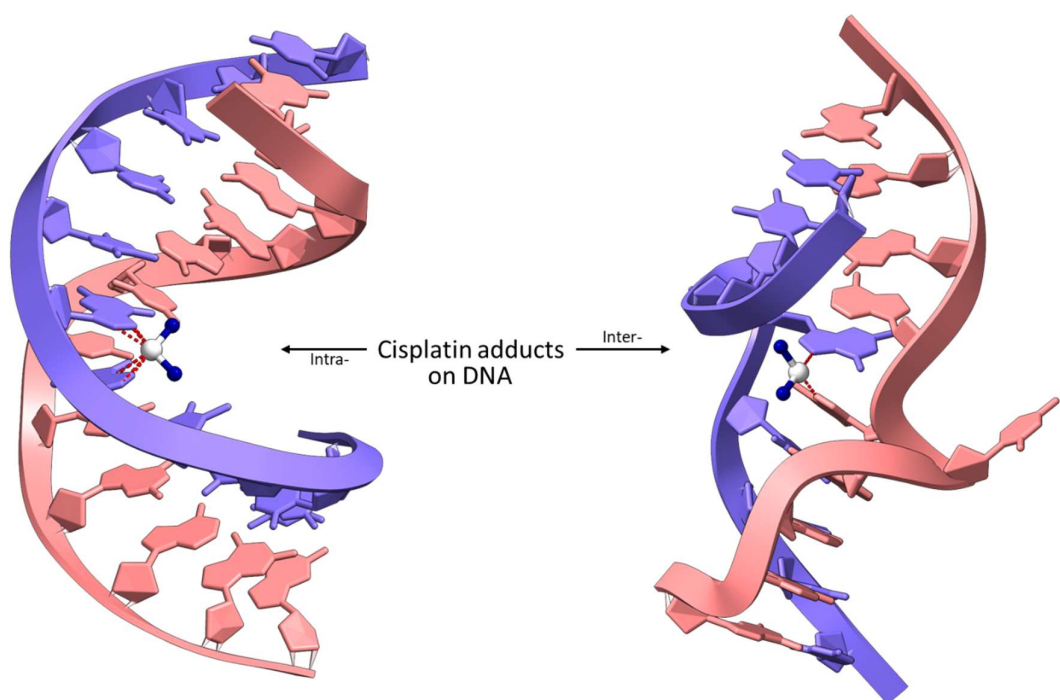


Figure 29: Intra- and inter-links formed by cisplatin and DNA. The figure shows the intrastrand (left) and interstrand (right) crosslink between one cisplatin molecule and two guanine bases, resulting in distortions of the DNA backbone. The structural data were retrieved from PDB (1A1O and 1A2A) (310, 311) and visualized in ChimeraX.

As already discussed in the introduction, DNA damage is catastrophic for cell survival and must be repaired to avoid cell death. This fact is easily demonstrated by the antimicrobial activity that leads to cisplatin discovery (301). However, bacteria can use the UvrABC system to detect cisplatin-DNA adducts and excise the damaged nucleotides. An article published in 1985 (312) directly evaluated the effects of bacterial NER on cisplatin-DNA complexes. The authors showed a 37% reduction in *E. coli* $\Delta uvrA$, *B* or *C* colony-forming

units when exposed to 30 µg/ml cisplatin for 1 h (in LB). In comparison, a 3h exposure could not kill the wild-type cells. Furthermore, they confirmed the NER-mediated cisplatin excision with a gel-based incision assay by detecting increased supercoiled-relaxed shifts on platin-treated DNA. This evidence was further confirmed (231, 313), proving NER's primary role in cisplatin-mediated resistance in bacteria. Therefore, we reasoned that the combination of this antitumoral drug with a molecule able to disrupt the UvrABC system would have been able to cause cell death by enhancing cisplatin toxicity to bacteria.

4.1.2 Combinatorial therapies

Any treatments can face obstacles due to resistance, toxicities or complex biological systems with redundant pathways. The concentration of a drug cannot be raised indefinitely to overcome these issues due to the unavoidable appearance of side effects. For example, we can look back at cisplatin as described before in chapter 4.1.1. The combination of the bacterial ability to resist its damage and the compound activity on eukaryotic cells render cisplatin ineffective as an antimicrobial due to the impossibility of reaching antibacterial concentrations without disastrous consequences for the patient. Combinatorial treatments aim to tackle these issues by co-administering multiple compounds targeting different cellular pathways contributing to the same final effect without raising the concentration of the single drug. In principle, this co-administration allows drugs to be maintained at concentrations below their toxic threshold while treating the disease synergistically, acting on multiple levels. Additionally, as mentioned in the introduction, combinatorial therapy makes the chances of developing resistance to the effects of two drugs much lower than using one drug alone, provided that the resistance mechanism is different for each drug (133).

Combining different chemicals to improve the efficacy of the treatment is not a new approach. The role of combinatorial therapies in medical treatments has been extensively reviewed over the years, from cancer and infections to cardiovascular diseases, psychiatric disorders and more (291–294, 314–319). Among those, the introduction of clavulanic acid alongside amoxicillin clearly demonstrates the potential of combinatorial prescriptions. Clavulanic acid is a penicillinase inhibitor with limited antibacterial activity, and amoxicillin is a β -lactam antibiotic which bacterial penicillinases can inactivate. The single use of the

two drug is ineffective in treating penicillinase-producing bacteria. However, when combined, they become potent against β -lactamase-producing pathogens (295, 320). The combination produces a dramatic decrease in ampicillin's MIC in ampicillin-resistant *Staphylococcus aureus* (320). This synergistic effect exemplifies how two drugs can successfully cooperate to enhance therapeutic efficacy, providing the rationale for developing other combinatorial therapies.

4.1.3 Summary

Infection is a heavy burden on patients battling cancer (as highlighted in Chapter 1.2.2.) who face increased exposure to multidrug-resistant bacteria found in hospitals (321). The degradation of physical barriers, including mucosal membrane degradation, surgical intervention, cancer growth (126) and severe chemotherapy-induced leukopenia (122, 322) elevate infection as one of the major leading cause of oncological mortality (124, 125). Therefore, developing new viable strategies to fight infections becomes essential.

Cisplatin cannot be used as an antimicrobial due to the bacterial ability to repair DNA damage through NER. At the same time, inhibiting NER without a DNA-damaging agent is not sufficient to kill bacteria. Following these premises, we aimed to develop a screening pipeline to allow the discovery of NER inhibitors working in conjunction with the anticancer cisplatin to kill bacteria. We screened an FDA-approved library in the presence and absence of cisplatin to isolate the antibacterial combinations. The compounds that were found able to inhibit *E. coli*'s growth were further screened by substituting cisplatin with UV, a well-known NER-activator. This additional screening step confirmed that the drugs targeted the DNA excision repair system. The selected compounds that maintained the activity were tested with a series of *in vivo*, *in vitro*, *in silico* and single-molecule assays to assess their mechanism of action. This pipeline culminated with identifying 5 potential leads, among which 3 were effective against a clinically relevant *E. coli* strain.

These findings represent the first step in the battle of co-infection during cancer chemotherapy, a major cause of concern among cancer patients. Furthermore, we open the door to a new therapeutical approach by targeting bacterial NER alongside DNA-damaging agents, laying the foundation for further developing new antimicrobials.

4.2 Results

4.2.1 Cisplatin activity on different strains

To investigate the combinatorial activity of cisplatin and NER inhibitors, we started the study by characterizing the compound in the new *E. coli* strains to quantify the ability to impair bacterial growth. In addition to the strains already tested, we also investigated *E. coli* EC958 (O25: H4-ST13) isolated in the Northwest of England (234) as a clinically relevant representative strain to assess for the relevance of our results. This strain has been well characterized and found to be responsible for globally disseminated multidrug-resistant infections (urosepsis) (234, 235). This strain possesses numerous virulence factors (e.g., adhesins, auto-transporters, and siderophore receptors) and antibiotic-resistance genes, which enable it to cause difficult-to-treat infections. Specifically, EC958 encodes for resistance genes reported to produce extended-spectrum β -lactamase, and cephalosporinase and be resistant to fluoroquinolones and ciprofloxacin (234, 235).

The solvent of choice for most of the compounds available in screening libraries is DMSO, which is not advised for cisplatin (323). We have shown previously that 2.5% DMSO, while being well below its MIC for MG1655 (as reported in the appendix, Figure 53) is still able to produce a ~4-fold loss of potency for cisplatin (324) as also showed in figure Figure 30 (4-fold shift in cisplatin MIC for EC958 $\pm$ DMSO). Therefore, we measured its MIC in the presence of 2.5 % v/v of DMSO regardless of the addition of drugs. We prepared a two-fold dilution of cisplatin dissolved in sterile 0.9 % w/v saline and used the colorimetric assay with BRS described previously. After the incubation for 20 h at 37°C (shaking), pictures were taken. Our results show (Figure 30) that in the presence of DMSO, the wild-type (MG1655) and the clinical isolate strain have an equal MIC (12.5 μ g/ml). Cisplatin exhibits growth inhibition in the MG1655 Δ tolC strain at a 6.25 μ g/ml concentration. Moreover, the double mutant Δ tolC Δ uvrA cannot grow at 3.13 μ g/ml, the same concentration previously found for MG1655 Δ uvrA (324). As already mentioned, in the absence of DMSO, cisplatin antimicrobial activity was found to be enhanced with a 4-fold shift in activity (12.5 to 3.13 μ g/ml for EC958).

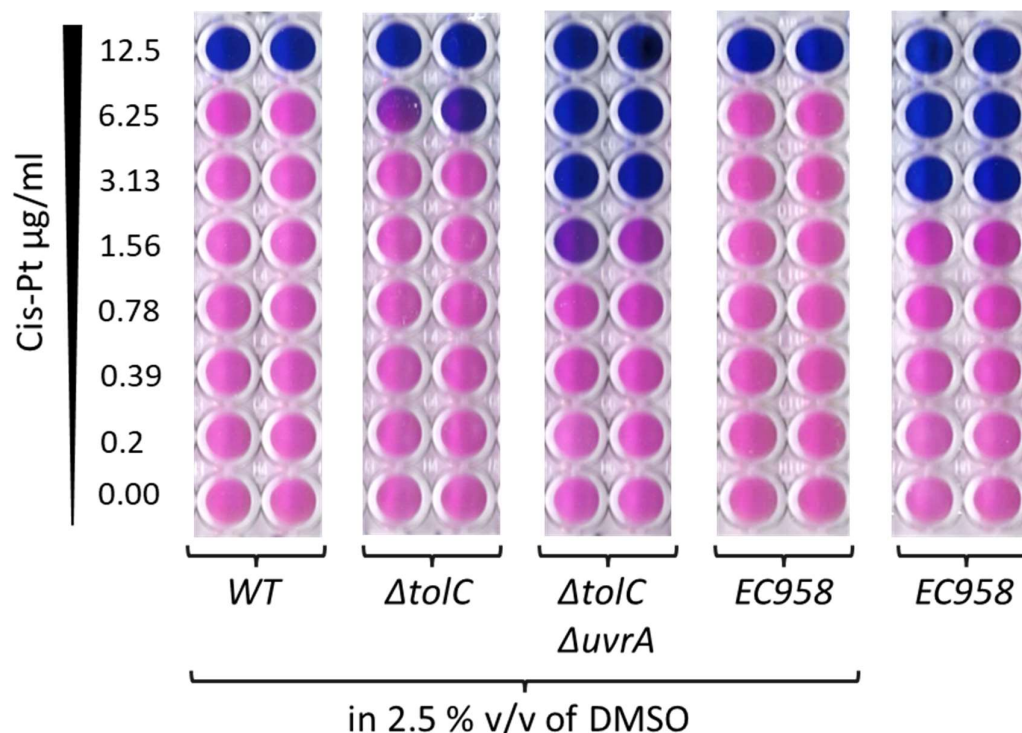


Figure 30: Survivability assay for cisplatin against different strains. Minimal inhibitory concentration for cisplatin in MG1655 (WT), MG 1655 $\Delta tolC$, MG 1655 $\Delta tolC \Delta uvrA$ and EC958 (in the presence of 2.5 % v/v DMSO). The wild-type and EC958 strains have a MIC of 12.5 $\mu g/ml$ if DMSO is present. The $\Delta tolC$ strain has a 6.25 $\mu g/ml$ MIC, while the double knockout $\Delta tolC \Delta uvrA$ shows a MIC of 3.13 $\mu g/ml$. Without DMSO, the MIC values for EC958 decreases to 3.13 $\mu g/ml$. This figure shows representative pictures of multiple independent replicates (≥ 3).

4.2.2 Screening an FDA-approved drug library

For our screening, we chose a library of compounds composed of 2731 FDA-approved and investigational drugs. We tested them simultaneously against MG1655 $\Delta tolC$ and against MG1655 wild type (Dr Arya Gupta did the wild type screening). Two plates were prepared in parallel, one containing only the FDA drugs and the other containing the combination of the FDA drugs and cisplatin. The concentrations chosen were 20 μM of the compounds, with and without 4 $\mu g/ml$ of cisplatin (cisplatin MICs in Figure 30). An example of the setup was previously shown in Figure 12. After the incubation and the addition of BRS, as described previously, the plates were inspected, and pictures were taken. 355 compounds reported antimicrobial activity against MG1655 $\Delta tolC$. When in the presence of cisplatin, further 108 compounds were found to inhibit the knock-out strain growth while being

inactive in the absence of the platinum compound. To further ensure a combinatorial activity against NER, we decided to re-test these 108 compounds replacing cisplatin with a sub-lethal dose of UV radiation (75 J/m^2) known to be sensitive to an impaired NER ($\text{MIC} \leq 5 \text{ J/m}^2$, as shown before in Figure 21). 21 of those compounds retained their activity and therefore were processed further.

Ultimately, the data acquired from the MG1655 ΔtoC screening was combined with the results collected from the wild type strain, and 5 hit compounds were picked for a complete characterization based on cross-activity, preliminary data, chemical profile, and availability. Figure 31 shows a flow chart summarizing the screening strategy. As shown in the figure, the compounds that did not make it through the chart were collected for future works and other evaluations. Some of those interesting compounds are described in the next chapter. Additional data were collected against MG1655 ΔtoC on compounds but with an unfinished profile. A table including those drugs and the work done can be found in the appendix (C – Additional compounds tested, Table 2).

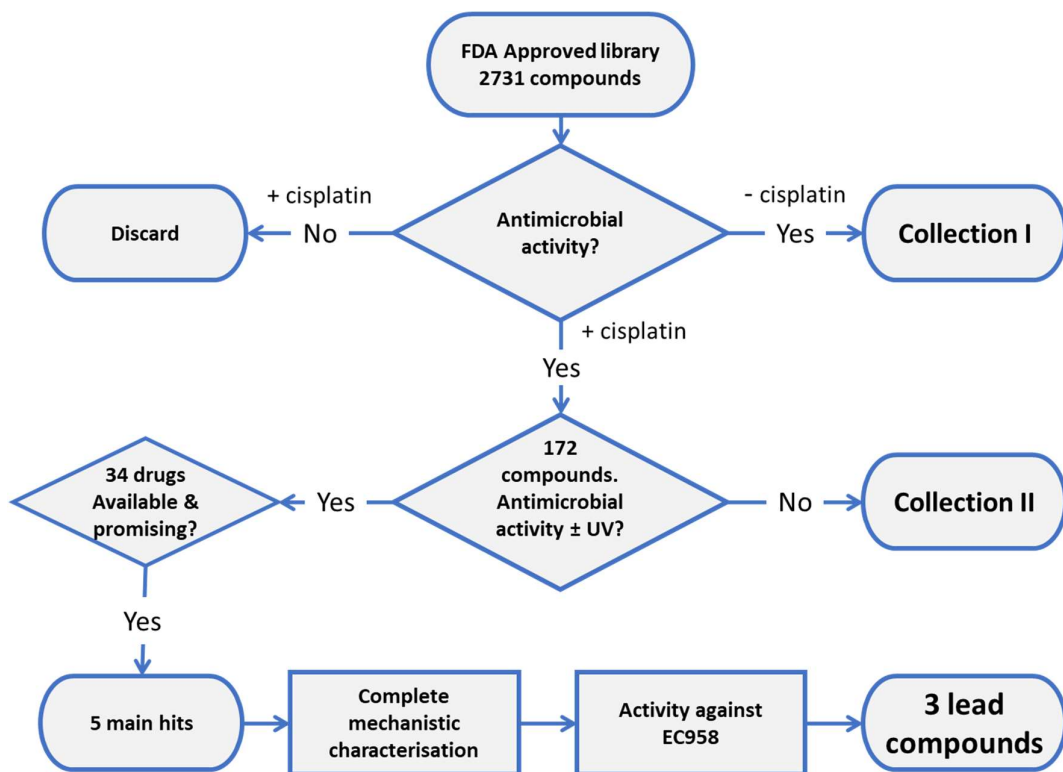


Figure 31: Screening strategy. The flow chart represents the screening strategy leading up to 3 active leads against EC958.

4.2.3 Testing the compound *in vivo*

5 compounds were chosen for an in-depth evaluation. This selection was composed by pirarubicin (4'-O-tetrahydropyranyl doxorubicin, THP) (325), mitoxantrone (326), dienestrol (327), L-thyroxine (328), and 9-aminoacridine (329). The results of the screening and the MICs for the hits are summarized in Table 1.

<i>Compounds</i>	Initial screening against MG1655 $\Delta toIC$			MICs	
	<i>w/o cisplatin</i>	<i>w/ cisplatin</i>	<i>w/UV radiation</i>	MG1655 $\Delta toIC$	MG1655 $\Delta toIC \Delta uvrA$
Mitoxantrone	Active	Active	Active	1.5 $\mu\text{g/ml}$	1.5 $\mu\text{g/ml}$
Dienestrol	Inactive	Active	Active	6.25 $\mu\text{g/ml}$	6.25 $\mu\text{g/ml}$
L-thyroxine	Inactive	Active	Active	100 $\mu\text{g/ml}$	50 $\mu\text{g/ml}$
Pirarubicin	Inactive	Active	Active	1.56 $\mu\text{g/ml}$	<0.78 $\mu\text{g/ml}$
9-aminoacridine	Partial	Active	Active	6.25 $\mu\text{g/ml}$	6.25 $\mu\text{g/ml}$

Table 1: Screening result and antimicrobial activity of the 5 major hits. The table reports the results obtained during the screening for the 5 significant hits (pink = alive, blue = dead) and the respective minimal inhibitory concentrations in MG1655 $\Delta toIC$ and MG1655 $\Delta toIC \Delta uvrA$. Every compound cause cell death when combined with a DNA-damaging agent. Among the compounds tested, Mitoxantrone and 9-Aminoacridine could impair bacterial growth without Cisplatin (in MG1655 $\Delta toIC$). The screening was repeated with a technical replicate, and the MICs measurements were repeated at least three times with one technical replicate each.

To test the degree of the combinatorial activity of the drugs with cisplatin we first needed to measure the MIC value in both MG1655 $\Delta toIC$ and MG1655 $\Delta toIC \Delta uvrA$ (Table 1). An example of the assay is shown for 9-aminoacridine in Figure 32 A (left).

Once we obtained the MICs, we could perform two-dimensional tests known as checkerboards. These assays allowed us to evaluate the combinatorial effect of the hits in the presence of cisplatin at different concentrations. As is shown in Figure 32 A (right), the x-axis reports the MIC for the compound tested (in this case, 6.25 $\mu\text{g/ml}$), while the y-axis displays the MIC for cisplatin (in this case, 12.5 $\mu\text{g/ml}$). The example shows that when the compounds are combined, we can observe multiple two-fold shifts in the concentrations needed to achieve the antimicrobial effect (follow the yellow line for the drug and the green line for the cisplatin). Every drug tested showed a cooperative activity combined with

the antitumoral drug with at least a 2-fold positive shift in inhibition. Among the compounds tested in MG1655 $\Delta toI/C$, 9-aminoacridine displayed the strongest cooperation with the platinum compound, with a 16-fold shift improvement in cisplatin antimicrobial activity and an 8-fold shift in 9-aminoacridine activity. Figure 32 C shows the results obtained for the wild-type strain (Dr Arya Gupta collected the wild-type data). The results show once again 9-aminoacridine/cisplatin as the best combination for antimicrobial activity with an 8-fold improvement in 9-aminoacridine maximum effect and a 4-fold shift for cisplatin.

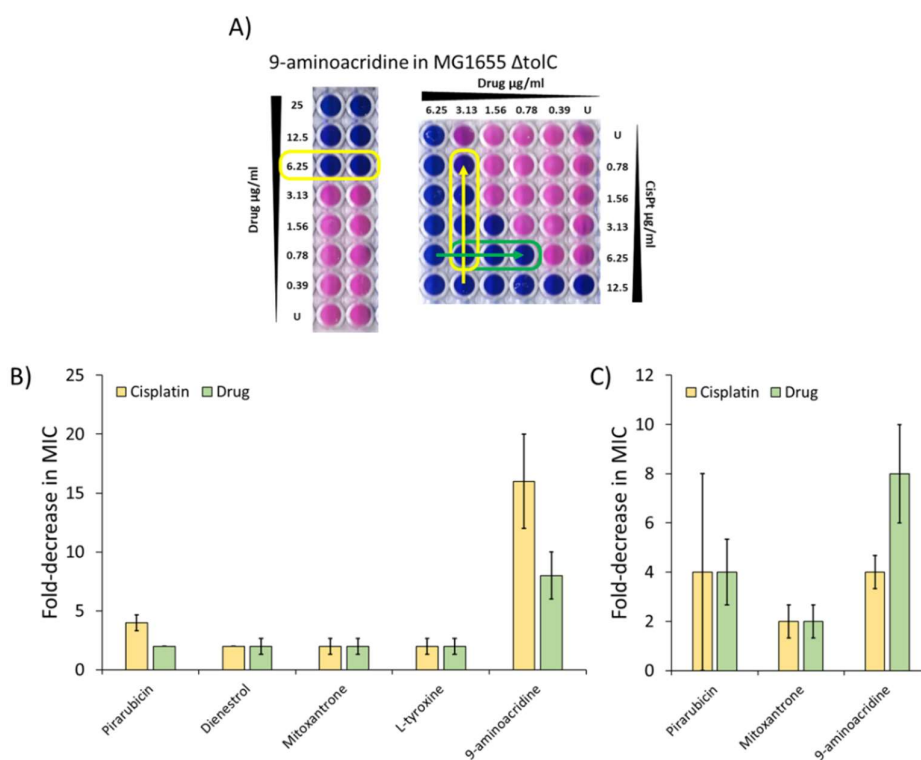


Figure 32: Checkerboard assay to test for the combinatorial effect of the selected compounds.

A) Representative picture of a MIC and checkerboard. On the left, the assay shows the minimal inhibitory concentration of 9-aminoacridine with a technical replicate in MG1655 $\Delta toI/C$. On the right is the checkerboard assay for the same drug in combination with cisplatin (in MG1655 $\Delta toI/C$). The green line represents the improvement of the antibacterial activity of the drug when combined, and the yellow the improvement of cisplatin when combined (U represent the untreated sample in the presence of equivalent DMSO). **B)** Bar chart showing the median of the fold decrease in MIC when the drugs are combined in the checkerboards assay for MG1655 $\Delta toI/C$ (green and yellow arrows). The data was collected in three independent experiments, and the error bars represent the standard error of the mean. **C)** The graph is equivalent to B but using MG1655 wild-type strain. Dr Arya Gupta collected the wild-type strain (C) data (left for clarity).

4.2.4 Confirming the activity against NER

Having assessed the cooperation of these drugs with UV and cisplatin *in vivo*, we proceeded to look at their ability to affect NER *in vitro*. We used a fluorescence-based assay to precisely assess the entity of the inhibition of UvrC-mediated incision ability. We also validated the results with a more traditional and established gel-based incision assay. As already described in Chapter 2.9, with this assay, we utilize the different melting temperatures of an intact oligo and a shorter incised oligo to drive the de-annealing after incision. This separation causes the dequenching of the fluorophore, which can be measured in real-time with a plate reader (Figure 15 reports the schematic representation of the system). The compounds were tested at 20 μ M to maintain a unified concentration in line with the initial screening. Compared to the untreated control (UvrABC), pirarubicin showed the most substantial effect with a maximum inhibition of ~97% (0.5 h incubation). Also, mitoxantrone and 9-aminoacridine reported good inhibition of the system with a maximum of ~82% (16 h incubation) and ~49% (2.5 h incubation), respectively. Dienestrol only showed marginal inhibition when significant (~25% after 16 h incubation). Ultimately, L-thyroxine did not seem able to impair NER in the fluorescence-based incision assay. The results are shown in Figure 33 (top).

Dr Arya Gupta performed a gel-based incision assay to verify the results obtained with the fluorescence experiments (Figure 33 bottom). Pirarubicin, mitoxantrone and 9-aminoacridine showed a significant inhibition consistently to results previously obtained (compare the U + UV with the D+ UV, an almost a complete disappearance of the nicked band can be observed when in the presence of the drugs). Furthermore, dienestrol displayed partial inhibition (the nicked band did not disappear, but residual DNA can be seen in the supercoiled position), once again agreeing with the fluorescence-based incision. Ultimately, L-thyroxine could not prevent the incision when incubated for 30 minutes. However, inhibition was observed when the incubation was reduced to 15 minutes (†).

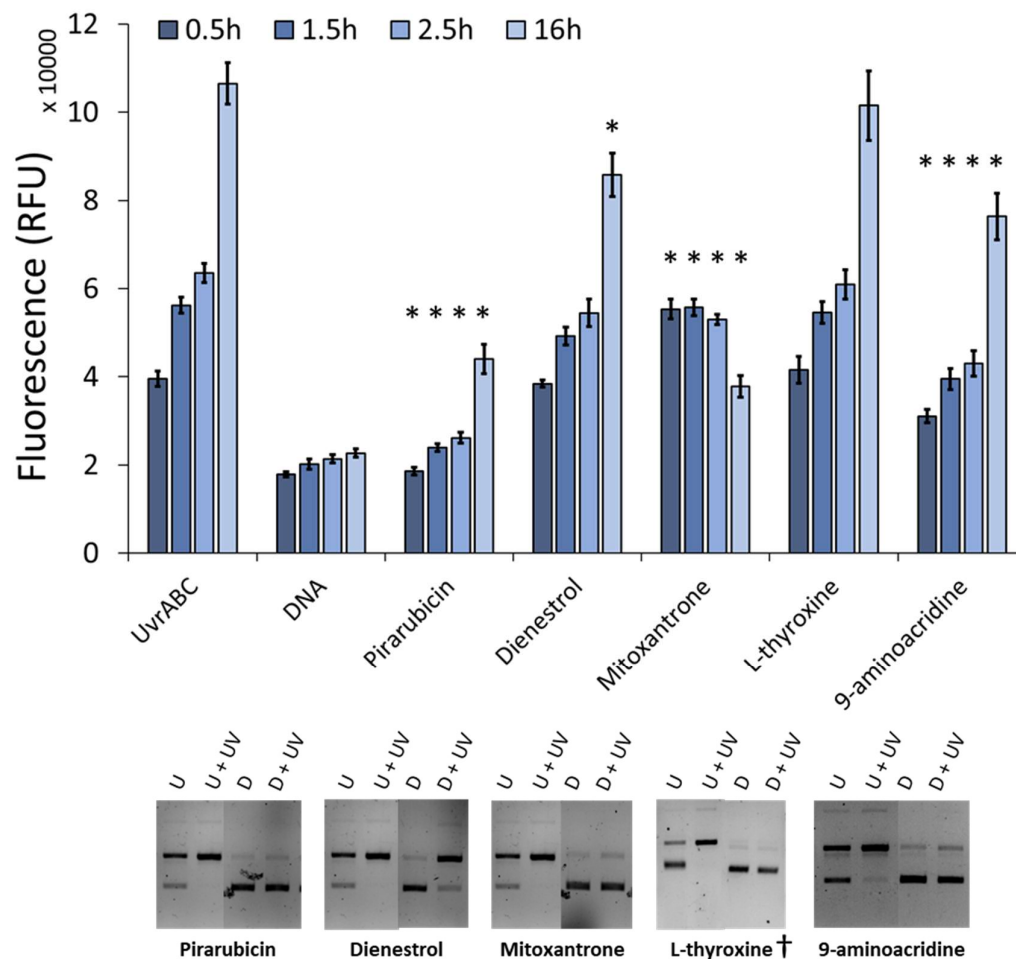


Figure 33: Incision assay to test for NER inhibiting activity in vitro. Two different incision assays were performed to evaluate the compounds' activity on the UvrABC complex. **A)** The bar chart reports the results of the fluorescence-based experiments. The graph shows the basal level of incision for the untreated sample (UvrABC, no drugs), the minimal fluorescence emitted by the oligos when quenched (DNA, no drugs or UvrABC), and the fluorescence emitted when the system was incubated with the drugs (schematic representation in Figure 15). The error bars represent the standard error of the mean ($n \geq 4$). The results were considered significant if $p \leq 0.05$ (*) (T-test) when compared to the untreated UvrABC. **B)** Gel-based incision assay performed by Dr Arya Gupta as further confirmation. The gel shows untreated pUC18 with the system UvrABC(U) and the same after UV irradiation (U + UV). The comparison is made with the equivalent of the two but when in the presence of the drugs (D and D + UV). The pictures are representative of two independent replicates.

4.2.5 UV-damage repair assay as further *in vivo* confirmation

In this section, Dr Arya Gupta tested some of the compounds in MG 1655 $\Delta tolC$ and defined the experiment's methodology.

To further assess the ability of these compounds to impair survivability *in vivo* acting on NER we performed an additional assay. With a UV-damage repair assay, we can evaluate the anti-NER activity by measuring the ability of transformant bacteria to repair a damaged plasmid coding for antibiotic resistance. In this experiment, cells are made competent and then transformed with a UV-damaged plasmid (200 J/m² UVC). During the recovery step, the cells are exposed to 2xMIC of the compounds (to account for a drastically higher number of cells and the use of rich media) before being washed and then plated in selective media. Successful inhibition of the system will render the cells unable to express the antibiotic resistance coded in the plasmid and therefore die when plated in selective LB-agar. The results are reported in Figure 34. All compounds tested inhibited cell growth (expressed in the number of colonies per ml of solution), corroborating the screening results. Among the hits, L-thyroxine was the least effective, with a 45% inhibition on the verge of significance ($p = 0.051$) in agreement with the lower effectiveness in the incision assay.

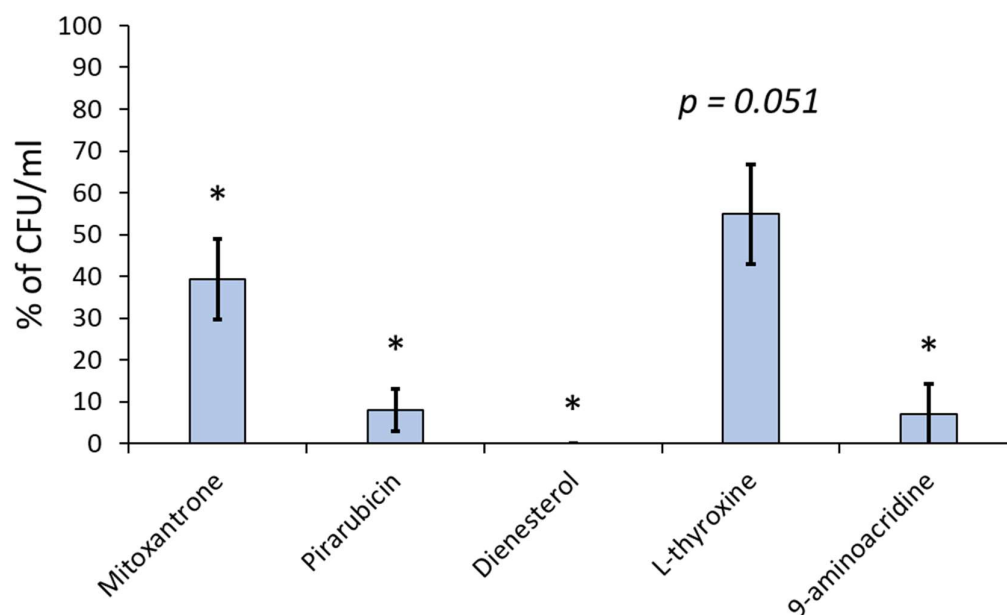


Figure 34: UV-damage repair assay evaluates the inhibiting activity *in vivo* against NER. The bar chart shows on the Y axis the percentages of colony-forming units per ml of solution when *E. coli* MG1655 $\Delta tolC$ is exposed to the compounds during recovery after being transformed with a UV-damaged plasmid (a schematic representation in Figure 17). The untreated control has been reported as 100%, and the bar chart of the compounds shows the percentage in CFU/ml when exposed to the drug during recovery. The error bars represent the standard error of the mean (n=3). The results were considered significant if $p \leq 0.05$ (*) (T-test) when compared to the untreated transformant. Dr Arya Gupta collected the data for some of the compounds.

4.2.6 Evaluating UvrA ATPase cassette as the possible molecular target

After the compounds were fully characterized against NER both *in vivo* and *in vitro*, we set out to evaluate UvrA as a possible molecular target. As shown previously, UvrA is responsible for damage location and UvrB recruitment, therefore, its impairment is detrimental to cell survival when exposed to DNA-damaging agents. We started by evaluating which one of the 5 main hit compounds maintained an effect against purified UvrA. The compounds displaying activity were then evaluated with an in-silico exploration of the protein's surface to identify possible binding sites. Furthermore, the best UvrA inhibiting drugs were evaluated in single-molecule to establish whether they could prevent DNA binding. Antoine Paris collected the single-molecule data and was not included in this thesis as not necessary for the comprehension of the study but can be found online (302).

NADH-coupled ATPase assay shows activity against UvrA

To investigate the effects of the compounds against UvrA, we went back to the NADH-couple ATPase assay. Once again, the compounds were tested at a concentration of 20 μM . Dienestrol and L-thyroxine were the compounds to exhibit the more potent effect. Dienestrol reduced UvrA's k_{cat} to ~24% and ~43% in the absence and presence of DNA. Similarly, L-thyroxine inhibited UvrA to ~27% and ~32% of the total activity (respectively, in the absence and presence of DNA). Following, pirarubicin and mitoxantrone inhibited UvrA reducing the total activity down to ~68% and 54% (respectively). Interestingly the compounds were not influenced by the addition of DNA. Ultimately, 9-aminoacridine was unable to inhibit UvrA significantly with or without DNA.

The compounds showing an effect on UvrA were then chosen for an in-silico exploration of the possible binding modes on the protein.

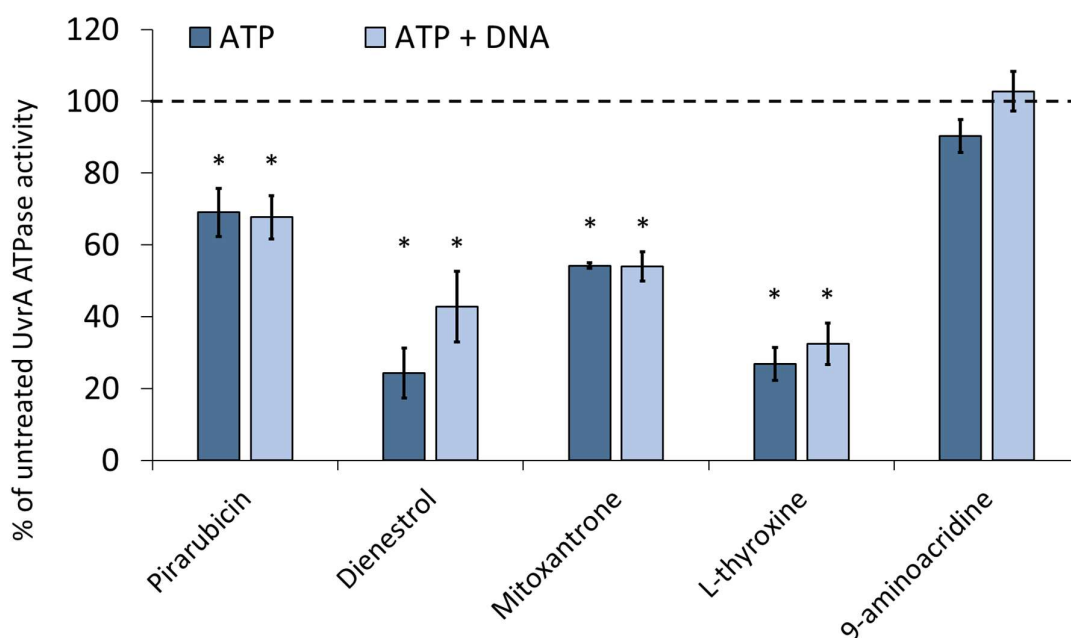


Figure 35: NADH-coupled ATPase assay on recombinant UvrA. The bar chart shows the effect of the 5 hits on the total UvrA ATPase activity expressed in percentage (dotted line). The readings were sequentially acquired after adding ATP and after further stimulation with 0.1 ng/ μl pUC18 DNA. The experiments were repeated three times. All the compounds were tested at 20 μM , and ATP was used at the saturating concentration of 1 mM (ratio ATP: drug = 50: 1). ADP gets regenerated to ATP in this assay and therefore doesn't accumulate. The error bars represent the standard error of the mean. The results were considered significant if $p \leq 0.05$ (*) (T-test) when compared to the untreated sample.

Studying the possible binding modes on UvrA

Having proved the reliability of the docking software AutoDock Vina (Chapter 3.2.5), we used it to investigate the potential binding sites of the compounds exerting an effect on the protein. We maximised the search space to include the entirety of the protein surface. Unlike the previous approach, we also dramatically increase the exhaustiveness (1000x), improving the software's freedom and, therefore, the chances of getting more accurate results. As suggested by Vina's authors, this adjustment was made to compensate for the unusually large search space that could increase the chances of getting false results (40).

We docked ATP alongside the hits to ensure the software could locate the two ATPase cassettes with the new parameter. Then we proceeded to evaluate the highest-scoring molecule given by the algorithm. Furthermore, we allowed AutoDock Vina to output more conformations than just the best-scoring results obtaining different binding possibilities reported in Table 4 (Appendix G – Hits' binding energies).

Our results (Figure 36) show the correct placement of ATP in UvrA's distal and proximal sites. Interestingly, it assigns a higher affinity to the distal pocket than the proximal one (-9.6 kcal/mol for the distal against -9.2 kcal/mol for the proximal) in agreement with recent experimental data reported (258). Furthermore, Vina could predict interactions with residues involved in ATP-binding (the specific residues-ATP interactions can be found in the appendix, Figure 61) (91). Among the compounds tested, pirarubicin (-9.8 kcal/mol) had the highest affinity for the protein compared to the other compounds docked. Mitoxantrone showed a more modest binding energy of 8.6 kcal/mol. Surprisingly, dienestrol and L-thyroxine (the two most active compounds in the NADH-linked ATPase assay shown in Figure 35) showed two preferential binding pockets (BP1 and BP2) distinct from the two distal and proximal sites. The binding energies reported were -8.7 and -7 kcal/mol, respectively.

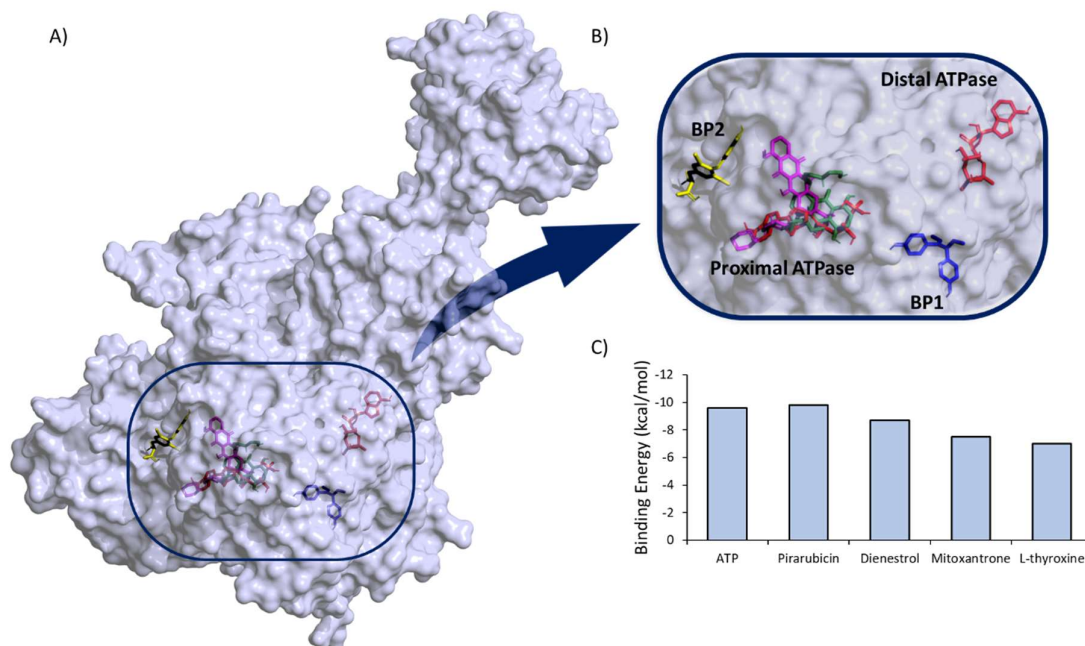


Figure 36: Studying possible binding sites on AlphaFold UvrA.

A) The structure for *E. coli*'s UvrA retrieved from AlphaFold and generated with PyMOL shows the molecular docking of the compounds exhibiting an effect on the ATPase through transparency. **B)** Zoomed-in section of the previous figure shows the docking of ATP (red), pirarubicin (magenta), L-thyroxine (yellow), mitoxantrone (green) and dienestrol (blue). Two compounds (L-thyroxine and dienestrol) bind to pockets distinct from the ATP binding sites. **C)** Graph showing the best docking conformation outputted by Vina with an exhaustiveness of 8000.

To complete the study on UvrA, dienestrol and L-thyroxine were tested by Antoine Paris on suspended DNA with fluorescently labelled UvrA to quantify their direct impact on DNA binding. Mitoxantrone and pirarubicin proved impossible to be imaged directly with this technique due to their intercalating effects. The result showed a high reduction in the number of binders when 20 μM of the compounds were incubated with the protein confirming the previous finding. The data is not shown here since not fundamental for the comprehension of the chapter but can be accessed on bioRxiv if interested (302).

4.2.7 Evaluation of the best hits on EC958 clinical isolate

Having fully characterised the compounds *in vivo* and *in vitro*, we decided to evaluate their activity against a commonly diffused infectant in clinical settings, *E. coli* ST131 EC958 (234). We decided to test the three compounds active in the MG1655 wild-type strain after the consideration that the multidrug-resistant EC958 would have carried an intact and functional TolC.

First, we measured the MIC values to guide the choices of concentrations for the checkerboards (the MICs experiments are reported in Figure 54 Appendix III - MIC values of the interesting compounds in EC958). Figure 30 shows MG1655 wild type and EC958 have the same MIC for cisplatin (12.5 µg/ml). The results of the combinatorial experiments are reported in Figure 37. Pirarubicin exerted the stronger combinatorial activity alongside cisplatin. The platinum compound had a 16-fold increase in its antimicrobial effect, while pirarubicin showed at least an 8-fold improvement in its killing ability. 9-aminoacridine saw a simultaneous 2-fold shift in MIC for the combination. Ultimately, mitoxantrone was able to improve cisplatin by a 4-fold factor. Also, mitoxantrone itself saw a 4-fold improvement when in the presence of cisplatin. Altogether, the drugs proved to be able to cooperate when combined with cisplatin, improving the efficacy of their antimicrobial activity against EC958.

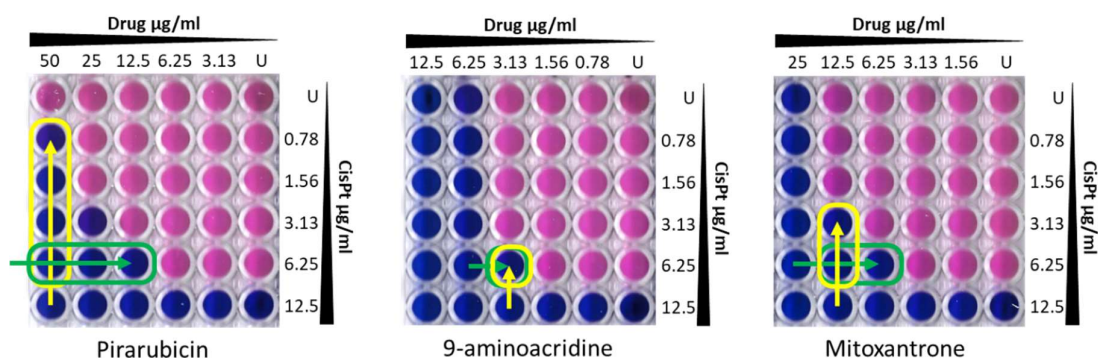


Figure 37: Three final hits are effective against the clinical isolate strain EC958. Checkerboard assays in EC958 show the combination with cisplatin. The yellow and green arrows show the shift of the MIC due to the combination. All the compounds exhibit cooperation, with pirarubicin displaying the most potent combinatorial effect. The pictures are representative of three independent replicates.

4.3 Discussion

Anticancer drugs are the cause of many toxicities, among which leukopenia is of primary concern. Coupled with the rise of antimicrobial resistance in bacteria, a hospital-acquired infection in immunocompromised patients can have catastrophic outcomes, even leading to death. We believe that adjuvant antimicrobial represents a promising approach to tackling infections in cancer patients escaping the rise of antimicrobial resistance.

We tested an adjuvant antimicrobial in combination with a well-established anticancer agent, cisplatin, targeting nucleotide excision repair to kill the infectant. From screening thousands of FDA-approved drugs, we evaluated these compounds with several techniques, from *in vivo* to single molecule. We proposed a mechanism of action, and we narrowed down three principal candidates to be progressed for pre-clinical evaluation.

4.3.1 FDA-approved screening

To our knowledge, the only study searching for molecules able to inhibit NER was published in 2011, focusing on mycobacterial NER (275). In this study, the authors aimed to find a molecule to help understand prokaryotic NER bypassing the need for mutants. Their screen yielded ATBC, which we characterised in chapter 3. However, the compound properties present a crucial limit in its applicability, and it is not surprising that we couldn't find mentions of the molecule after its discovery. Therefore, our attention went to FDA-approved drugs which already possess good drug-like properties. Furthermore, Mazloum's study (275) utilized UV radiation as the DNA-damaging agent. Despite being a potent NER activator (193), as we showed in Figure 21, radiation has limited applicability in clinical therapy. We then decided to move to cisplatin, an exceptionally well-characterized drug used to treat many cancers (103) and known for its link to excision DNA repair (312).

Looking at what was done before us (275), the investigators identified only 1 hit after a screening on ~40 thousand drug-like molecules. Therefore, we decided to start with the relatively high drug concentration of 20 μ M. However, this strategy might have caused the misclassification of the more potent compounds sufficiently strong to act independently, which would have been placed in collection one (Figure 31) for further testing. Having to screen thousands of compounds, some trade-offs must be made. Moreover, we

standardized the approach and maintained a fixed, uniform pipeline to process these drugs efficiently. We decided to maintain a standard concentration and buffer conditions for all the drugs during the biochemical testing. This mandatory trade-off does not consider eventual requirements or incompatibility of the single compound, potentially causing activity loss on some of the hits. Nonetheless, we were able to identify 5 promising leads able to efficiently inhibit NER *in vivo*, causing cell death upon cisplatin (and UV) exposure. Furthermore, all the compounds effectively inhibited incision in our biochemical tests representing optimal candidates for further evaluations.

4.3.2 Characterization of the 5 leads

It is not surprising that these leads are currently used for different applications in endocrinology and oncology, as this is the case when repositioning established pharmaceuticals. However, the advantage of repurposing these drugs is that it provides an opportunity to accelerate the discovery process towards pre-clinical evaluation. The anticancer agents are especially promising since they could require a simple change in prescription to achieve immediate benefits on infection rates.

Furthermore, three lead compounds exhibit improved antimicrobial activity in a clinically relevant strain of *E. coli*, providing further evidence for pre-clinical testing.

9-aminoacridine has been used in therapy as a topical antiseptic; however, the compound has recently been investigated for anticancer therapy for its action on the PI3K (330). Furthermore, the drug has also been used to derivatize cisplatin to enhance DNA damage (331). 9-aminoacridine showed good anti-NER activity being able to inhibit incision significantly but could not disrupt UvrA when directly tested. The possible interactions with the other components of the UvrABC system are fundamental to precisely understanding its molecular target.

Pirarubicin and mitoxantrone are well-known topoisomerase inhibitors acting through DNA intercalation (98). Therefore, we wondered if the action we were measuring was just due to their ability to intercalate DNA. However, we have shown how both pirarubicin and mitoxantrone were effective on UvrA without DNA. Furthermore, their activity is not enhanced after the stimulation of pUC18 DNA, demonstrating their direct action on UvrA.

The molecular docking confirmed these observations placing the two molecules in the ATP-binding pockets of the protein. Furthermore, we found pixantrone (mitoxantrone analogue) and various members of the camptothecin family (other topoisomerase poisons also acting through intercalation) to be unable to prevent incision (Appendix D & F). These observations clearly indicate that intercalation cannot be the sole mode of action.

AutoDock Vina does not restrict the docking with artificial limits such as the search space size or its number of runs allowing the user to determine the depth of the exploration. Furthermore, it doesn't operate by biasing the search on the input conformations (40). Therefore, we extended the approach used previously for ATBC. We maximised the search space to include the entirety of the surface and dramatically increased the exhaustiveness (x1000), allowing the algorithm to probe different binding poses accurately. This approach increases the chances of obtaining more representative results and can give insight into the nature of the interactions. As shown in appendix H, AutoDock Vina can correctly place ATP in UvrA's binding pockets and even identify crucial interactions with the ligand. When our hits were docked, we were surprised to find that l-thyroxine and dienestrol, the compounds showing higher ATPase inhibition (>70%), were placed in allosteric pockets previously unidentified. We could confirm this action in single molecule (data collected by Antoine Paris and not shown here because not essential for comprehension but available online (302)), measuring a dramatic decrease in DNA binding. These new pockets could represent new highly specific druggable sites in UvrA that could be exploited to develop new adjuvant antimicrobials.

It is important to note that the repurposed drugs identified in the study may need to be co-administered with cisplatin to be clinically valuable. This can limit their therapeutic windows depending on the drug's pharmacokinetics. However, we show through our checkerboards that the combination remains effective at lower concentrations, especially regarding pirarubicin and 9-aminoacridine. These results provide the starting point for further and more detailed investigations.

4.4 Future work

The work done up to this point is not the journey's end but only the beginning. We were able to generate a vast amount of data that can be mined for more insight into NER and combinatorial activity with cisplatin.

As mentioned, our compounds were tested at a relatively high concentration (20 μ M) following a precursor screening on excision repair (275) and to pick up even the weaker drugs. However, this choice leads to the possible loss of the more potent compounds by appearing bactericidal on their own, ending in collection 1 (Figure 31). This collection could be re-tested at a lower concentration, both alone and with cisplatin, to surface new possible hits. Furthermore, the screening was conducted in parallel between *E. coli* Δ tolC and the wild-type strain (by Dr Arya Gupta). Comparing the antimicrobial activities of these drugs in the absence of cisplatin provides new screening data on Δ tolC substrates.

Collection 2 (Figure 31) represents another pool of exciting compounds that were not investigated. These drugs showed combinatorial activity with cisplatin but not with UV, possibly indicating cooperation affecting other essential pathways for cell survival, such as the recombination repair system (313). These compounds could be examined singularly by picking up the most promising member to be further tested.

Due to time and resources restriction, we could not fully characterise every compound that displayed anti-NER activity, and we focused on the more promising ones. In the next chapter and the appendix, we have reported the additional work done on two of the many compounds we set aside for further characterization. Furthermore, we believe that drugs like apigenin or hydroxyprogesterone (Appendix E) should be further investigated to understand their cooperation with cisplatin and their possible role in therapy.

We have thoroughly examined the action of these compounds on UvrA, the first protein to make contact with the lesion during excision DNA repair (200). We have shown that 9-aminoacridine could not affect UvrA ATPase activity (Figure 35), raising the question of what could then be its possible molecular target in NER. An activity against UvrA not linked to its ATP-binding pockets remains possible; however, the compound could affect other system members. For example, UvrB possesses an UvrA stimulated ATPase required to

progress the excision repair (220). The hits could be tested for the UvrAB complex ability to hydrolyse ATP for further insights into the mechanism.

Another question that remains unresolved is what precise interactions are the main responsible for the activity. We tried to answer it by looking at the molecular dockings. The experiments indicate that two allosteric binding pockets could be involved in the anti-UvrA activity of l-thyroxine and dienestrol. Co-crystallising the protein while bound to these drugs would provide direct empirical evidence to confirm the impact of these two sites.

A central part of drug development is the chemical improvement of the hits to enhance efficacy. We have begun our investigation, testing other members of the rubicin family against UvrA as well as a mitoxantrone analogue, pixantrone (Appendix F). However, the work remained unfinished. Progressing with the study and comparing how different members of the same chemical family exhibit different potency will be the first step to defining a quantitative structure-activity relationship that could be invaluable for the rational design of more potent and specific drugs.

To conclude, we could successfully demonstrate the combinatorial activity between cisplatin and 5 hits, with 3 active on a clinically relevant strain. Having screened FDA-approved drugs provided us with the advantage of having “safe” and clinically applicable compounds without the need for years of basic research. As discussed previously, the prospect of advancing these candidates to pre-clinical testing on patients isolates remains tantalizing, representing an immediate step forward in fighting nosocomial infections.

4.5 Conclusions

Starting from the combinatorial screening of an FDA-approved drug library on *E. coli* $\Delta toIC$ (and wild-type) using a sub-MIC quantity of cisplatin, we highlighted possible compounds acting by inhibiting NER. We further tested this anti-NER activity by repeating the inhibition tests utilizing a different DNA-damaging agent (UVC). The compounds maintaining their antimicrobial activity were then characterised through a wide range of microbiological and biochemical techniques. We confirmed their ability to impair NER biochemically with two different incision assays and by exploiting UV damage in an *in vivo* test. Furthermore, we directly measured their action against UvrA, the system's first responder, both empirically and computationally. This pipeline ultimately led to 5 compounds, among which 3 showed enhanced antibacterial activity when combined with cisplatin in a clinically relevant strain. Furthermore, we identified their action mechanism and described two unknown allosteric UvrA regions.

In doing so, we have proposed NER as the molecular target for a new kind of antibacterial treatment that could ease the burden of infection in cancer patients. Ultimately, we have provided 3 candidates that could be explored immediately in a pre-clinical setting, laying the foundations for developing new antimicrobials targeting NER in combined therapy for cancer patients.

Chapter 5 – Screening outliers: exploring compounds with interesting properties

This chapter includes small sections containing works done by Antoine Paris (data collection, analysis and methodology on the optical trap) and Dr Arya Gupta (data collection and analysis on the gel-based incision). External contributions were left for clarity and are specified in the figure legend and text.

5.1 Summary

During the screening, we began the exploration of additional compounds that presented promising characteristics but did not fit among the exclusion criteria used for selecting the hits in the previous chapter. This work could not be explored fully due to insufficient time or resources and remains unfinished. However, our finding shows interesting preliminary data on several different compounds. The details and the overall progress are summarized in Table 2 (Appendix C – Additional compounds tested) and Appendix F – Unfinished work on different hits analogues.

In this chapter, we wanted to highlight the results obtained on two of the more investigated compounds, the anticancer bleomycin and the proteinase inhibitor aprotinin.

5.2 Bleomycin

5.2.1 Introduction

Bleomycin A₂ is a drug with a peculiar structure (Figure 38) and mechanism of action discovered in Japan in the early 1970s and quickly entered the anticancer arsenal. The compound isolated from *Streptomyces verticillus* acts by binding to DNA as a “mini-enzyme”, forming complexes that result in DNA breakage (332). It has been well documented that bleomycin can bind to DNA (preferentially around 5' GC 3' or 5' GT 3' sites) and induce specific lesions by generating free radicals (catalyzed by a metallic atom). The lesions can result in single and double-strand breaks compromising cell replication and survival (333, 334).

This “mini-enzyme” comprises 3 principal domains (Figure 38). The metal-binding domain forms a complex with an iron atom (Fe II) able to reduce molecular oxygen to a free radical believed to be responsible for DNA damage (98, 114). The DNA binding domain is composed of a bi-thiazole moiety attracted by the phosphodiester backbone of the DNA molecule (114). Ultimately, the carbohydrate domain is believed to be responsible for compound systemic distribution (114).

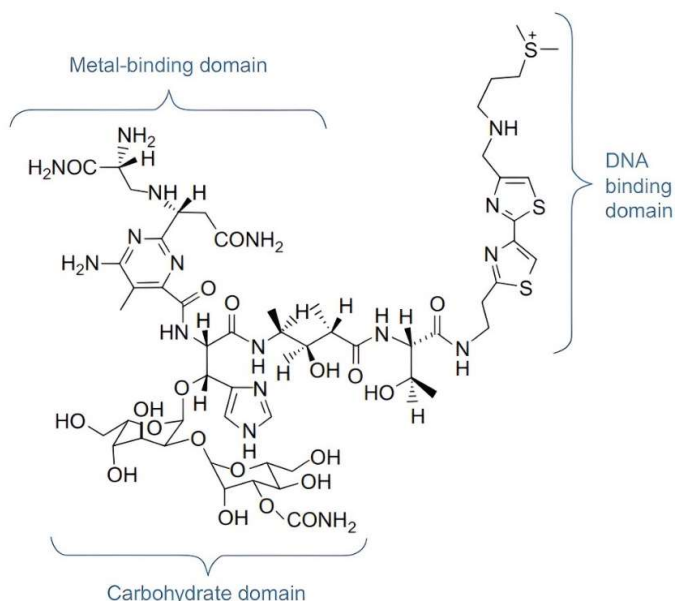


Figure 38: Bleomycin A₂ structure. The figure from Madkour (114) shows bleomycin's structure and domains.

Bleomycin has been approved for treating several types of cancers (335), both alone and in combination with other drugs. A study from 2004 evaluated the co-administration of bleomycin/cisplatin and other antitumoral agents with positive results for cancer patients with intermediate or poor prognoses (336). In their trial, the researchers investigated the effects of intense administrations of bleomycin, cisplatin and vincristine, alternating with bleomycin, cisplatin and etoposide, on 43 patients. Their finding showed that these combinations were well tolerated leading to good survival and progression-free rates during the patients' follow-up.

Like many other anticancer drugs, bleomycin has a narrow therapeutic index and can cause side effects, such as pulmonary toxicity, mucositis or alopecia. However, unlike most other cancer chemotherapeutics, bleomycin is unexpectedly safe against the bone marrow and does not contribute to myelosuppression (98, 332). This characteristic reduces the haematological toxicity and the likelihood of bacterial infections in cancer patients, a significant concern during cancer chemotherapy (as discussed in the previous chapters).

With these premises, we set out to evaluate bleomycin combinatorial activity with cisplatin and its effects on nucleotide excision DNA repair. We started by measuring the ability of bleomycin alone and in combination with NER to promote DNA incision, confirming what was already established and measuring additive effects. Furthermore, we tested the significance of these results *in vivo* against two *E. coli* strains using cisplatin as the DNA damaging agent (MG1655 $\Delta toIC$ and EC958), measuring the synergy between the two agents. Ultimately, we re-confirmed bleomycin's cooperative action with NER using an additional *in vivo* assay, providing the rationale for further explorations.

5.2.2 Bleomycin shows an opposite effect against NER

The fluorescence-based incision assay measures DNA cleavage over time, giving insights into the process. When 20 μ M Bleomycin was incubated with DNA, we measured a total increase in fluorescence significantly stronger than the one produced by UvrABC alone (as shown in Figure 39 A, compare UvrABC to Bleomycin). After the first reading (0.5 h), bleomycin alone released only ~65% of the oligos released by UvrABC, but at the second reading (1.5 h), the two measurements were not significantly different. However, bleomycin maintained a more consistent rate throughout the assay, reaching a total incision 160 % higher than the maximum value reached by UvrABC (16 h).

When the proteins are incubated with Bleomycin and the UvrABC system simultaneously, we observe a clear additive effect, as shown in Figure 39 A, with a further increased release in fluorophore.

These results were then confirmed with a gel-based incision assay (Figure 39 B). The assay visualizes the incision through the tendency of the DNA to switch from its supercoiled state (bottom band) to its linear or open circular form (upper band) when nicked (Dr Arya Gupta collected the data for UvrABC). It appears clear in Figure 39 B that the DNA shifts from the supercoiled state to the nicked one when in the presence of either UvrABC or 20 μ M bleomycin. Furthermore, only when UvrABC and bleomycin are combined during the incubation, the DNA band fades into a smear indicating multiple DNA breakage.

As previously done, we assessed for a possible effect against UvrA. We performed an ATPase assay (as already described) and found that bleomycin cannot significantly affect UvrA either in the presence or absence of DNA (Figure 39 C).

It is important to mention that to closely mimic *in vivo* biochemistry, a redox system should be used in the *in vitro* experiments to prevent self-inactivation of the compound over time due to auto-oxidation (337–339). This system was not present in our assays. However, the self-inactivation occurs only in the absence of DNA since the degradation of the drug is linked to the DNA damaging activity (337, 338). Therefore, the compound would have only been minimally affected during the incision experiment explaining the results in Figure 39 A&B. Ultimately, despite the ATPase assay was carried over a short period of only ~15

minutes, we cannot exclude that the complete lack of activity (Figure 39 C) could be partially due to the absence of the redox cycling system preserving bleomycin's integrity.

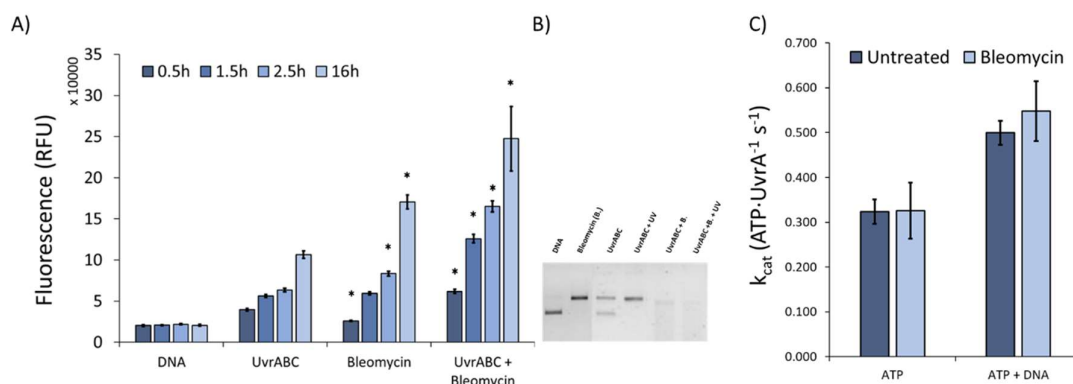


Figure 39: Bleomycin acts directly on the DNA. **A)** Fluorescence-based incision assay showing the base level of fluorescence of the untreated oligos (DNA), the NER complex without the drug (UvrABC), the drug without UvrABC (Bleomycin) and the combination of the two (UvrABC + bleomycin). The results show bleomycin's ability to release the oligos with a comparable rate of UvrABC (comparing UvrABC and bleomycin). When the DNA is in the presence of the combination, an increase in incision is measured. The results were compared to the UvrABC samples and considered significant if $p \leq 0.05$ (*) (T-test). **B)** The gel-based incision as further confirmation (Dr Arya Gupta collected the data for the gel-based incision in the presence of UvrABC). The gel shows the ability of both bleomycin and UvrABC to incise the DNA on their own. However, the effects are amplified when the compound is incubated with the system. **C)** NADH-coupled ATPase assay shows no significant effect on UvrA's ATPase when 20 μ M Bleomycin is in solution.

5.2.3 Bleomycin is strongly synergistic with cisplatin in clinical isolates

After demonstrating this unusual cooperation with NER, we wanted to evaluate this effect *in vivo*. We then set out to characterise its antimicrobial activity by measuring its MIC in different strains. Bleomycin showed remarkable antimicrobial activity in MG1655 $\Delta toI/C$ and MG1655 $\Delta toI/C \Delta uvrA$ (48.8 nM for both strains), implying that the compound does not cause damage repaired by NER (Figure 40 A). When the compound was tested in the MDR strain EC958, the concentration required to prevent growth was raised to 3.13 μ M (Figure 40 A).

With this data, we performed the 2D MICs (checkerboard assay) shown in Figure 34 B. The results indicate strong cooperation with cisplatin, especially in the clinical isolate strain, despite the need for higher concentrations. In MG1655 $\Delta toI/C$, cisplatin increased its maximum antimicrobial activity by ~8-fold as well as bleomycin.

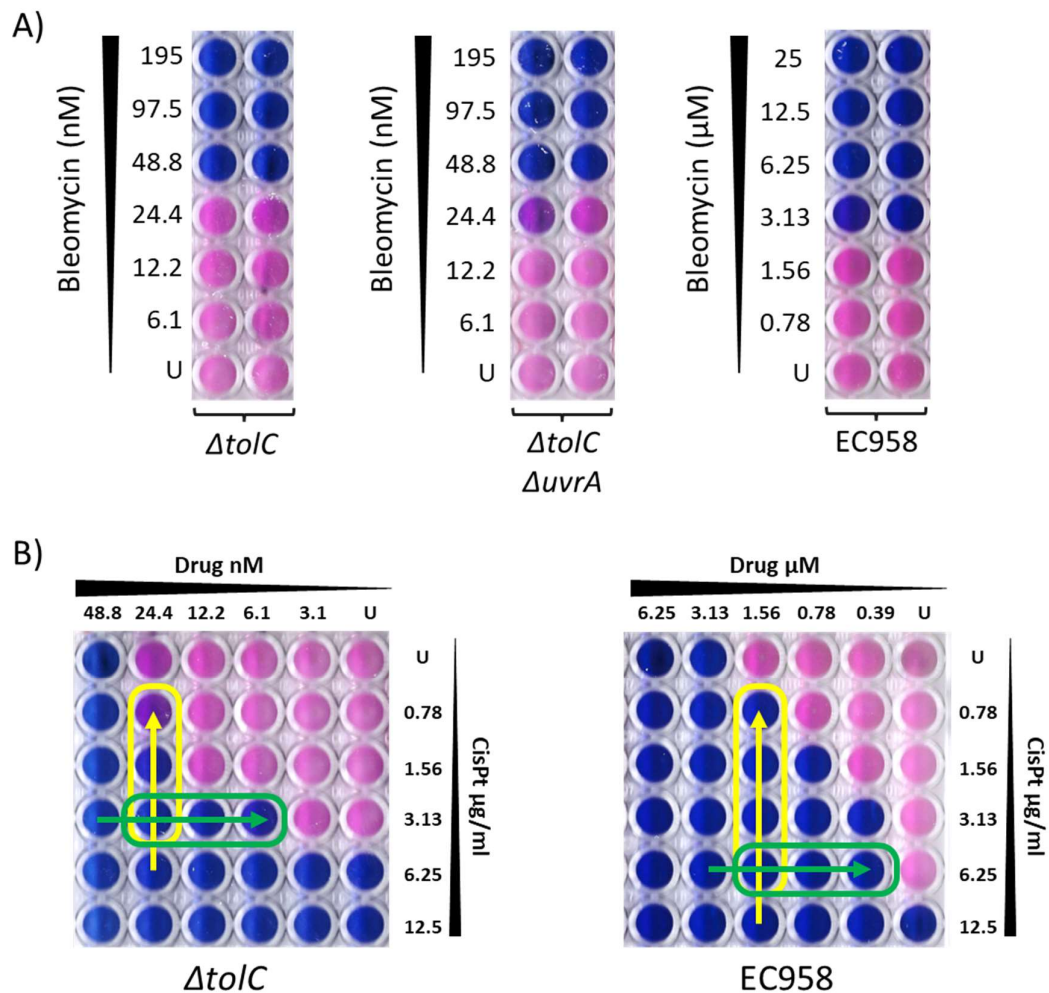


Figure 40: Survivability assay for Bleomycin and combinatorial effects with cisplatin. A) MICs for bleomycin in different strains, *E. coli* MG1655 $\Delta tolC$ and $\Delta tolC \Delta uvrA$, exhibit the same MIC (48.8 nM) for the compound, while EC958 shows a MIC of 3.13 μM . **B)** The combinatorial effect with cisplatin shows an important improvement in the killing ability of both compounds (yellow and green arrows). Remarkably in EC958, we can observe strong cooperation with a major shift for cisplatin (16-folds, yellow arrow) and an important shift for bleomycin (8-folds, green arrow). The pictures are representative of three independent replicates.

5.2.4 UV-damage repair assay confirms its anti-NER activity

To further confirm that the antimicrobial activity was through a combined effect between bleomycin and nucleotide excision repair, we directly tested the ability of the compound to prevent NER from rescuing bacterial survivability after DNA damage. In this assay, the plasmid chosen carried a Cm^{R} due to the high resistance of EC958 to ampicillin. After the incubation period, the plates were imaged, as shown in Figure 41. A significant reduction in CFU is immediately visible when comparing the two plates, indicating a substantial effect on NER.

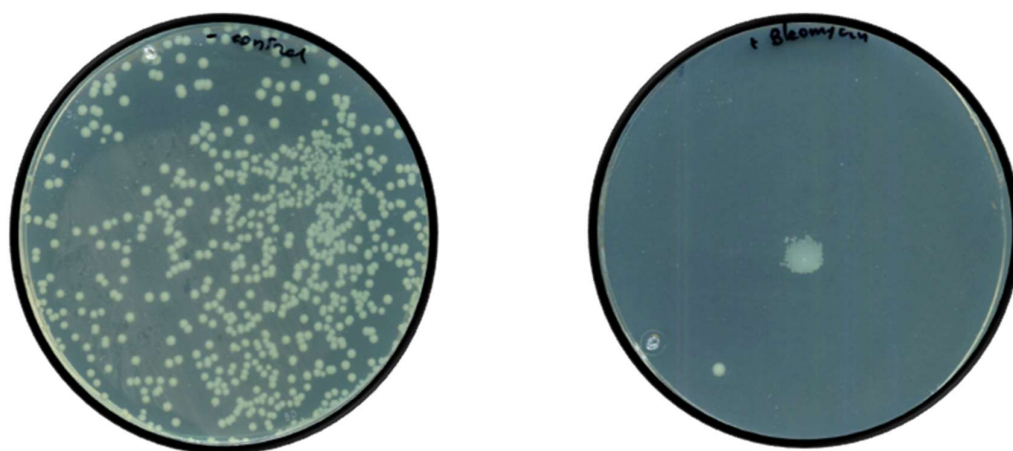


Figure 41: UV-damage repair assay with Bleomycin in EC958. Representative pictures showing the CFU after the incubation (at 37°C) following the transformation of a UV-damaged (UVC, 200 J/m²) plasmid carrying the Cm^{R} in the absence (left) or presence (right) of bleomycin during the recovery step. A drastic reduction in the number of colonies can be observed. The pictures are representative pictures of three replicates.

5.2.5 Discussion and conclusions on bleomycin

Bleomycin surfaced as an interesting compound during a preliminary evaluation of a reduced panel of FDA-approved compounds (~1000) done by Dr Alexandra Moores on MG1655 *E. coli*. In that previous evaluation, the bacteria were inoculated at a much higher concentration (initial OD₆₀₀ 0.1), while the drugs were used at a reduced quantity (1 µM).

Upon testing bleomycin's MIC in MG1655 $\Delta toI/C$, we found the drug to be heavily toxic with a minimal inhibitory concentration of ~49 nM (Figure 40). This fact explains why the compound showed killing activity in our screening (drugs tested at 20 µM) even in the absence of cisplatin or UV radiation. However, despite not fitting our exclusion criteria reported in Figure 31, the drug showed promising characteristics and apparent synergism with cisplatin. The combined action between cisplatin and bleomycin has already been studied in the past through incision assays (340). The authors proposed a cooperation that sees cisplatin responsible for creating DNA adducts guiding bleomycin in additional sites on the DNA, thus facilitating DNA cleavage (340). Similarly, NER and bleomycin could cooperate to cause bacterial death by creating additional DNA cleavage and non-specific incisions leading to catastrophic dsDNA breaks. To verify this hypothesis, we measured the combination of UvrABC and bleomycin's incision levels, comparing it with the basal incision of the two agents alone (Figure 39 A). We could show how bleomycin can produce nicks *in vitro* which are comparable to the incision level produced by NER in a fluorescence-based incision assay (Figure 39). Furthermore, when UvrABC and bleomycin are incubated together the combination clearly indicates a cooperative action. We could confirm the results with a traditional gel-based assay. Additionally, the gels showed a more synergistic effect than the one measured through the fluorescence-based incision. When the UvrABC protein was alongside the drug, the DNA disappeared into a smear, indicating the formation of dsDNA breaks (see the disappearance of the bands in Figure 39 B) possibly representing the cause of the harmonious behaviour *in vivo*.

To evaluate the possible clinical significance of this cooperation, we looked at the impact on bacterial survival of the simultaneous exposure of cisplatin (used to activate NER) and bleomycin (Figure 40). The improved antibacterial effect was evident in both the strain tested (MG1655 $\Delta toI/C$ and the clinically relevant EC958). When cisplatin is co-administered

with bleomycin in *E. coli*, we see a drastic increase in antibacterial activity, up to 16 and 8-fold (respectively for cisplatin and bleomycin) in the MDR strain EC958. These results showed potent cooperation between cisplatin and bleomycin, offering promising future prospects.

Despite having good evidence showing how NER is affected by bleomycin, we set out to confirm it further through a UV-damage repair assay (Figure 41). In this experiment, only a functional NER can rescue the transformant cells carrying a damaged plasmid able to promote antibiotic survival. The experiment clearly shows how the transformant EC958 cannot efficiently repair the DNA lesions when recovered in the presence of bleomycin, demonstrating the drug effects on NER.

The benefits of combining bleomycin and cisplatin to treat cancer patients with poor prognoses have already been shown (336, 341). Specifically, bleomycin is still used in combination with etoposide and cisplatin (BEP) for the treatment of testicular or ovarian cancers (341, 342). However, similarly to other anticancer drug, bleomycin shows a narrow therapeutic profile with pulmonary damage among the most severe form of toxicities (332, 343). These side effects are well-documented and can even be fatal. Furthermore, lung damage can represent an increase in the infection risk (343). However, bleomycin doesn't exert any bone marrow toxicity (332), counterbalancing the increase in susceptibility to infection due to pulmonary damage and representing a safer option compared to other anticancer drug. These considerations and further assessment on bleomycin's toxicology profile both alone and in BEP will be essential in future investigations.

In this study, however, we do not assess their potential in cancer treatment but evaluate their effects on bacterial survival. We show how bleomycin, cisplatin and DNA excision repair are involved in a cooperative system which synergistically leads to bacterial cell death, even in a multidrug-resistant *E. coli* strain. We argue that the combination of cisplatin and bleomycin could be beneficial in preventing or treating multi-drug-resistant infections that often arise during hospitalization in cancer patients. Considering that both cisplatin and bleomycin are well-established chemotherapeutics, and their pharmacokinetics are available, new studies using clinically relevant conditions could be

enough to justify a change in the prescription regimen in cancer patients to achieve immediate benefits.

5.3 Aprotinin

5.3.1 Introduction

Aprotinin is a bovine pancreatic trypsin inhibitor (BPTI) composed of only 58 AA as a single and tightly compact chain (Figure 42). Its tertiary structure renders the molecule stable against temperature, solvents or degradations, characteristics which allow for numerous laboratory applications (344).

The small polypeptide can efficiently inhibit several serine proteases (such as trypsin, chymotrypsin and plasmin) by forming stable complexes in solution with their catalytic sites through its loop region (Figure 42 green) (344). These interactions are thought to require minimal conformational adjustments representing the reason for the formation of stable aprotinin-protease complexes (344). Aprotinin has been investigated for several applications (a summary is listed in Figure 42). Principally, the peptide is used to protect proteins from degradation (as a component in several protease inhibitor cocktails) or as a reference weight marker due to its good stability in solution (344, 345). Aprotinin has also been extensively investigated for clinical applications (344) ranging from a possible antimicrobial activity (346) and more recently for cancer chemotherapy (347) and even as an antiviral drug against SARS-CoV-2 (348), remaining the subject of repositioning studies aiming to find new uses for the peptide. In particular, the molecule was extensively evaluated for the treatment of blood loss after surgery for its major impact on the coagulation system, mainly due to the ability to inhibit plasmin- and kallikrein-mediated fibrinolysis (349). Marketed as Trasylo[®] (Bayer), aprotinin demonstrated effectiveness in reducing transfusion needs and blood loss in patients undergoing coronary artery bypass, heart valve replacement or other kinds of surgery (349). Furthermore, early clinical trials generally reported it to be well tolerated with no significant incidence of serious adverse effects (349). For example, a relatively recent study based on *in vitro* and *in vivo* (on a rat model) observations shows aprotinin's multiple effects on coagulation by inhibiting fibrinolysis and rat bleeding while doubling coagulation times and preventing thrombus formation (350).

Aprotinin was officially withdrawn from therapy later in 2007 following the results of a clinical trial showing an increased risk of death associated with the peptide treatment (351), blocking its use in therapy until recent years when the peptide was re-approved for uses in the European market for the treatment of patients undergoing coronary bypass with an associated high-risk of major blood loss (352).

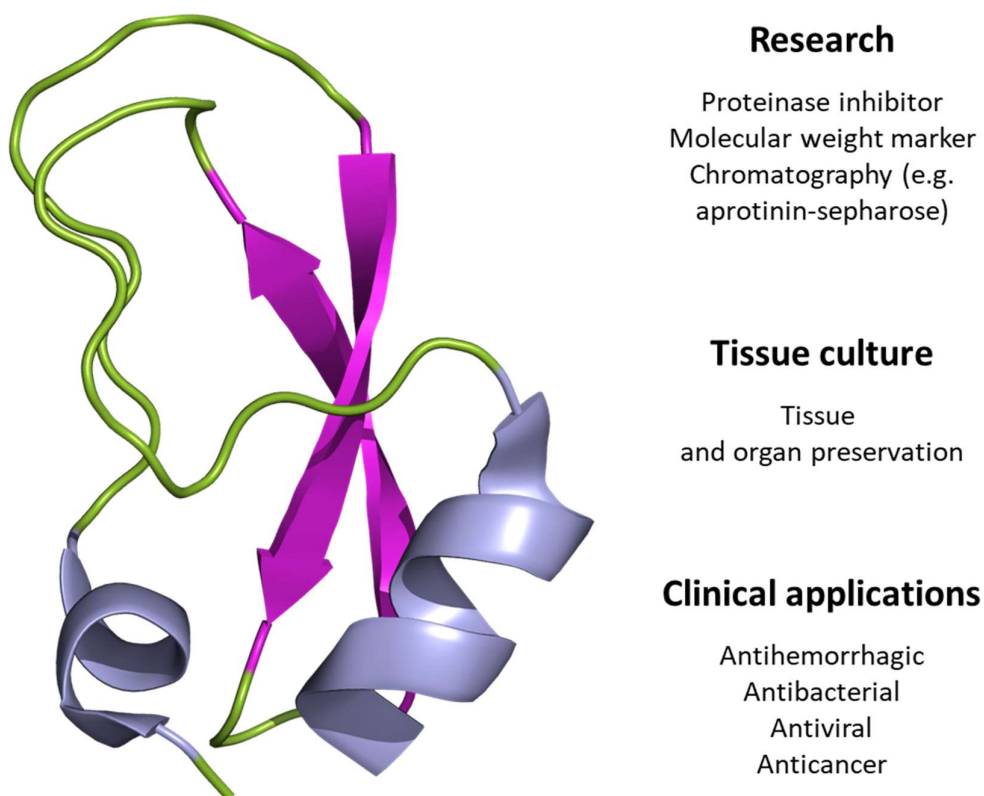


Figure 42: Summary of aprotinin`s explored applications. The figure shows the structure of aprotinin retrieved from PDB (PDB:3LDJ) and visualized with PyMOL and a summary of its principal used and investigated applications.

During our FDA-approved screening (chapter 4), aprotinin appeared active against *E. coli* $\Delta toIC$ when combined with cisplatin and maintained the activity even when we switched to UV radiation as the damaging agent (however, the compound was excluded for the reasons reported in the discussion at the end of this chapter). Therefore, we set out to study the compound by measuring its minimal inhibitory concentration alone and combined with cisplatin. The simultaneous presence of both drugs displayed a more potent antibacterial

activity, and the drug was found to be able to inhibit NER-mediated incision. Furthermore, the peptide acted on UvrA directly, diminishing its ATPase activity and severely modifying its binding properties. Interestingly UvrA was observed to possess a dramatically increased binding ability opposite to what was previously found for other UvrA inhibitors. The mechanism of action remains unclear, and further investigations are necessary to elucidate how aprotinin affects UvrA and its possible role in therapy.

5.3.2 Aprotinin impairs NER while causing a tremendous increase in DNA binding

Interestingly aprotinin didn't display any toxicity against MG1655 $\Delta toI/C$ and $\Delta toI/C \Delta uvrA$ at the concentrations tested, as shown in Figure 43 A (left). However, when combined with cisplatin (in MG1655 $\Delta toI/C$), the antitumoral compound increased its antibiotic activity by ~4-fold while aprotinin had at least a 2-fold shift in its MIC Figure 43 A (right). However, aprotinin showed limitations in solubility, necessitating ultrasonication for proper dispersion in solution. Consequently, reaching higher concentrations for the tests was impossible.

Having established a cooperative action between the compound and cisplatin *in vivo*, we evaluated its effects on DNA incision. We performed a fluorescence-based incision assay following the previously established pipeline of experiments for an anti-NER characterization. In the assay, 20 μ M of the peptide could inhibit incision only at the 16h incubation point. The compound reduced the incision level by ~52% of the total untreated sample activity (Figure 43 B).

Having shown the compound's anti-NER properties *in vitro*, we assessed UvrA ATPase as the possible molecular target of the peptide. Our findings in Figure 43 C show that 20 μ M aprotinin cannot significantly inhibit UvrA. However, when the peptide concentration is raised to 50 μ M, we recorded a significant reduction in the ATPase activity with a k_{cat} dropping by ~67 and 87%, respectively, when in the absence and presence of pUC18 DNA.

The following experiment using the optical traps was performed by Antoine Paris, who also designed the method and analysed the data.

We have previously shown how an impairment to UvrA's ATPases also caused deficiencies in DNA binding according to previous observations (212, 244). Unexpectedly, the number of UvrA binders drastically increased when UvrA was incubated with 50 μ M Aprotinin before being visualized onto DNA. The number of binders per minute is reported in the bar chart in Figure 43 D.

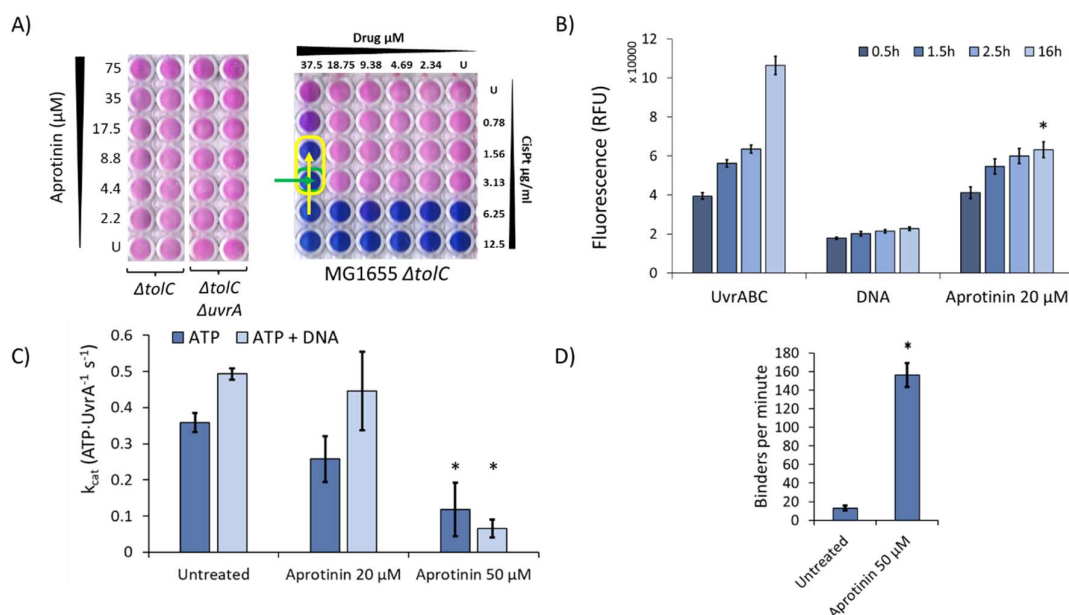


Figure 43: Aprotinin can impair NER. Aprotinin's activity evaluation. **A)** On the left, we show the MIC for the compound in MG1655 $\Delta toI/C$ and $\Delta toI/C \Delta uvrA$. No MIC could be observed at the concentration tested. On the right, the combinatorial effects with cisplatin in MG1655 $\Delta toI/C$ are shown. Cisplatin improved its antibacterial effect by ~4-fold (yellow line), while aprotinin had an indefinite (but ≥ 4 -fold) shift (green line). The pictures are representative of three independent replicates. **B)** Fluorescence-based incision assay showing significant inhibition of the UvrABC system (~50% reduction) at the 16h time point when compared to the untreated sample (UvrABC). The error bars represent the standard error of the mean ($n \geq 6$). The results were considered significant if $p \leq 0.05$ (*) (T-test) when compared with UvrABC. **C)** NADH-coupled ATPase assay with and without 20 or 50 μM aprotinin. The k_{cat} only shows a reduction in UvrA activity with the higher compound concentration. The error bars represent the standard error of the mean ($n \geq 3$). The results were considered significant if $p \leq 0.05$ (*) (T-test) when compared with the untreated sample UvrA. **D)** Data on optical trap collected and analysed by Antoine Paris shows a tremendous increase in binders per minute on suspended DNA when UvrA is treated with aprotinin. The error bars represent the standard error of the mean ($n \geq 3$), and the results were considered significant if $p \leq 0.05$ (*) (T-test).

5.3.3 Discussion and conclusions on aprotinin

In Chapter 4, we presented the most promising hits that surfaced during the screening. However, other compounds with limited availability or reduced activity were also investigated. Among those compounds, aprotinin could impair the growth of *E. coli* MG1655 Δ tolC only in the presence of cisplatin or UV radiation. When re-tested, the polypeptide proved to be non-toxic with no measurable MIC up to 75 μ M (Figure 43 A). Despite these facts, aprotinin had limited availability and presented solubility issues (required ultrasonication), which, coupled with its high molecular weight, rendered reaching the necessary concentration challenging, forcing us to prioritize other compounds.

Nonetheless, our preliminary *in vivo* data showed an antibacterial activity only in the presence of DNA damage with both cisplatin and UV, suggesting a cooperative action against NER. Therefore, we re-tested it through a checkerboard assay finding significant cooperation between cisplatin and aprotinin. Electron microscopy observations have shown that aprotinin can lead to the cytoplasm's disintegration with bacterial body swelling (346). Therefore, it is possible that this severe damage could overwhelm the bacteria rendering them unable to repair the cisplatin's DNA damage efficiently. However, it is important to point out that we have shown direct effects against NER and, specifically, UvrA.

Specifically, in the incision assay, aprotinin could significantly delay DNA nicking after 16 h of incubation (but not in the other time points), either indicating a slow action on the proteins or that the concentration of 20 μ M was insufficient to inhibit incision effectively. In agreement with the latter statement, when aprotinin was tested against UvrA, 20 μ M could not significantly inhibit the ATPase activity. However, a significant inhibition was measured when the concentration was raised to 50 μ M. Furthermore, 50 μ M aprotinin drastically affected the DNA binding abilities of UvrA *in vitro*, increasing the number of binders per minute $\sim$ 100 times and prolonging the protein lifetime on DNA by $\sim$ 25% (preliminary data collected and analysed by Antoine Paris). The reasons for this phenomenon are still unclear. Additional tests would be required to understand how the

interaction between the two molecules can disrupt the UvrA ATPase function while multiplying the number of binders.

An initial evaluation of the interaction could be obtained utilizing docking software (such as HDOCK or AlphaFold multimer) to predict protein-protein binding. In principle, feeding the UvrA₂-DNA (PDB:3PIH) or the UvrA₂ (PDB:3UX8) structure and aprotinin (PDB:3LDJ) to the software might help in understanding the nature of their interaction by simulating their possible complex. However, docking results without empirical evidence are difficult to interpret and must be analysed with caution. Assays aimed at understanding aprotinin-UvrA interactions would be necessary to clarify the mechanism and validate the computational data. For example, crosslinking aprotinin with a thermostable UvrA (such as *Thermotoga maritima*) (353) would create a crystallizable complex providing empirical data and precise insights on aprotinin-UvrA interactions.

Despite the results shown against NER, aprotinin doesn't represent an immediately viable candidate for in depth clinical studies. Some concerns emerged about aprotinin's safety profile, and in 2007-08, the UK and EU suspended the license for the drug (354). However, these safety reports were later opposed by contrasting evidence, and aprotinin was ultimately reintroduced in clinical use for high-risk patients (352, 354, 355). These strict requirements might not be a limit for an eventual reposition of aprotinin for adjuvant cancer chemotherapy, considering the high risk represented by infections. However, the concentration required for the anti-NER effect represents a more concrete limit for the peptide applicability in therapy. Aprotinin was found active only at high concentrations, even using the efflux pump knockout $\Delta toI/C$ needed 50 μ M of the peptide in the conditions of the experiments.

Therefore, unlike other drugs discussed in this thesis, the journey to bring this molecule into clinical use is only at the beginning. Nonetheless, we believe that aprotinin represents an interesting candidate for further investigations to better understand the interaction with UvrA, its DNA binding stimulating activity and its possible role as an adjuvant antimicrobial.

Chapter 6 – Characterization of bemcentinib as a NER inhibitor acting as an adjuvant antimicrobial against a clinical isolate strain.

Small sections of this chapter include works done by Antoine Paris (data collection, analysis, and methodology on the optical trap) and Dr Arya Gupta on the gel-based incision (data collection and analysis). External contributions were left for clarity and are specified in the figure legend and text.

6.1 Introduction

After having characterized UvrA as the molecular target of the drug-like NER inhibitor ATBC (chapter 3) and proposed the ATP-binding pockets as the inhibition sites, we set out to target UvrA directly. We specifically targeted the distal and proximal sites using a computational approach parallel to the manual screening (chapter 4).

Following the suggestion that bigger libraries yield better results than smaller ones (356), we prepared the most extensive selection of compounds our system could handle. The library was populated with ~170000 drug-like molecules (among which ~120000 unique compounds), including molecules reported active *in vitro* and available for sale (downloaded from ZINC and prepared with OpenBabel) (25, 250). Ultimately we performed molecular docking simulations to predict their activity using the computationally generated *E. coli* UvrA (AlphaFold) (16).

The virtual screening was performed using the open-source AutoDock Vina (19) and a custom Python code to increase the automation and the level of parallelization. The results were sorted by their computed docking score (binding energy), and one compound was chosen to be fully characterized. The findings show that bemcentinib can bind to UvrA₂, drastically impairing its ATPase activity and disrupting DNA binding. This biochemical evidence results in a heavily reduced *E. coli* survivability when the compound is co-administered with DNA-damaging agents. Furthermore, this activity was proven to be extended to the multidrug-resistant clinical isolate EC958 providing the rationale to bring the compound forward for additional studies in more relevant clinical setting.

6.2 Results

6.2.1 Screening time improvements with higher levels of parallelization

AutoDock Vina uses multiple CPUs to speed up the docking (19, 40). However, opening/reading and writing files are discrete operations requiring a fixed amount of time that could benefit from increased parallelism. Moreover, many small molecules with few torsional degrees do not require intensive computational power to be docked by the software. Therefore, we evaluated a possible time-saving strategy allocating multiple jobs simultaneously and limiting the CPU available per job. We wrote a Python code (with the collaboration of Jake McGreig, Dr Robert Charman and Antoine Paris) allowing timed and sequential multi-molecule submission with the possibility of changing the number of CPU cores allocated for each singular submission. We prepared two random sets of molecules containing 500 and 10000 items. In doing so, we wanted to evaluate possible slowdowns in handling different entry volumes and have a generic pool of compounds not biased by chemical similarity (the number of torsional freedoms impacts the docking speed). The results are reported in Figure 44 as a plot of the total run time over the number of CPUs allocated for a single job. Our machine has an ordinary 8-core processor (Intel i7-9700) that was able to finish the docking with total times of ~1h and ~21 h, respectively, for the 500 and 10000 sets of molecules. Therefore, the software took an average of ~7.5 seconds per molecule, unaffected by the size of the set tested. Splitting the available processing power to 4 CPUs per job submitted and doubling the simultaneous docking instances, the total run time decreased by 24% with an average docking-per-molecule of ~5.7 seconds. Further cutting in half the available processing power while increasing the number of tasks saved a further 8% of total run time (32% shorter compared to the original settings) with an average of ~5.1 seconds per molecule. As evident in Figure 44, only minimal increments were achieved when the CPUs were further divided, with minuscule improvements from 1/16 to 1/32 CPUs per job. The maximum velocity peaked at ~4.6 seconds per molecule, saving 38% on the total run time in both conditions.

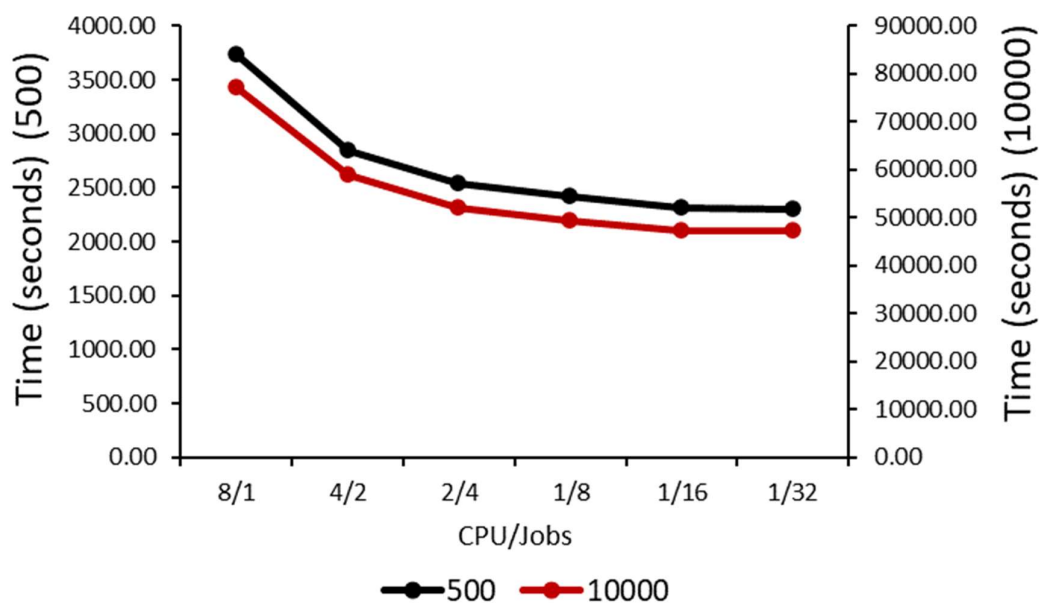


Figure 44: Evaluating the efficiency of multiple jobs submission against the available computing power. Multiple docking speed tests to evaluate the time improvement. Two sets of random molecules containing 500 and 10000 compounds were docked. The total time was plotted against the CPU used per job (CPU/Jobs). With the limited amount of CPU available in the machine, we can observe a reduction of ~24% passing from 1 to 2 jobs submitted simultaneously and a maximum time reduction of ~38% when the system is saturated.

6.2.2 Virtual screening highlights a possible new hit

Having established UvrA as a viable molecular target to cause deficiencies in NER (as shown in Chapter 3), we started a fast and efficient computational approach to identify possible hits. The protein structure of choice was the AI-generated *E. coli* UvrA (retrieved from AlphaFold (16, 35)), and the docking software chosen was AutoDock Vina (19, 249).

A library of drug-like molecules reported active *in vitro* and available for purchase was downloaded from ZINC, one of the largest chemical databases (17). The library used was comprised of ~170000 compounds (among which 117760 unique structures) and then converted in bulk to the appropriate format (.pdbqt). The protein and the search space, including the distal and proximal sites, were defined with the open-access software MGL tools 1.5.7 and the compounds were screened with the custom code reported in the appendix (L – Code for virtual screening).

Figure 45 A reports the screening results as a histogram of the number of binders per the different binding energies the software gave. The figure shows that ATP was docked alongside the other compounds to establish a baseline for proficient binding energy. The endogenous ligand ATP scored in position 13452 with a reported - 9.6 kcal/mol binding energy in its highest affinity mode.

To minimize the number of compounds purchased, we examined the 50 highest-scoring binders for interesting features in search of possible candidates to be tested *in vitro* against purified UvrA, as done previously (357). Among these high-scoring molecules, we found phosphates compounds (such as cGAMP, Cyclic di-AMP, Myo-inositol trispyrophosphate) as well as very low specificity compounds (such as tetracosafuorophenanthrene), which would likely bind multiple ATPase cassettes. Interestingly in position 35 (-12.4 kcal/mol), we found Hinokiflavone (or biapigenin), an apigenin analogue that surfaced as an exciting compound acting against NER in the screening (chapter 4) and reported in the appendix (E). Furthermore, the compound N-(9-Fluorenylmethoxycarbonyl) doxorubicin, a pirarubicin analogue active even against EC958 in the previous chapter, scored in position 42 (-12.3 kcal/mol).

Ultimately, we settled on bemcentinib (R428) as a promising hit (position 46, -12.3 kcal/mol) able to bind both the ATPase sites (Figure 45 B) with higher affinity than ATP. We mainly chose this compound due to its failed use as a highly specific AXL inhibitor as an adjuvant in anticancer therapy and its availability.

Before testing the compound *in vitro*, we re-ran it virtually using the same search space but increasing the exhaustiveness by 1000x (as described previously in chapter 4.2.6) to control for a false positive and increase the chances of more reliable data. The docking confirmed the previous result showing a propensity of the compound to bind the proximal site (- 12.3 kcal/mol) better than the distal one (- 11.6 kcal/mol).

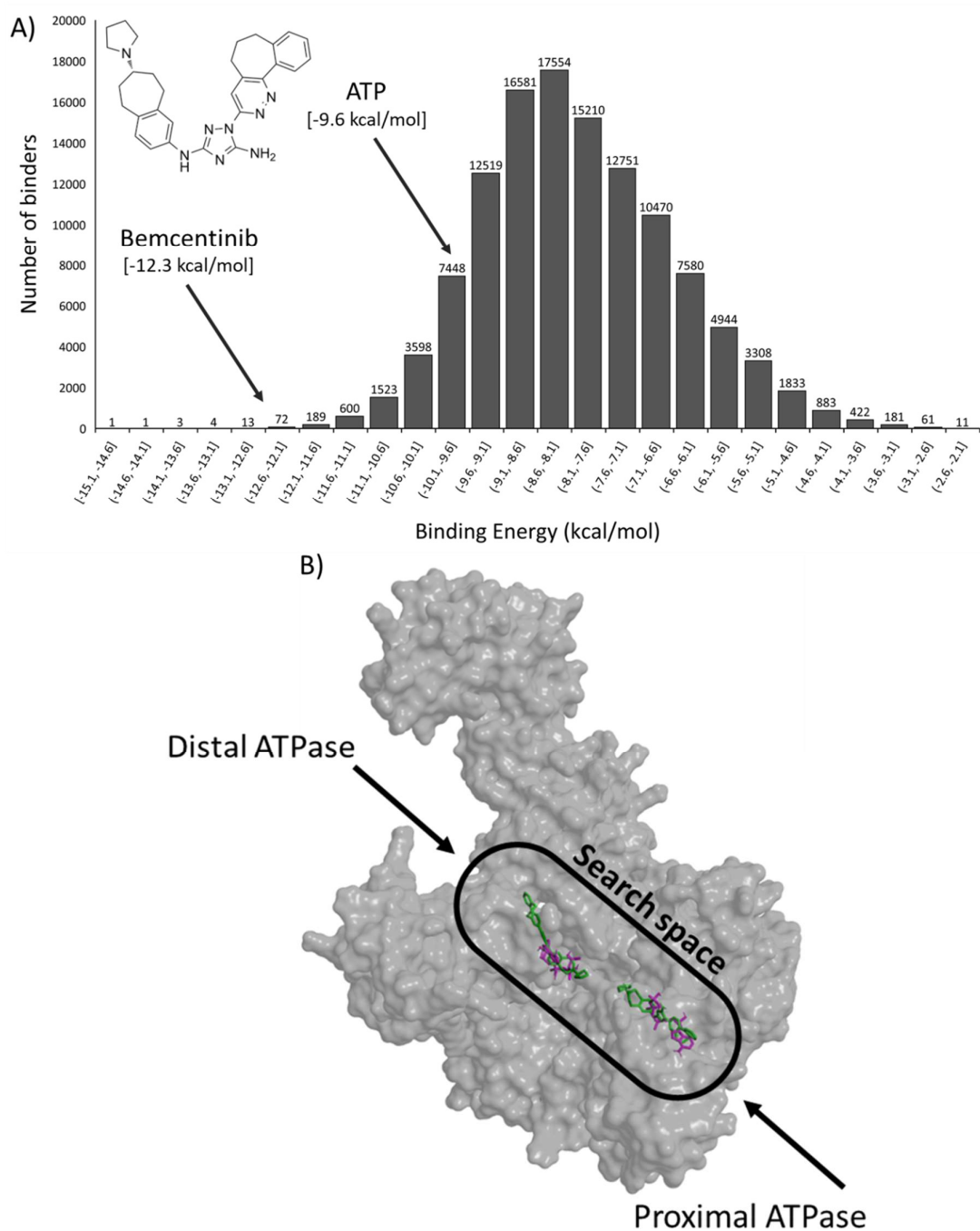


Figure 45: In-silico screening shows a possible hit of ~120000 candidates. A) Histogram reporting the number of binders over the binding energy. The first 50 candidates over the total amount of compounds screened showed a binding energy considerably lower than the one for the endogenous ligand (ATP) docked as control. **B)** *E. coli* UvrA 3D surface retrieved from AlphaFold and generated using PyMOL shows the highest-scoring conformations in the two ATPase of both ATP (magenta) and Bemcentinib (green).

6.2.3 Bemcentinib effectively inhibits UvrA *in vitro*

Having good computational proof pointing to bemcentinib as a possible UvrA inhibitor, we decided to test the compound directly against purified UvrA. In solution, UvrA forms a homodimer UvrA₂ (referred to as UvrA for simplicity) with 4 total ATP-binding pockets cooperating with a complex and yet not fully understood mechanism (213, 220, 244, 258). We titrated bemcentinib with 50 nM UvrA in a saturating ATP environment in the presence and absence of pUC18 DNA and calculated the respective k_{cat} . The data was plotted over the concentrations used and then fitted to the Hill equation (246) to indicate the nature of the inhibition. In this experiment, we first measure the catalytic activity after adding ATP to start the reaction. The observed decrease in k_{cat} (Figure 46 A) fitted through the hill equation shows a sigmoid-like relationship with a small Hill coefficient (~ 1.8). This fit also provides an IC_{50} of $7.49 (\pm 0.69) \mu M$. The nature of the fit indicates cooperativity between two possible sites.

Interestingly when pUC18 DNA was added to the solution to stimulate the ATPase activity as previously reported (244), we noticed a shift to strongly cooperative binding (figure 2A). The sigmoidal nature of the fit and the calculated Hill coefficient up to ~ 3.6 suggest multiple sites involved in the inhibition. The presence of DNA also changes the IC_{50} for the compound to $9.78 (\pm 0.23) \mu M$.

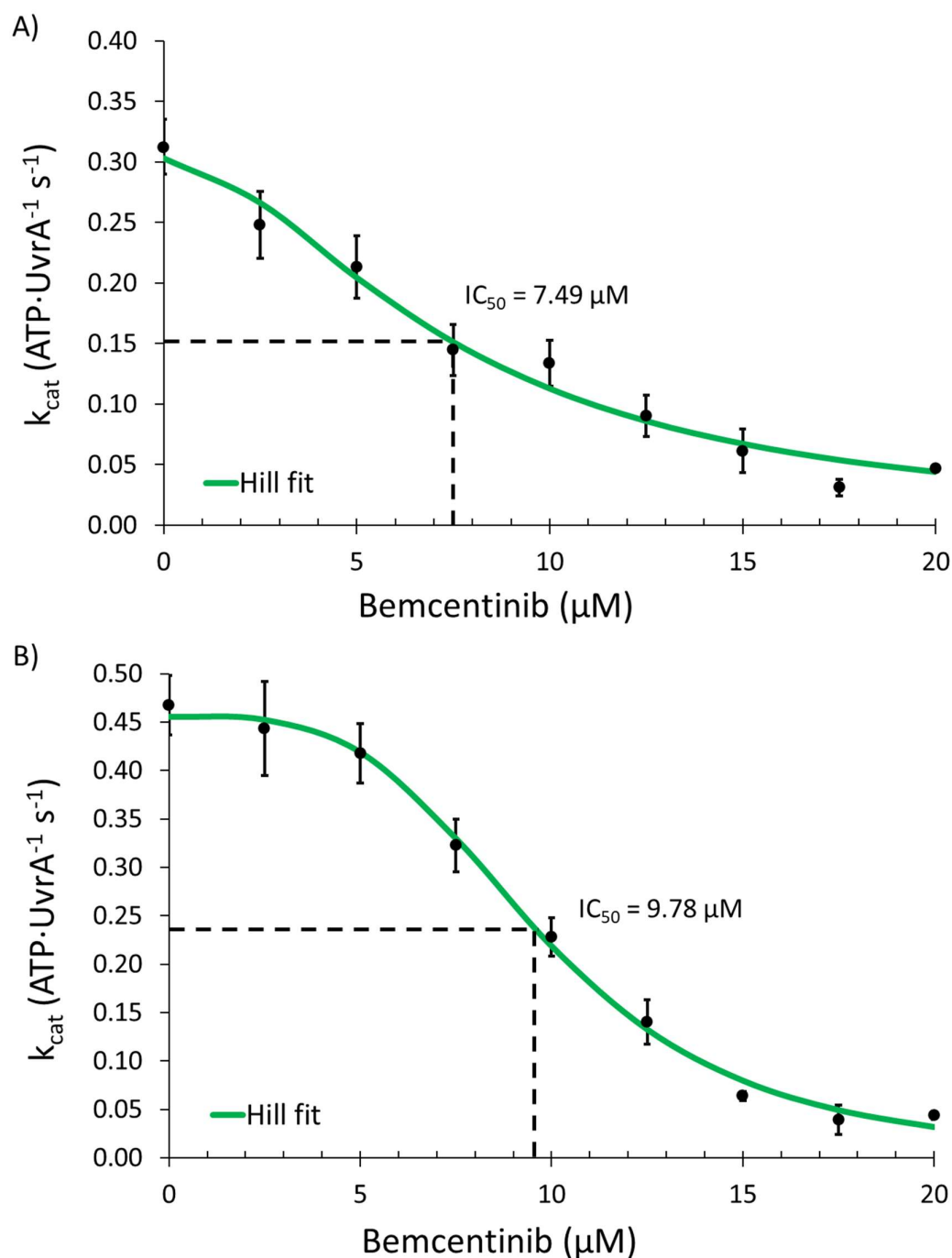


Figure 46: Real-time NADH-coupled assay to confirm the molecular docking. Titration of bemcentinib inhibitory effect on 50 nM purified UvrA. **A)** Bemcentinib can inhibit ATP turnover showing an IC_{50} of 7.49 ($\pm$ 0.69) μ M. The green fit represents the Hill coefficient equation (Hill coefficient $\sim$ 1.8) calculated in Excel through data solver. **B)** ATPase rate calculated after further stimulation with 0.1 ng/ μ l pUC18 DNA. The IC_{50} 9.78 ($\pm$ 0.23) and Hill coefficient ($\sim$ 3.6) show robust cooperative behaviour with multiple sites in opposition to what is seen in the absence of DNA. The experiments were repeated three times, and the error bars represent the standard error of the mean (n=3).

UvrA DNA binding ability is compromised when in the presence of bemcentinib

The data in this section was collected by Antoine Paris, who also defined the methodology. The results are only included for clarity.

In this experiment, we can trap DNA among two silica beds and directly visualize fluorescently labelled. We used UvrA₂ tagged in the C-terminally with mNeonGreen to visualize DNA binding (Figure 47, the DNA is unlabelled; therefore, we only see binders) and then repeated the experiment in the presence of 50 μ M bemcentinib. This significantly decreased the binding to the DNA strand (Figure 47). In order to quantify the inhibitory effect of bemcentinib, we then reduced its concentration to 20 μ M to enable enough binders to calculate a relative binding affinity. We observed a reduction by $\sim 90\%$ of the number of binders at this concentration compared to untreated samples (Figure 47, n. untreated: 824, n. treated: 99).

The results were consistent with the evidence collected during the ATPase assay and proved an intense activity on UvrA's ability to function correctly.

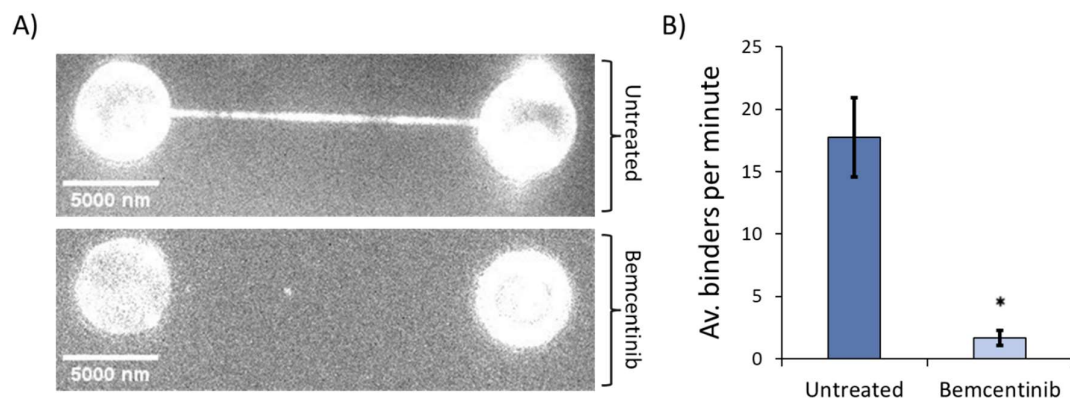


Figure 47: Real-time investigation of UvrA binding ability on suspended DNA. The optical trap data collection and analysis were done by Antoine Paris A) Unlabelled double-stranded DNA suspended between silica beads in the presence and absence of 50 μ M bemcentinib. The top pictures clearly show a functioning UvrA mNeonGreen able to coat the DNA in the absence of the drug completely. The bottom picture shows only sporadic binders when bemcentinib is present in the solution. B) Quantification of the average binders per minute when bemcentinib concentration is lowered at 20 μ M. A reduction of $\sim 90\%$ can be observed. The results were considered significant if $p \leq 0.05$ (*) (T-test) when compared to the untreated sample. The error bar represents the standard error of the mean (n=6).

6.2.4 Bemcentinib inhibits DNA incision *in vitro*

Having confirmed bemcentinib's ability to inhibit UvrA's ATPase and DNA binding, we evaluated its action against the entire NER system *in vitro*. We applied the already established fluorescence-based incision assay in the presence and absence of 20 and 50 μM bemcentinib. The untreated sample (UvrABC) shows the time evolution of the de-quenched fluorescence. No significant change was observed when the proteins were treated with 20 μM R428 compared to the untreated control. However, when the concentration of the inhibitor was raised to 50 μM , a significant reduction in incision was measured at 1.5, 2.5 and 16h up to $\sim 76\%$ for the last timepoint observed (16 h). To further confirm this result, we measured the shift from supercoiled to open circle DNA caused by incision (Dr Arya Gupta collected and analysed the data for the gel-based incision assay). This experiment clearly showed that 50 μM of the compound significantly inhibits incision.

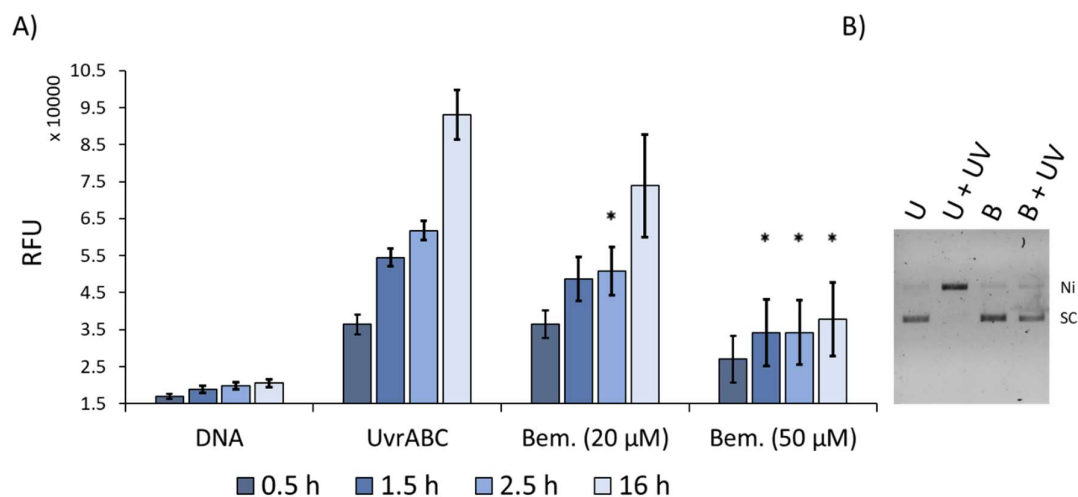


Figure 48: Incision assay shows the activity of the compounds on NER. **A)** Fluorescence-based incision assay shows the ability of bemcentinib to inhibit UvrABC incision to a maximum of $\sim 76\%$ (16h timepoint) when used at 50 μM , 20 μM of the compound was not enough to significantly inhibit the system in the condition used for the experiment. The results were considered significant if $p \leq 0.05$ (*) (T-test) when compared to the untreated sample (UvrABC). The error bars represent the standard error of the mean ($n \geq 6$). **B)** Gel-based incision assay performed by Dr Arya Gupta and incubated for 15 minutes with 50 μM bemcentinib. The gel shows untreated pUC18 with the system UvrABC(U) and the same after 200 J/m^2 UV irradiation (U + UV). The comparison is made with the equivalent of the two but when in the presence of 50 μM bemcentinib (B and B + UV). The results confirm the fluorescence-based incision. The gel is representative of three independent experiments.

6.2.5 Bemcentinib significantly impairs bacterial survivability

Having proven that bemcentinib inhibits NER *in vitro* through multiple assays, we set out to evaluate its *in vivo* activity. Firstly, we measured the minimal inhibitory concentration of the compound in *E. coli* MG1655 Δ tolC, *E. coli* MG1655 Δ tolC Δ uvrA (Figure 49 A) to assess for its antimicrobial activity with and without a DNA-damaging agent. Both MIC values were 3.13 μ g/ml, indicating that the compound does not generate NER substrates. We then chose 4-NQO to detect if bemcentinib can impair bacterial growth in the presence of DNA damage (Figure 49 B). When the Δ tolC strain was exposed to either 4-NQO (10 μ M) or bemcentinib (1.56 μ g/ml) at sub-MIC concentrations (compare blue to black lines and green to black lines), no critical changes in growth were observed. However, when the two compounds were used together, a significant impairment was observed, leading to minimal growth after 20 hours of observation (Figure 49 B; compare red vs black lines).

To bring our results closer to a clinical application, we investigated the inhibitory effects of R428 on a multi-drug resistant *E. coli* strain (EC958). Comparing the response of wild-type MG1655 with EC958 (Figure 49 C) to bemcentinib revealed that in wild-type, the MIC value was 25 μ g/ml, half that of EC958 (50 μ g/ml). We had previously measured the MIC of cisplatin (MICs in different strains in Figure 30), a common antitumoral agent used in chemotherapy (103), which alkylates DNA requiring NER for survival. For this assay, we incubated the bacteria with a sub-MIC concentration of bemcentinib (25 μ g/ml), measuring a modest ~2 h delay in growth (compare blue to black lines in Figure 49 D). Similarly, we incubated the cells with cisplatin at 1/4th of its MIC (3.13 μ g/ml), recording another modest ~1 h delay in growth (compare green to black lines in Figure 49 D). However, when the compounds were combined, cell growth was prevented throughout the entire observation (compare red to green and blue lines in Figure 49 D).

The experiment indicates strong effects of the combination even in the multidrug-resistant bacteria used (EC958).

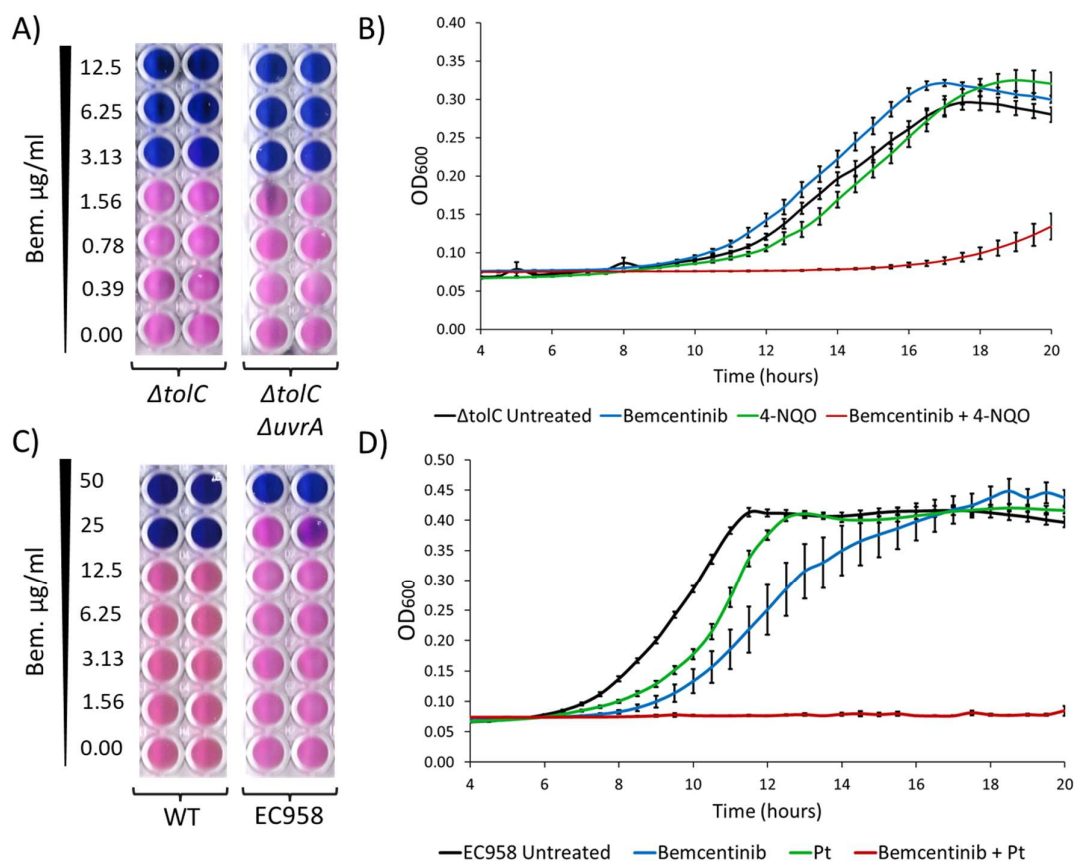


Figure 49: In vivo characterisation of bemcentinib. Survivability assay against different strains treated with bemcentinib. **A)** Minimal inhibitory concentrations in (MG1655) *ΔtolC* and *ΔtolC ΔuvrA* show a MIC of 3.13 $\mu\text{g/ml}$ with no effect when the bacteria have an impaired NER. The result shows that bemcentinib does not produce damages repaired by UvrABC. **B)** Growth curves acquired in a plate reader (Spectramax id5) incubating at 37°C with vigorous shaking. The samples containing sub-MICs quantity of bemcentinib (1.56 $\mu\text{g/ml}$) and the DNA damaging agent 4-NQO (10 μM) do not exhibit important deviations from the untreated sample. However, a significant growth impairment can be observed when the two compounds are combined. **C)** Minimal inhibitory concentrations in wild type (MG1555) and the clinical isolate strain (EC958) show a respective 8- and 16-fold increase when compared to the strain lacking the efflux pump *ΔtolC* (respectively 25 and 50 $\mu\text{g/ml}$). **D)** Growth curves showing the combinatorial effect between cisplatin and bemcentinib. 1/4th of cisplatin MIC (3.13 $\mu\text{g/ml}$) and half of bemcentinib MIC (25 $\mu\text{g/ml}$) can only minimally affect growth. However, when used simultaneously, the combinatorial effect is enough to prevent growth for the duration of the experiment entirely. The data was acquired with multiple independent measurements. The error bars represent the standard error of the mean (n $\geq$ 3).

6.3 Discussion

6.3.1 In-silico study identifies bemcentinib

The fast development of both hardware components and software is enabling more and more research groups to access vast arrays of computational tools for computer-aided drug design (358). These advancements open several doors in the first stages of ligand finding. They can allow for a free evaluation of even billions of compounds (17, 18), provide reliable AI-generated protein structures otherwise unavailable (16) and enable the search for structural similarities on massive and free databases (20, 359). Therefore, it is not surprising to see numerous examples of drugs being designed thanks to the aid of computational methods (29, 30, 360–363).

It has been suggested that using ultra-large libraries (up to billions of compounds) is beneficial in virtual screening (356). However, the computational requirements are unavailable for most academic research teams. Therefore, to decrease the number of compounds and enhance the chances of success, we searched for a library of compounds already reported active *in vitro* and available for purchase. 173234 compounds (the most extensive library our system could handle), among which 117760 unique drugs were downloaded from ZINC (17). In this study, we used AutoDock Vina as our open-source choice. The software uses multiple CPUs to speed up the docking (19, 40). However, opening/reading and writing files are discrete operations requiring a fixed amount of time that could benefit from increased parallelism.

Moreover, many small molecules with few torsional degrees do not require intensive computational power to be docked by the software. Using a custom Python code (Appendix L – Code for virtual screening), we could exploit the CPU to the fullest, increasing the total speed of the docking by ~40%. Our system had a total of only 8 cores and therefore was quickly saturated, measuring marginal improvements after having assigned 1 job/core. Therefore, we believe that systems with more cores would see even better improvements when assigning multiple jobs simultaneously.

Molecular docking can speed up the screening procedures and cut costs but at the price of precision. Researchers have to accept numerous approximations in favour of speed.

Common compromises involve the 3D structure of the protein (the computationally generated model from AlphaFold was used as a rigid model for the docking), the absence of water (which could play a role in facilitating hydrogen bonds *in vivo*) and the preparation of the ligands (converted automatically in bulk through OpenBabel). Therefore, there should not be a pretence for the docking algorithm to be able to rank the molecules realistically but to successfully prioritize ligands with a higher possibility of success from the rest (33, 41).

To ensure the reliability of the screening, we visually inspected the first 50 compounds. We were pleased to find Hinokiflavone (or Biapigenin) and N-(9-Fluorenylmethoxycarbonyl) Doxorubicin among the best-scoring molecules in excellent agreement with the results obtained during the screening described in Chapter 4. Among the other compounds, due to availability, poor chemical characteristics and time constraints, we selected bemcentinib (R428), an easily accessible compound being investigated as an anticancer agent and for a possible role against COVID-19 (364–366). Furthermore, bemcentinib belongs to the family of AXL - tyrosine kinase inhibitors (364, 367), and interestingly, Nintedanib (another tyrosine kinase inhibitor (368)) surfaced during the previous screening (but was not fully characterized due to lack of availability (Appendix C – Additional compounds tested)).

AXL receptors are involved in tumour migration, proliferation and cell survival, therefore, their inhibitors have been developed for cancer treatments (367). Among them, bemcentinib seems to be able to bind competitively to the kinase ATP-binding pocket (364, 367). To verify bemcentinib specificity for UvrA, we searched for analogues reported to act by inhibiting the ATP-binding pockets among the molecules screened. Numerous other AXL inhibitors were among the compounds screened with poor scores. The second top-scoring compound (after bemcentinib) was amuvatinib (another AXL inhibitor (367)) in position 8188 with a binding energy of -9.9 kcal/mol (bemcentinib -12.3 kcal/mol, ATP -9.6 kcal/mol). Other AXL inhibitors were ranked with even poorer scores (e.g. gilteritinib pos.13652, -9.5 kcal/mol; crizotinib pos. 15557 -9.4 kcal/mol: TP-0903 pos. 24585 -9.1 kcal/mol, sunitinib 46257 -8.4 kcal/mol) (367).

As the last confirmatory test, we increased the exhaustiveness of the docking algorithm (1000x) and docked the compound again in the same search space in search of more precise

results. We confirmed the previous docking and found that bemcentinib preferentially binds the proximal site instead of the distal one (-12.3 kcal/mol and -11.6 kcal/mol, respectively) in opposition to ATP's preferential binding (-9.2 kcal/mol and -9.6 kcal/mol, respectively). Nonetheless, bemcentinib seems to have better affinity than ATP in both sites.

Taken together, these were solid indications for a specific activity directed against the UvrA ATP-binding pocket thus, we decided to evaluate the compound *in vitro* and *in vivo*.

6.3.2 Bemcentinib is effective against different strains of *E. coli*

When tested against purified UvrA₂ in an NADH-coupled ATPase assay, we could immediately confirm the *in silico* results, measuring a reduction up to ~90% of its total activity in the presence of 20 μ M of the compound with a good IC₅₀ in the μ M range (~7.5 and ~9.8 μ M respectively in the absence or presence of DNA). Moreover, when bemcentinib was titrated at different concentrations, we could fit the reactions to a Hill slight sigmoidal curve with a Hill coefficient of ~1.8, indicating that the nature of the inhibition had a degree of cooperation between different sites. However, upon adding pUC18 DNA, previously reported to enhance UvrA₂ catalytic activity (244), the reactions changed to a strong sigmoidal trend well represented by a Hill fit with a Hill coefficient of ~3.6. Sigmoidal fits are usually typical of cooperative interactions (e.g. haemoglobin, GroEL, etc) (369, 370) therefore, our results imply a potent cooperative inhibition with multiple different sites involved.

Traditionally the Hill coefficient has been used as an indicator of the number of sites involved in the reaction after the observation that the sigmoidal behaviour of oxygen-haemoglobin binding was best described by a Hill coefficient of 4 (246). Although this mathematical approach has limitations and it is not always accurate, it provides a starting point to speculate on how bemcentinib is showing these 2 different profiles of inhibitions.

In chapter 3.1.1, we have already discussed the recent findings about UvrA and the role of the ATPases. As already stated, their specific functions are not yet fully understood; however, the distal and proximal sites seem to cooperate, possibly through an exchange of phosphate groups through a hollow cavity connecting the two sites. Moreover, the distal site seems responsible for fast ATP turnover, while the proximal site is able to modulate the distal site activity (220, 244, 258). It was observed that when UvrA₂ is in the absence of DNA, the distal ATPase is mainly responsible for the catalytic activity, with minimal activity in the proximal sites (258). However, when DNA is introduced, both the distal and proximal sites get activated, resulting in a complex system influenced by the distortions caused by DNA binding (244, 258).

We argue that our findings indicate that in the absence of DNA, only the distal sites catalyse ATP; therefore, bemcentinib would exert its inhibiting action on 2 sites resulting in a Hill

coefficient of ~ 2 . However, upon the addition of DNA, the 2 proximal sites contribute to the activity, too, raising the active sites to 4 as indicated by the new Hill coefficient (~ 4). A schematic representation of the inhibition in the presence and absence of DNA is summarized in Figure 50.

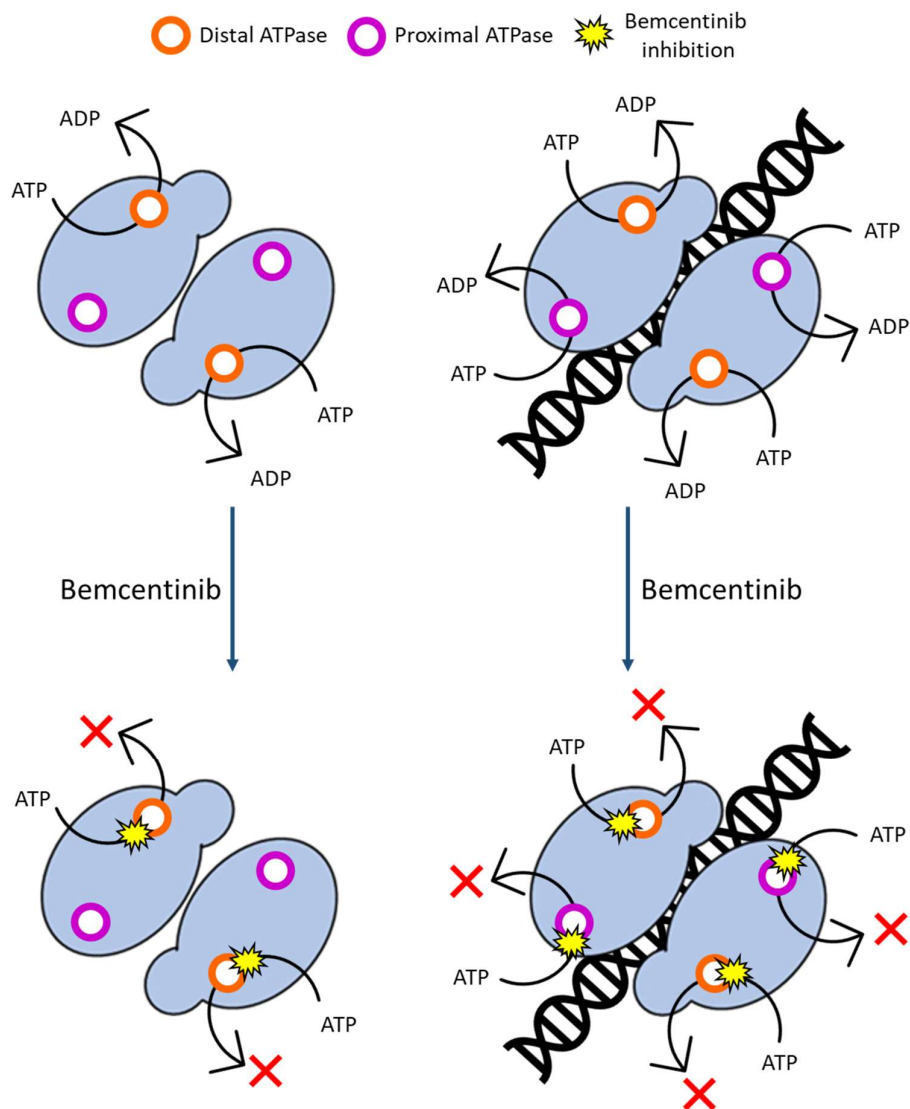


Figure 50: Representation of bemcentinib cooperative inhibition. The figure shows how bemcentinib inhibits the ATPase activity of the dimer UvrA₂ when in the absence (left) and presence (right) of DNA. Since only the distal sites (orange circles) are actively contributing to ATP hydrolysis when DNA is not in solution, the inhibitions appear moderately cooperative (with a measured Hill coefficient of ~ 2). When DNA is present, the proximal sites (purple circles) contribute to the reaction raising the number of active sites (and the Hill coefficient) to 4.

While the precise nature of the inhibition remained to be further studied, we could successfully validate the virtual screening results against pure UvrA₂. We then moved to study the drug's effect on the ability to disrupt DNA binding. UvrA₂ was incubated alone and with bemcentinib (50 and 20 μ M) before being imaged on suspended DNA. The experiment was conducted and analysed by Antoine Paris and, therefore, not discussed in detail. However, it is essential to mention that the results showed a drastic reduction in binders, reaching up to a ~90% difference when compared to the untreated UvrA sample. These results agree with what was already found for ATBC in Chapter 3, pointing to a deficient DNA binding ability as a possible molecular mechanism of action of these types of inhibitors.

Having assessed the drug activity on UvrA, we tested bemcentinib against the entire system *in vitro*. Our fluorescence-based incision assay showed that 20 μ M was insufficient to impair NER significantly. As measured with the previous assays, 20 μ M bemcentinib is able to reduce UvrA activity dramatically, however, the protein is able to catalytically load UvrB onto the lesions (219) therefore, even few active molecules would be able to allow the reaction to proceed *in vitro* if given enough time. However, when the concentrations were raised to 50 μ M, a total of ~76% inhibition was measured (at 16h), showing bemcentinib's ability to impair NER. This result was then confirmed with the traditional gel-based incision assay when 50 μ M were incubated with the proteins and then ran for 15 minutes.

Once the activity against NER was confirmed *in vitro*, we looked at the drug's effect on *E. coli*. As previously done for ATBC, we paired bemcentinib with the potent NER activator 4-NQO (286, 287) in MG1655 Δ tolC using sub-MIC concentrations. The two chemicals alone could not affect growth significantly, but the bacteria were severely affected when exposed to the combination, demonstrating bemcentinib's efficacy on NER *in vivo*.

Having fully proven the compound's activity with multiple assays, we explored a possible real-life application for repurposing bemcentinib. Therefore we moved to the antitumoral cisplatin as a DNA-damaging agent and NER activator (103, 312) and to a clinically relevant strain, the multidrug-resistant *E. coli* ST131 (EC958) (234) for our *in vivo* tests. EC958 showed resistance to the drug compared to the efflux pump knockout with a MIC of 50 μ g/ml (compared to 3.13 μ g/ml in Δ tolC). However, when a sub-MIC concentration of the

drug (half of its MIC) was combined with only 1/4th of cisplatin MIC, the multidrug-resistant strain could not grow for the duration of the experiment showing a high degree of cooperation.

6.3.3 Bemcentinib/cisplatin for infections during cancer chemotherapy

Unfortunately, cancer chemotherapy carries numerous side effects, including neutropenia, disruption of anatomical barriers and often lacerations due to surgical treatments (126). These combined effects hinder the patients' immune systems leaving them often highly susceptible to hospital infections (124, 126). A standard approach to prevent complications is using large-spectrum antibiotic prophylaxis, which causes problems on its own due to the intrinsic toxicity in the antimicrobials (122, 125, 371, 372). Additionally, the overuse of antibiotics leads to a rapid insurgence of multi-drug resistance bacteria, especially in hospitals, causing difficult-to-treat infections in patients already immunocompromised (125, 128, 373, 374). Therefore, it is unsurprising that bacterial infections during cancer chemotherapy are a significant problem for their well-being.

The development of new combinatorial therapies has been proposed among the possible strategies to tackle this burden (375). As discussed in the introduction, numerous studies on combinatorial therapies are available in literature. These strategies have been investigated and used for different applications, from cancer therapy to cardiovascular diseases, rhinitis, pain medications, psychiatric disorders and antimicrobial therapies (291–295, 314–319, 376–379).

In this study, we evaluated the combination of the widely used antitumoral agent Cisplatin with the AXL inhibitor bemcentinib in cancer therapy to fight infections.

Since It has been reported that *E. coli* UTIs are among the most common infections during hospitalizations and often due to drug-resistant organisms, we specifically used a multi-drug-resistant *E. coli* ST131, one of the most widespread causes of UTIs and bloodstream infections worldwide (122, 234, 235, 380, 381).

Furthermore, cisplatin is used for the treatment of bladder cancers and is excreted in urine, where it can accumulate in high concentrations where it usually exerts its toxicity (382, 383). Therefore, the possibility of utilizing this side effect in an advantageous way to prevent UTIs could be promising. We successfully showed that when cisplatin is coupled with bemcentinib, the clinically relevant strain cannot grow to indicate solid antimicrobial

cooperation between the two compounds. We believe co-administering the two drugs during cancer therapy could reduce multidrug-resistant infections (especially UTIs), minimizing the need for antibiotic prophylaxis.

AXL inhibitors are now studied for lung cancer treatment, where AXL is usually overexpressed (384, 385). These diseases are often treated with platinum-based antitumoral drugs (386–389) whose activity might then be enhanced by AXL inhibitors through the reduction of drug resistance (390, 391).

For these reasons, it could also be beneficial to keep investigating the combination of cisplatin/bemcentinib to improve the cisplatin effect against cancer while preventing or treating the insurgence of multi-drug resistant bacteria infections in a clinical setting.

6.4 Future work

Due to resources and time, we could not properly explore other compounds that surfaced in the screening. Considering the success obtained with bemcentinib seems reasonable to explore more drugs reported to be active against UvrA.

As an example, a particularly interesting compound is Lolitrem B, scoring in position 14 (-12.7 kcal/mol). Lolitrem B is a mycotoxin which accumulates in the brain, causing neurological problems. The compound molecular structure is complex and, from our preliminary analysis, appears to bind to the distal ATP-binding pocket and extend through the channel connecting the two sites (Appendix I – Lolitrem B). The lower affinity modes produced by the docking algorithm also show a propensity for the drug to bind inside that hollow region. A higher exhaustiveness virtual analysis and a biochemical confirmation of the results could shed light on the compound's preferred binding modes and the possible channel residues involved in the interaction. This information would contribute to the rational design of a novel, highly specific, nontoxic inhibitor and potentially help clarify the channel's role.

The cooperative nature of the inhibition between the two ATPase sites with bemcentinib is unclear. Understanding the precise mechanism of their interaction was beyond the scope of this study; however, additional experiments could be undertaken to help define the roles of the distal and proximal sites in bemcentinib inhibition. The use of mutants lacking an ATPase activity (K37A and K646A (91)) could provide insights into which site is most affected by the presence of the inhibitor through comparative ATPase measurements. Moreover, analysing and mutating the residues supposed to be interacting with the drug in the different ATP-binding pockets will provide interesting data on the compound binding modes. An additional experiment would be modulating the ATP concentrations when measuring the inhibition to have an insight into the competitive nature of bemcentinib activity. Ultimately, crystallizing the protein while bound to the molecule would provide clear and empirical information on their interactions.

An additional step that could be taken for bemcentinib would be extending the test to other bacterial species causing infections in humans. *Staphylococcus aureus* and *Enterococcus*

faecium could be chosen since they are both reported to be among the most widespread MDR hospital-acquired infections (392). Their NER proteins could be purified and tested *in vitro* against the drug, and the bacteria evaluated *in vivo* with the combination of cisplatin/bemcentinib to have data on the spectrum of the possible treatment.

Ultimately, the effect of the combination of cisplatin/bemcentinib should be tested in more representative clinical settings using different clinical isolates and possibly in patient serum to have the best representation in physiological conditions.

6.5 Conclusions

We have developed a drug strategy rooted in computational techniques enabling the evaluation of hundreds of thousands of compounds against a novel drug target. We have highlighted several possible compounds that can bind to the catalytic sites by targeting the critical protein UvrA₂ required to repair a broad spectrum of DNA damage types efficiently. We have chosen bemcentinib, characterizing it through a series of biochemical assays. We validated the virtual screening showing the ability of the drug to inhibit UvrA₂ catalytic activity with an ATPase assay, further confirming it, and showing the impairment in binding derived from the exposure to the drug. We have shown how bemcentinib is able to prevent efficient DNA incision through a fluorescence-based incision assay and then confirmed it with the more traditional gel-based incision. Ultimately, we explored the drug's ability to affect bacterial growth in two different strains. We have successfully demonstrated bemcentinib's ability to cooperate with the antitumoral cisplatin to prevent growth against the multi-drug resistant *E. coli* strain EC958 and advanced a viable repurposing strategy for our hit. These results show how bemcentinib is a promising candidate as it is or as a template for chemical improvements to be used in combinatorial therapy alongside cisplatin.

Chapter 7 – Last remarks and conclusions

7.1 General discussion

The burden of infection among cancer patients is undeniable (122, 124–126, 128). Specifically, *E. coli* has been reported to be pathogenic in cancer patients and represent one of the organisms causing difficult-to-treat infections due to the spread of multidrug resistance in bacteria (171, 234, 393). Therefore, we focused our studies on two main strains of *E. coli*, a K12 wild-type strain and a clinically relevant EC958 (234).

With this premise, we decided to look at this known problem from a different angle. We devised a novel antimicrobial approach by developing an adjuvant drug with the ability to activate the antibacterial activity of the cancer agents already used during cancer chemotherapy by impeding the bacteria's defences.

We developed these ideas with a broad investigation incorporating *in vivo*, *in vitro* and *in silico* approaches. We highlighted several compounds with promising characteristics and began the study of many more. As previously discussed, some of these candidates could be close to an eventual application (such as pirarubicin or bemcentinib), while some others represent a template for further development aiming to achieve a better understanding of how the inhibition works and for the rational design of selective inhibitors (such as ATBC or dienestrol).

However, these results represent only a starting point. For this pilot study we chose cisplatin as the representative anticancer agent. However, several other antitumor drugs exert their therapeutic effect by damaging DNA (as described in the introduction), which could represent more potent candidates and better NER activators than platinum-based drugs and are worth exploring in future studies. Furthermore, numerous other bacterial strains contribute to the burden of infections, such as *S. aureus*, *P. aeruginosa*, *K. pneumoniae* or *E. faecium* (171, 392). Therefore, another step in establishing the spectrum of the combination between NER inhibitors and antitumoral agents would be exploring their activity across these different strains to assess their eventual impact in therapy. An even more interesting possibility would be testing our most promising compounds on a variety of clinical isolates from infectious species, ideally using patients' blood samples, to

assess the ability of our combinations to overcome pathogens with more real-life conditions.

Some of the most common challenges when undertaking a drug discovery project often are the unrealistic expectations of immediately identifying potent drugs, especially with limited access to good chemical libraries, and the "artificial" compartmentalization of the approach (e.g. cell-based versus biochemical characterization) (394). In agreement with this statement, the pilot study on ~40k compounds screened against mycobacterial NER (275) highlighted a single lead, ATBC, which we then characterised, finding it unsuitable for clinical use. Principally the compound showed poor biochemical characteristics and stability that could significantly impact its applicability. However, its importance went beyond its possible use in therapy. The study on the compound allowed us to validate UvrA as a possible molecular target for NER inhibitors and guided the planning of the following screenings. Furthermore, the molecule still offers an optimal template for chemical optimizations that can drive the design of potent analogues.

Drug discovery is expensive (1), therefore, to make the most of our resources, we selected a library of a wide range of drugs already optimised for human use and available for repurposing. We reasoned that the advantage of fishing from a pool of compounds easily accessible would have minimized the chances of identifying unavailable or challenging to synthesize molecules. Another advantage of using already-approved drugs lies in the available information on the compounds. At the stage of our research, we didn't require knowledge of the bioavailability and pharmacokinetics of the drugs tested, however, that data would be of great use for any further investigation.

For example, a study published in 2016 (395) examines cisplatin's plasma concentration after 1 or 3h long infusions in cancer patients. The findings show a max concentration of 5.37 (± 1.47) $\mu\text{g/ml}$ and 9.03 (± 1.93) $\mu\text{g/ml}$ in the 1 and 3h infusion with total exposure to the drug (AUC) of 12.5 (± 3.7) mg.h/L and 75 (± 29.9) mg.h/L . These concentrations are significantly higher than those required for our experiments, especially considering the several-fold improvements associated with NER inhibitors. Furthermore, these data should be available for the other compounds discussed in this thesis, providing more information on the feasibility of their combination for antimicrobial chemotherapy.

Despite this approach however, we could still not retrieve all the compounds we would have wanted to test, being particularly constrained when selecting the hits to test from the virtual screening. Specifically, compounds such as the mycotoxin lolitrem B (Figure 62) were either too expensive or unavailable for purchase.

We could still generate a tremendous amount of data in the project's early process and decided not to limit the search for our leads using a single approach. It is important to remember that when repositioning already used drugs, a previous activity is implied. That activity has to be taken into account and if relevant has to be addressed (as discussed for the intercalating effects in Chapter 4). As eloquently stated by Bender (41), the controls are not made to ensure success but to guide in case of problems and prevent obvious sources of failure. Therefore, we developed a pipeline of assays to test the molecule's effect on nucleotide excision DNA repair from multiple angles. We tested the combinatorial activity of the hits in three *E. coli* strains (wild-type, *ΔtolC* and EC958) with two different DNA-damaging agents to ensure effects against NER. Then we confirmed it directly with three different approaches, 2 different incision assays (fluorescence and gel-based) and one *in vivo* test (UV-damage repair assay). Ultimately, we probed UvrA as a molecular target by looking at two necessary aspects of its activity, ATP hydrolysis and DNA binding. These results together paint a clear picture of the activity of the target compounds. Furthermore, this approach allowed us to validate observed results from *in silico* studies. Historically, such approaches can be plagued by false positives amongst their other limitations (33), however, in our study they correlated well with the anti-UvrA activity measured with the ATPase, which was further confirmed independently through the inability of the protein to bind to suspended DNA.

Regarding our computational exploration, we chose AutoDock Vina, designed to be unbiased by the input and position conformations (19). Its algorithm is based on several steps, alternating mutations and local optimizations in parallel, taking advantage of multithreading (19). However, molecular docking remains an approximate approach. The use of a rigid receptor, fixed charge distribution and absence of solvent creates a simplified model of the actual complex in solution. Therefore, we didn't aim to define the best binding conformation but we used this tool for a less traditional "blind docking" (39) in which the search space was maximised either to locate the binding sites (Chapter 3 and 4) or for

virtual screening (Chapter 6), where the precise interaction between the ligand and the protein are superfluous. Furthermore, we controlled for random binding docking of the endogenous ligand (ATP) along with our tested molecules, verifying if the software could locate the appropriate binding sites. Interestingly ATP was docked with remarkable precision, even discriminating preferential sites and contacts with critical residues reported in the literature (215, 258). However, the precise energy values of the docking are not yet understood and should be used as an internal indicator to guide experiments more than absolute values. We decided not to overanalyse multiple bound conformations or the distal/proximal preference for the ATP binding pocket without an empirical validator to ensure the accuracy of the docking.

However, the new allosteric binding sites allow for some speculation. The presence of those sites and their ability to control for ATP hydrolysis and DNA binding could represent an excellent target for highly selective inhibitors. At the moment, the specific function of these new sites is not yet understood, and the docking is not validated by empirical evidence. However, our findings interestingly show that the molecules binding to the allosteric sites (L-thyroxine and dienestrol) score poorly against ATP while displaying a strong ATPase inhibition confirmed with a tremendous reduction in DNA binding (302). This phenomenon could be due to the fact that we carried out our investigation using a rigid protein (also computationally generated) with no solvent present able to stabilize the interactions. However, we found remarkable accuracy across previous docking experiments, even picking up 1 active molecule out of ~120 000 unique compounds (bemcentinib). Therefore, we think that their possible binding should be investigated more. Our molecular docking hints at the possible residues involved in the formation of the complex between the protein and the drugs (Figure 51). A possible approach would be to mutate the charged residues involved in the interactions (such as His786 or Thr860, for example, to the small and non-polar alanine), which should reduce or even eliminate the affinity of the compounds for the protein, demonstrating those sites as responsible for the interactions.

ATP binding domains are highly conserved across species and fairly common. Therefore, it is reasonable to wonder about the specificity of a compound that aims to inhibit their action. However, it is worth mentioning that selective ATPase inhibitors are being

developed for cancer and antimicrobial therapy (396, 397), proving that therapy against these targets is possible. Furthermore, the region surrounding the nucleotide-binding site is not conserved and can be exploited to design specific inhibitors. Ultimately, new-developed drugs could aim at the allosteric sites (Figure 51), as previously mentioned, to increase the specificity of the treatment.

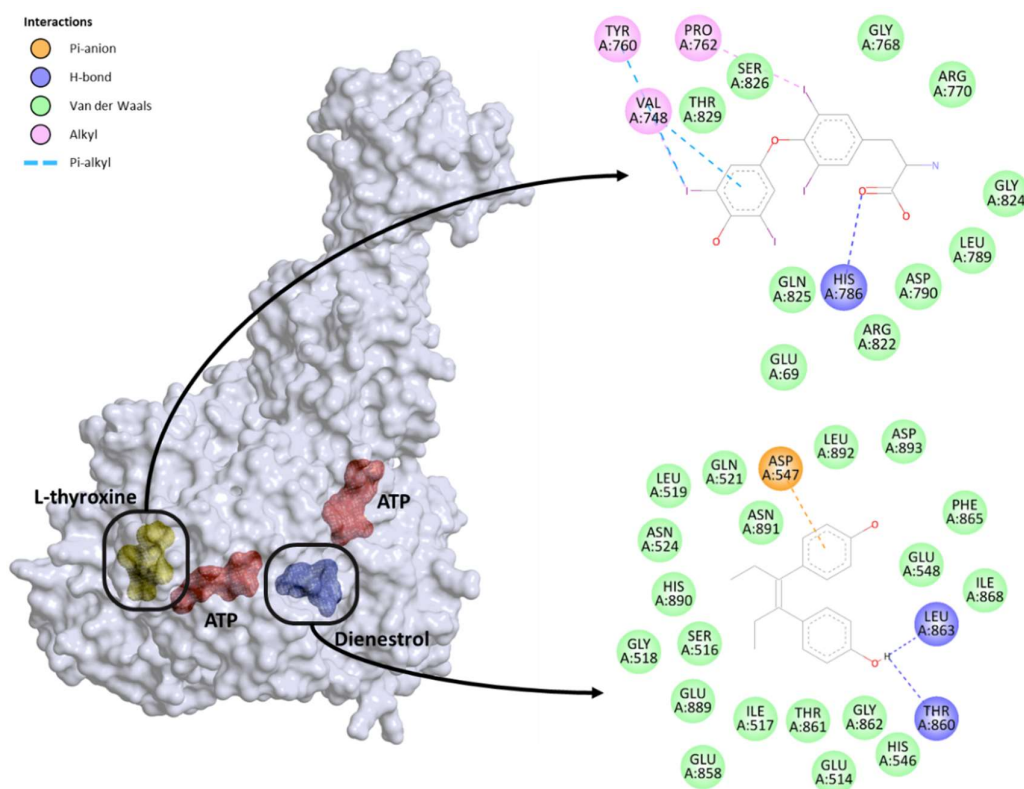


Figure 51: L-thyroxine and dienestrol docking in the allosteric sites. On the left, the figure shows the surface of AlphaFold UvrA (through transparency) while bound to ATP (red), L-thyroxine (yellow) and dienestrol (blue). On the right is reported the 2D representation of the binding generated through Biovia studio visualizer, showing the residues interacting with the ligands.

A last question that remains to be answered regards the involvement of the other members of the NER pathway in the inhibition. We could only fully test UvrA, the initiator of the repair pathway and the one responsible for the first contact with the lesion (268). However, the protein matchmaker UvrB could also be picked as a molecular target for its central position in the pathway. The protein possesses an ATP binding pocket (UvrA stimulated) (220) that could be targeted through an *in silico* screening looking for inhibitors, similarly as it was done for UvrA in Chapter 6.

To conclude, drug discovery projects usually don't lead to potent molecules ready to be used in therapy. However, our findings represent the foundations for additional studies aimed at understanding our leads' viability and, more importantly, for the rational design of novel antimicrobials. We believe that we have proven how NER could represent an optimal molecular target for adjuvants in cancer chemotherapy and showed examples that can be taken as a template for further improvements.

7.2 Conclusions

This thesis explores NER as a novel drug target for combinatorial drug therapy. We began the research from ATBC, the first excision repair inhibitor discovered for *mycobacteria* (275). We confirmed its effects on *E. coli*, characterised the molecule and proposed UvrA as its target. We then devised two different approaches to discover new inhibitors. We computationally screened ~120000 unique compounds against UvrA's ATP binding pockets targeting the initiator of the system directly. We also manually screened ~3000 compounds alone and in combination with cisplatin as the DNA-damaging agent. From these screens, we have found several exciting compounds affecting NER *in vitro* and *in vivo*. We have characterised the activity of 8 of those drugs and proposed possible mechanisms of action. Ultimately, we have highlighted 5 promising main leads (pirarubicin, mitoxantrone, 9-aminoacridine and bleomycin from the manual screening and bemcentinib using computational tools) that could be combined with cancer chemotherapeutics, specifically cisplatin, to treat or prevent co-infection during cancer chemotherapy. In doing so, we have opened the door for the development of a new class of antimicrobials.

Appendix

A – Minimal inhibitory concentration controls

I - Resazurin colour changes when used at different volumes

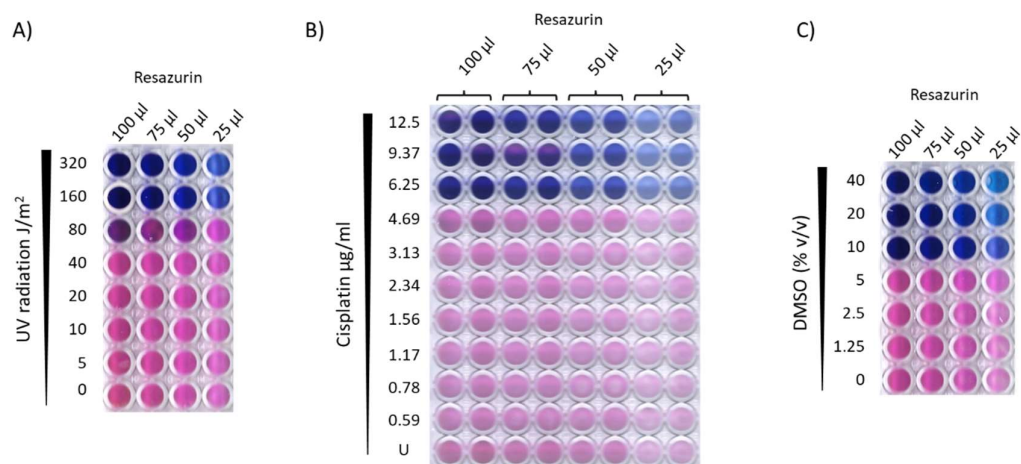


Figure 52: Different volumes of resazurin do not affect the result. Results of minimal inhibitory concentrations of three different antimicrobial agents after adding different volumes of resazurin show that the amount of dye used does not affect the final result.

II - DMSO impact on growth

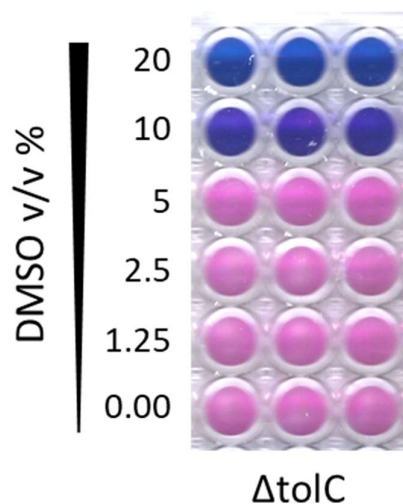


Figure 53: DMSO's MIC in MG1655 $\Delta tolC$. The minimal inhibitory concentration of DMSO in MG1655 $\Delta tolC$ was incubated for 20 h (16 h + 4 h with resazurin) in the dark while shaking. The maximum amount of DMSO tolerated by the cell is 5%. Representative plate with 3 technical replicates (visible in the figure) of 3 biological replicates.

III - MIC values of the interesting compounds in EC958

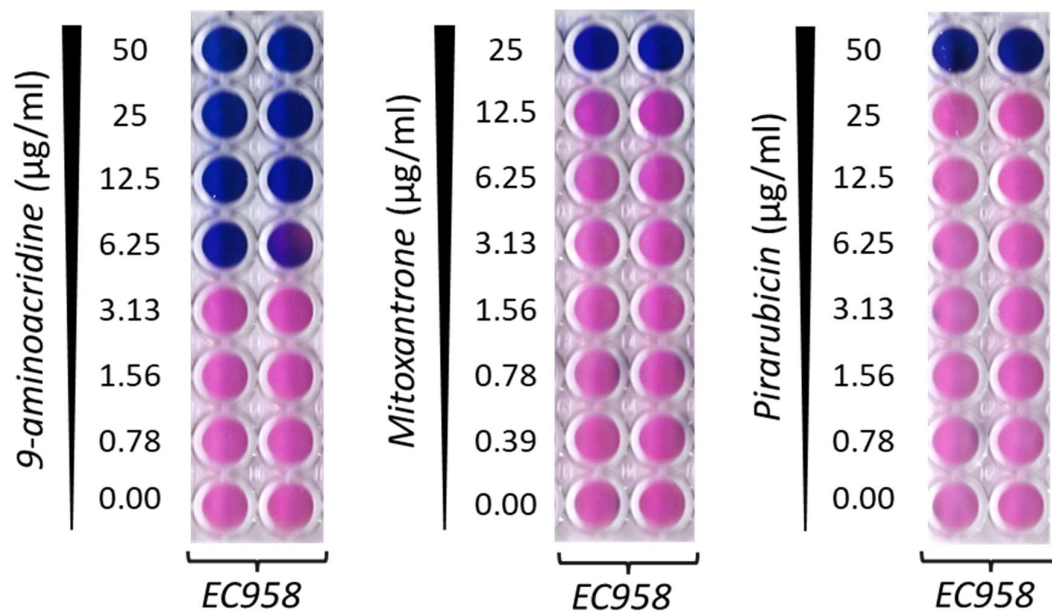


Figure 54: MICs experiments in EC958. MICs value recorded for the 3 main leads (9-aminoacridine, Mitoxantrone and Pirarubicin). The MIC value represents the first well which reports an inhibition of growth (blue). The last well from the top (0.00) shows the untreated sample and shows unaffected growth (pink). The experiments were repeated at least three times with a technical replicate (visible in the figure). The results report MIC values of 6.25, 25, and 50 µg/ml for, respectively, 9-aminoacridine, mitoxantrone and pirarubicin.

B – Growth curves comparing MG1655, MG1655 Δ tolC, MG1655 Δ tolC Δ uvrA

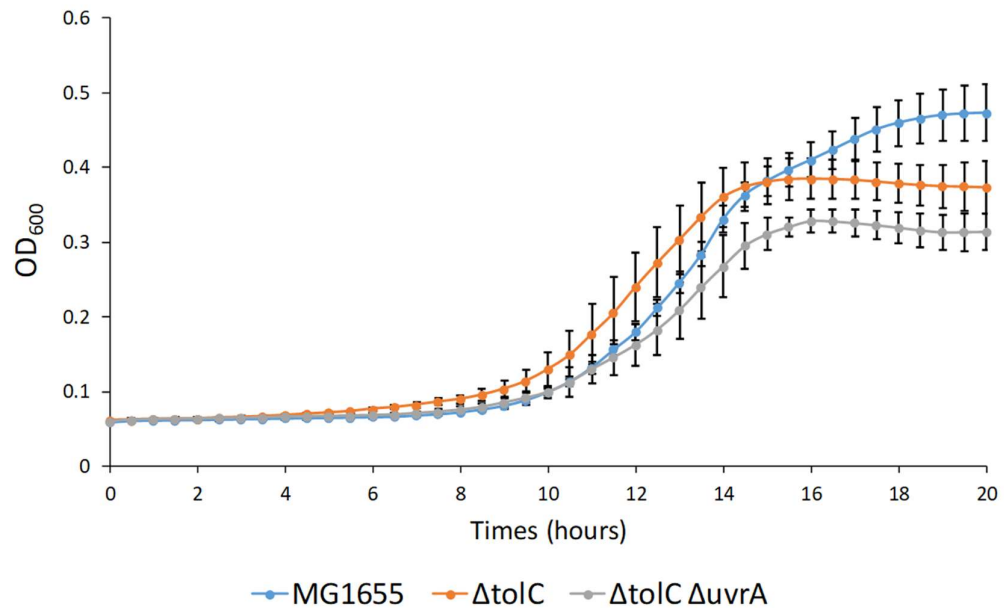


Figure 55: MG1655, MG1655 Δ tolC and MG1655 Δ tolC Δ uvrA growth curves. Growth curves comparing MG1655, MG1655 Δ tolC, MG1655 Δ tolC Δ uvrA incubated in the plate reader (id5 Spectramax, Molecular Devices) in the dark at 37°C while shaking. Only minimal difference in growth can be observed among the three different strains. The error bars represent the standard deviation between the technical and biological replicates (3 biologicals with 3 technical each).

C – Additional compounds tested

Compounds	screening against MG1655 ΔtolC			ATPase assay (% of UvrA activity)				Fluorescence-based incision (% of total activity)								MIC		Checkerboards against MG1655 ΔtolC	
	w/o ciprofloxacin	w/ ciprofloxacin	w/UV damage	% ATP	% ATP + DNA	ATP T - test	ATP + DNA T - test	0.5h	1.5h	2.5h	16h	0.5h T - test	1.5h T - test	2.5h T - test	16h T - test	MG1655 ΔtolC	MG1655 ΔtolC ΔuvrA	ΔtolC Checkerboard (fold decrease for drug)	ΔtolC Checkerboard (fold decrease for CIS)
Sulfacarbamide	Inactive	Inactive	Partial	81	94	S	NS	105	107	98	92	NS	NS	NS	NS	6.25 μg/ml	12.5 μg/ml	no change	no change
Valacyclovir	Active	Active	Active	101	111	NS	NS	93	84	77	75	NS	NS	NS	NS	6.25 μg/ml	6.25 μg/ml	2-fold antagonist	no change
Amonafide	Inactive	Active	Inactive	94	104	NS	NS	89	69	65	68	NS	NS	NS	NS	25 μg/ml	25 μg/ml	2-fold	no change
Kaempferol	Inactive	Active	Active	80	121	NS	NS	98	88	89	104	NS	NS	NS	NS	12.5 μg/ml	12.5 μg/ml	2-fold	2-fold
Berberine	Inactive	Active	Inactive	94	138	NS	NS	112	94	84	76	NS	NS	NS	S	50 μg/ml	50 μg/ml	2-fold	2-fold
Flufenamic acid	Inactive	Active	Inactive	94	114	NS	NS	116	101	91	70	NS	NS	NS	S	25 μg/ml	25 μg/ml	4-fold	2-fold
Nomegestrol	Inactive	Active	Inactive	79	116	S	S	114	95	86	74	NS	NS	NS	S	25 μg/ml	25 μg/ml	2-fold	4-fold
Tafuprost	Inactive	Active	Inactive	81	89	S	NS	113	89	80	43	NS	NS	NS	S	25 μg/ml	25 μg/ml	2-fold	4-fold
Danazol	Inactive	Active	Active	83	86	NS	S	117	98	97	92	NS	NS	NS	NS	>25 μM	>25 μM	no change	no change
Ixazomib	Inactive	Active	Active	85	103	S	NS	91	67	66	30	NS	S	S	S	N			
Nintedanib	Inactive	Active	Active	71	98	S	NS	124	93	82	36	NS	NS	NS	S				
Mepivacaine (HCl)	Inactive	Active	Active	87	90	NS	S	86	77	67	51	NS	S	S	S				
Indacaterol	Inactive	Active	Active	74	107	S	NS	115	97	86	79	NS	NS	NS	NS				
Cobimetinib	Inactive	Active	Active	79	82	S	S	125	94	85	75	NS	NS	NS	NS				
(+)-Ketoconazole	Inactive	Active	Active	78	87	S	S	123	102	95	76	NS	NS	NS	NS				
Drospirenone	Inactive	Active	Active	80	85	S	S	91	80	77	74	NS	NS	S	S				
Dabigatran etexilate	Inactive	Active	Active	67	108	S	NS	113	108	98	91	NS	NS	NS	NS				
Rolapitant	Inactive	Active	Active	60	63	S	S	132	119	117	100	NS	NS	NS	NS				
Dibucaine	Inactive	Active	Active	82	104	S	NS	116	98	95	87	NS	NS	NS	NS	97.5 nM	97.5 nM	2-fold	4-fold
Zidovudine	Active	Active	Active	77	80	S	S	127	98	101	106	NS	NS	NS	NS	1.56 μM	390 nM	no change	no change
Tebipenem pivoxil	Active	Active	Active	80	73	NS	S	123	88	80	86	NS	NS	NS	NS	N		N	N
Gatifloxacin	Active	Active	Active	58	60	S	S	100	94	85	72	NS	NS	NS	S	12.5 μM	3.13 μM	8-fold	8-fold
Hygromycin B	Active	Active	Active	N				104	84	80	104	NS	NS	NS	NS	195 nM	195 nM	4-fold	8-fold
Azithromycin	Active	Active	Active	73	89	NS	NS	101	78	79	82	NS	S	NS	NS	12.9 nM	12.9 nM	2-fold	2-fold
Nadifloxacin	Active	Active	Active	125	130	S	S	110	88	89	110	NS	NS	NS	NS	N		N	N
Riboflavin	Inactive	Inactive	Inactive	N				N								<500 nM	N	N	
Mitomycin c																>250 μM			
Cyclophosphamide																			

Table 2: Summary of the additional compounds tested while working on Chapter 4. The table shows the compounds initially tested but not pursued for different reasons. The screening section reports the results obtained in the screening. Inactive (pink) indicates living bacteria, while active (blue) indicates an antimicrobial activity of the compound/combination. The wild type data collected by Dr Arya Gupta is not shown. The three columns report the results for the drug alone, the combination with cisplatin and the combination with UV radiation. The screening was repeated with a technical replicate. The second section from the left shows the ATPase results expressed in percentage of the untreated UvrA activity when in the presence of ATP or ATP + DNA. The experiment where repeated 3 times and considered significant if $p \leq 0.05$ (*) (T-test) when compared to the untreated sample. The third section includes the results obtained from the fluorescence-based incision reported in percentage of the untreated NER activity. The experiments were repeated at least twice with two technical replicates and considered significant if $p \leq 0.05$ (*) (T-test) when compared to the untreated sample. The fourth section reports the MIC values measured for those compounds, as shown in Figure 10. The fifth section shows the results obtained from the checkerboard assay reported in folds of inhibition for the drugs and cisplatin, as seen in Figure 32. The microbiological experiments reported here were acquired with at least two independent measurements. The significance is reported with an S when the results were statistically significant and NS when non-statistically significant ($p\text{-value} \geq 0.05$). The significant results are highlighted in celeste to enhance accessibility. N = not tested due to poor activity, availability, time or other criteria.

D – Different intercalators show that DNA intercalation alone does not affect the incision assay.

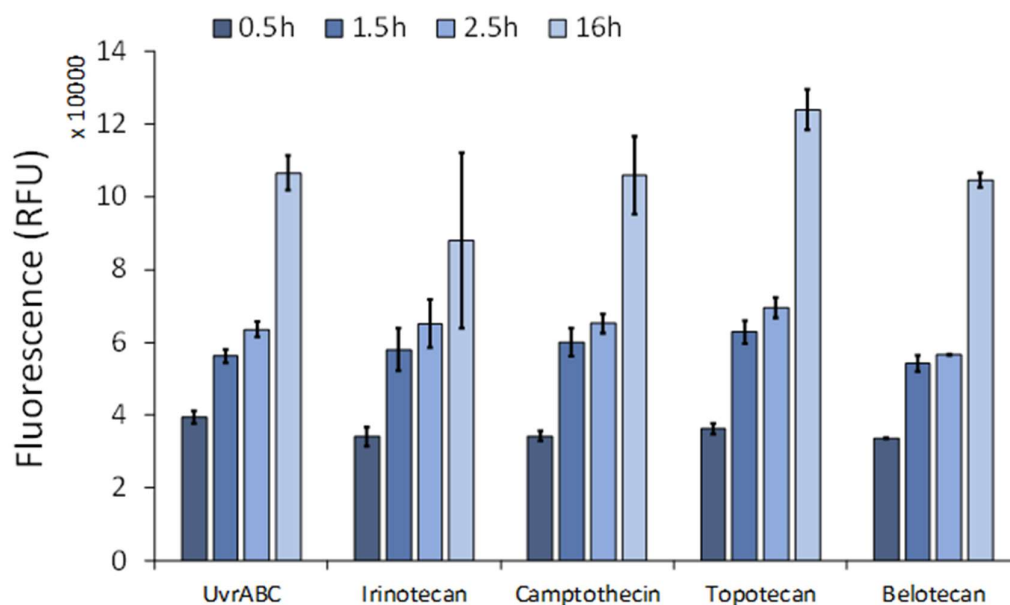


Figure 56: Intercalating agents do not affect the incision mediated by NER. Fluorescence-based incision assay using known intercalators (398–400). The graph shows that none of the compounds tested can produce significant inhibitions compared to the untreated UvrABC. Dr Arya Gupta further confirmed the results with a gel-based incision assay (data not shown). The data shows that the intercalating effects are not directly responsible for NER inhibition. This conclusion is further supported by the lack of inhibition reported by pixantrone, another intercalating agent, in Figure 60. The error bars represent the standard error of the mean (n≥4). The results were considered significant if $p \leq 0.05$ (*) (T-test) when compared to the untreated UvrABC.

E - Apigenin, Hydroxyprogesterone and Meisoindigo exhibit synergism with cisplatin

<i>Compounds</i>	Initial screening against MG1655 ΔtolC		
	<i>w/o cisplatin</i>	<i>w/cisplatin</i>	<i>w/UV damage</i>
Apigenin	Inactive	Active	Active
Hydroxyprogesterone	Inactive	Active	Inactive
Meisoindigo	Inactive	Active	Inactive

Table 3: Screening results for the three compounds. The table shows the screening results obtained for the three compounds. The three drugs were not toxic on their own but cooperated successfully with cisplatin to cause cell death. Among those three, apigenin retained its activity when tested again using UV radiation as the source of DNA damaging agent, strongly pointing towards an anti-NER activity. The cisplatin screening was done with one technical replicate, and the UV experiments were tested with a biological replicate.

When the compounds were tested singularly in MG1655 Δ tolC they did not exhibit antimicrobial properties at the concentrations used in the assay (Figure 57 A). However, when combined with cisplatin, they strongly affected bacterial growth. Apigenin (Figure 57 B) had at least a 32-fold shift in antimicrobial activity, while cisplatin saw an 8-fold shift in its MIC. Furthermore, it appears that increasing apigenin's concentration has a detrimental effect on combining the two drugs. Hydroxyprogesterone (Figure 57 C) showed a tremendous synergistic effect with cisplatin, with 16-fold and 8-fold maximum shifts for the drug and the platinum compound, respectively. Ultimately, meisoindigo (Figure 57 D) displayed a 32-fold shift in MIC when combined, while cisplatin had a 2-fold improved antibacterial activity.

Unfortunately, the work to fully characterize these promising molecules remained unfinished due to insufficient time.

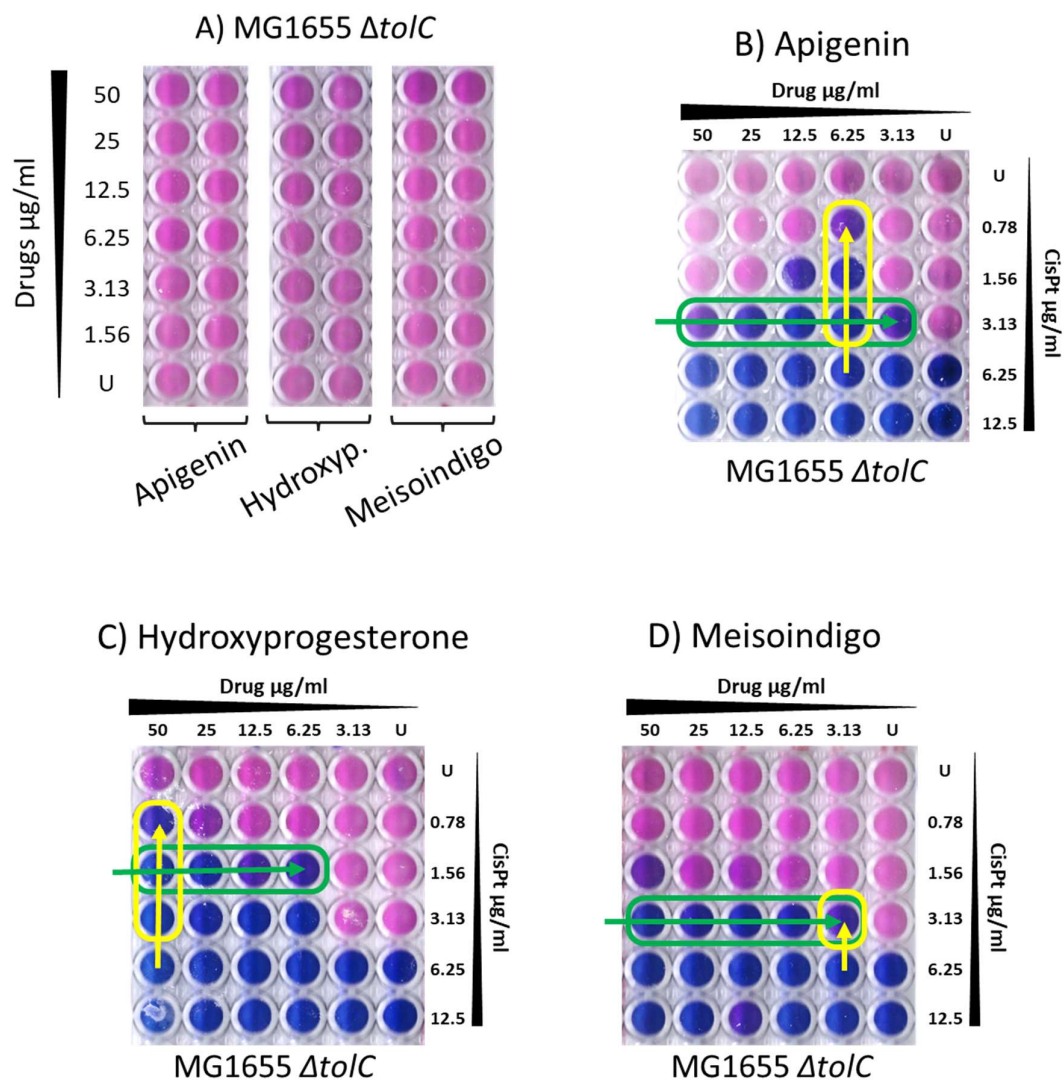


Figure 57: Survival assay with three interesting compounds. Unfinished works show MICs with different compounds not further explored after the screening. **A)** The three compounds do not show antimicrobial activity on their own up to a concentration of 50 $\mu\text{g/ml}$. **B)** Apigenin shows drastic improvements when combined with cisplatin with 32 and 8-fold maximum shifts in MICs, respectively, for apigenin and cisplatin. **C)** Hydroxyprogesterone sees a 16 and 8-fold improvement in the drug's and platinum's antimicrobial maximum activity, respectively. **D)** meisoindigo improves cisplatin activity by only 2-fold, but its maximum effect against the bacteria was increased by at least 32-fold when in the presence of the platinum drug. The experiments were done in MG1655 $\Delta tolC$, and the pictures represent three independent experiments.

F – Unfinished work on different hits analogues

I - Pirarubicin analogues show different degrees of activity

Figure 58 shows that almost every analogue retains a degree of activity against the untreated sample UvrABC. However, among the ones tested, valrubicin could not effectively inhibit NER with minimal changes (when significant) compared to the untreated control at the 0.5, 1.5 and 2.5 h time points. The 16 h timepoint for the compound revealed a significant impairment of the system (46 % of the untreated incision) with an unusual variability (as displayed by the bigger error bars compared to the other compounds).

On the other hand, doxorubicin and epirubicin scored as the most active members of the family. The compounds completely prevented incision when the DNA was incubated for up to 2.5h. When the samples were measured after 16 h incubation, only Doxorubicin showed a statistically different reading from the untreated DNA allowing for a minimal level of inhibition of up to (9% of the untreated incision).

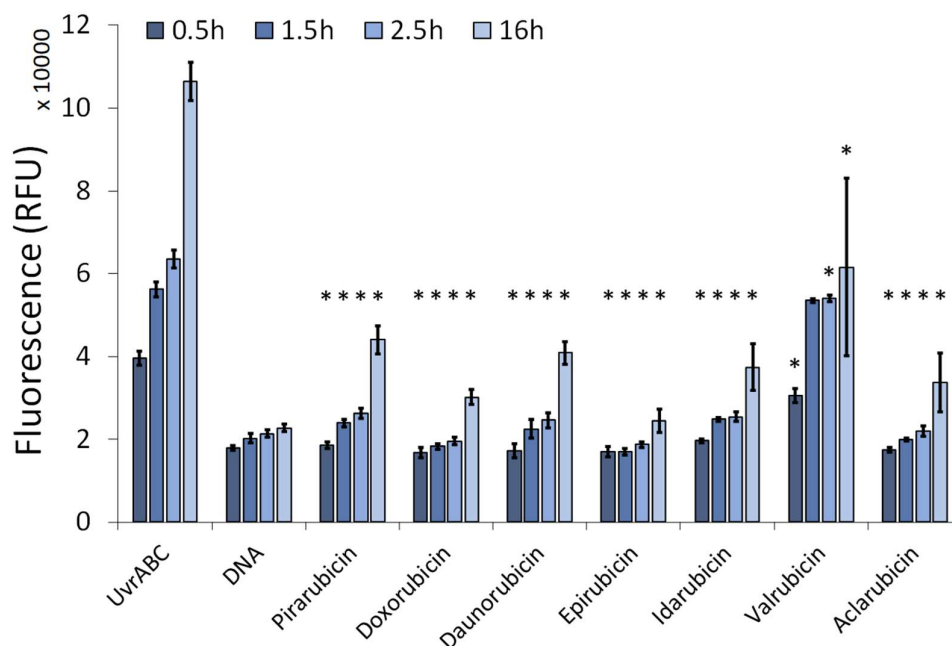


Figure 58: anti-NER activity of the rubicin family. Fluorescence-based incision assay on Pirarubicin analogues shows different degrees of inhibition, with Epirubicin being the most active and completely inhibiting incision to Valrubicin, the least active. The results were considered significant if $p \leq 0.05$ (*) (T-test) when compared to the untreated sample UvrABC. The error bars represent the standard error of the mean ($n \geq 4$).

Among these compounds, we tested doxorubicin against UvrA to verify if the activity already shown for pirarubicin was maintained. Therefore, we tested 20 μM of the compound with an NADH-coupled ATPase assay as done previously. The results in Figure 59 showed the percentage of untreated UvrA ATPase activity when the compounds were incubated with the protein. The catalytic activity of the protein was reduced to ~51 and ~58 % of the basal level, respectively, when in the absence and presence of pUC18. The results align with the previous finding for pirarubicin showing partial inhibition of ATP consumption.

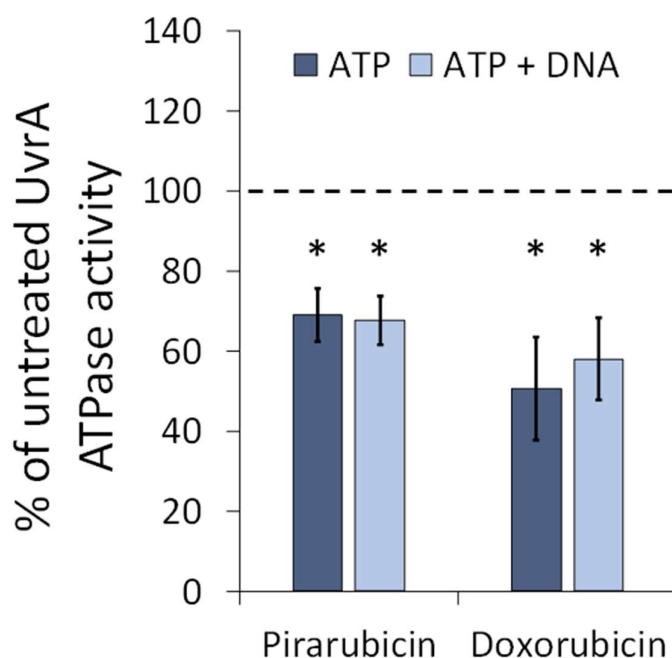


Figure 59: Doxorubicin inhibits UvrA similarly to Pirarubicin. NADH-coupled ATPase assay compares the percentage activity between Pirarubicin and Doxorubicin. The compounds exert similar activity against UvrA. The results were considered significant if $p \leq 0.05$ (*) (T-test) when compared to the untreated sample. The error bars represent the standard error of the mean ($n \geq 3$).

Further exploration to investigate the biological reasons for these effects and their significance on NER was not completed due to insufficient time.

II - Pizxantrone, a non-active Mitoxantrone analogue

The results are shown in Figure 60, including the previous data for mitoxantrone for reference. When 20 μ M Pizxantrone were incubated with UvrABC during the assay, the result showed a drastically different behaviour from Mitoxantrone's effect. The incision level was significantly slowed during the first two observations to respectively 49 and 78 % of the total activity. However, the inhibition was not maintained over time, with results at 2.5 and 16 h not significantly different from the untreated control.

We cannot exclude that the intercalating effect might be playing a role in disrupting NER, but pizxantrone's lack of activity, further demonstrates that the intercalating effects cannot be the only responsible for the inhibition in accordance with the one shown for other intercalators (Figure 56).

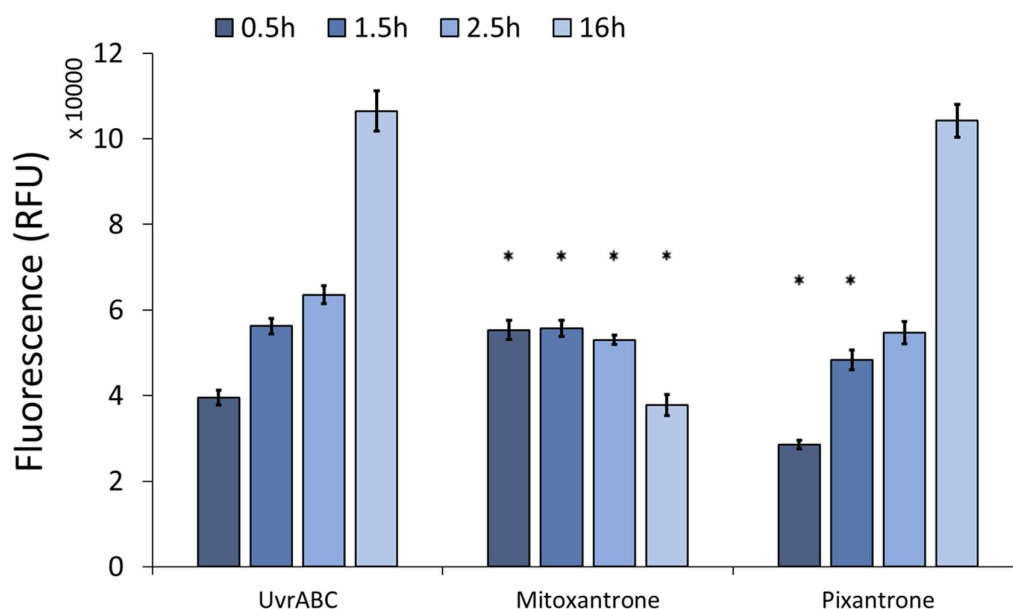


Figure 60: The analogue Pizxantrone doesn't maintain anti-NER activity. Fluorescence-based incision assay to evaluate the ability of Pizxantrone, a Mitoxantrone analogue, to inhibit UvrABC incision. The compound shows only modest inhibiting activity during the first two time points and no significant difference with the untreated control for the 2.5 and 16h. The results were considered significant if $p \leq 0.05$ (*) (T-test) when compared to UvrABC. The error bars represent the standard error of the mean ($n \geq 4$).

G – Hits` binding energies

	Binding energy (kcal/mol)		
	Distal	Proximal	Allosteric
ATP	-9.6	-9.2	
Pirarubicin	-9.6	-9.8	
Dienestrol	-7.4		-8.7
Mitoxantrone	-7.4	-7.5	
L-Thyroxine	-6.9	-6.9	-7

Table 4: Hits` binding energy. Results obtained from the molecular docking on AlphaFold UvrA. The table shows the docking of the compounds in three different binding sites, the ATP-binding pockets (distal and proximal) and eventual allosteric sites (BP1 and BP2). Noticeably, ATP was found able to bind to the ATPase cassette with a major affinity for the distal site in agreement with recent experimental evidence (258).

H – ATP interactions with the distal and proximal sites

A)

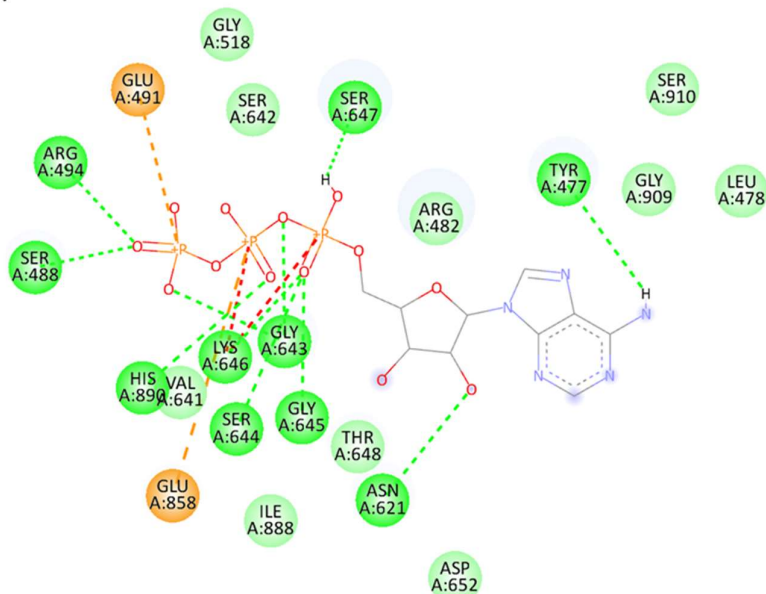
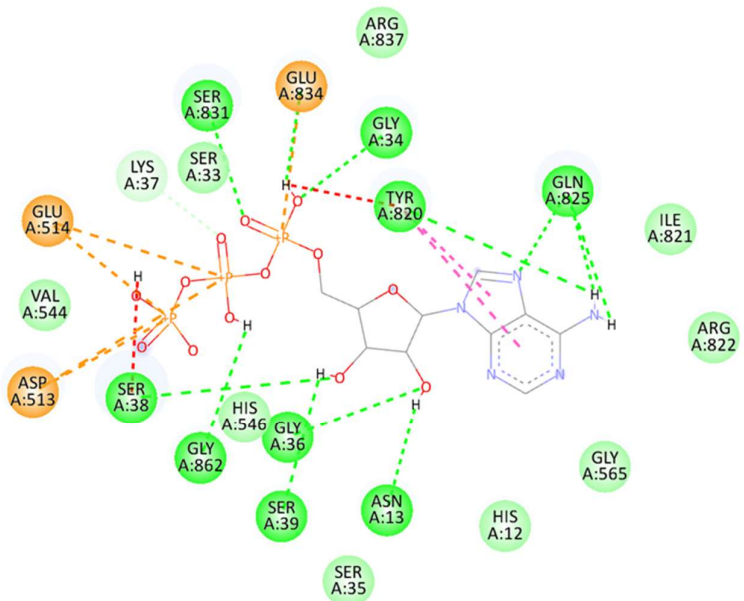


Figure 61: UvrA-ATP docking interactions.

The figure generated with Biovia Studio Visualizer shows the interaction between the ATP and the residues in the protein after an all-surface molecular docking (Vina) with increased exhaustiveness (8000).

A) The image shows the residues predicted to interact with ATP in the higher affinity distal site. Noticeably, the residues 643 and 646 have been highlighted to interact with the compound in agreement with experimental findings (91, 258). **B)** The image shows the binding in the proximal site. In this case, AA 37 is shown to participate in the binding with ATP, as shown previously with empirical data (91).

B)



Interactions

van der Waals	Carbon Hydrogen Bond	Unfavorable Donor-Donor	Unfavorable Positive-Positive
Attractive Charge	Conventional Hydrogen Bond	Pi-Pi T-shaped	

I – Lolitrem B

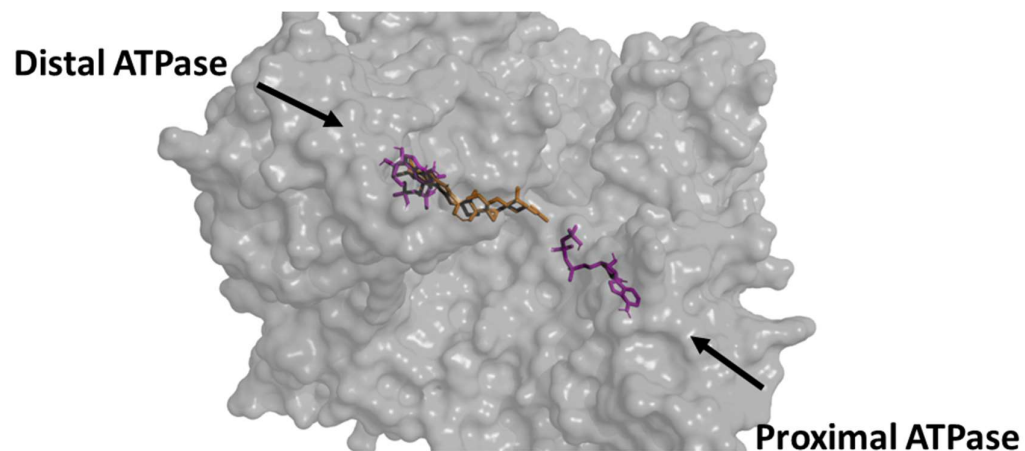


Figure 62: Molecular docking of Lolitrem B on UvrA. UvrA 3D surface used for the virtual screening shows the binding of ATP in the ATP-binding pockets as a reference (magenta) and Lolitrem B in its highest binding mode (orange). The binding energy measured by AutoDock Vina was -12.7 kcal/mol.

L – Code for virtual screening

```
import os
import time
import subprocess
import pandas as pd

def gen_sys_calls(path):
    calls = []
    for x in os.listdir(path):
        if x.endswith(".pdbqt") and x != "protein.pdbqt":
            call = "vina --receptor protein.pdbqt --ligand %s --center_x XX --center_y XX --center_z XX --size_x 25 -
-size_y 25 --size_z 25 --exhaustiveness 8 --cpu 2 --out outfiles_models/%s_models.pdbqt --log
outfiles/%s_vina_out" % (
                path + x, x, x)
            calls.append(call)
    return calls

#AutoDock Vina settings, 25 Angstrom search space with coordinates XX, with an exhaustiveness of 8, using
2 CPU per 1 job

def job_submit(maxNumOfJobsSubmitted, sleepTime, jobFilePath, start_time, n_ligands, lig_Count):
    """submit only when max jobs isn't exceeded"""
    numberOfJobsSubmitted = int(subprocess.check_output("ps -A | grep vina | wc -l", shell=True).strip())
    while numberOfJobsSubmitted >= maxNumOfJobsSubmitted:
        time.sleep(sleepTime)
        numberOfJobsSubmitted = int(subprocess.check_output("ps -A | grep vina | wc -l", shell=True).strip())

    os.system(jobFilePath + " &")

    print('--- Ligand %s out of %s finished at: %s seconds ---' % (lig_Count, n_ligands, time.time() - start_time))

def check_run_ended(sleepTime):
    current_jobs = int(subprocess.check_output("ps -A | grep vina | wc -l", shell=True).strip())
    while current_jobs > 0:
        time.sleep(sleepTime)
        current_jobs = int(subprocess.check_output("ps -A | grep vina | wc -l", shell=True).strip())

def submitter(path, start_time, n_ligands):
    #Check queue system every 5 seconds for completed processes and submit 2 jobs at the same time
    lig_count = 1
    for x in path:
        job_submit(2, 5, x, start_time, n_ligands, lig_count)
        print(x)
        lig_count+=1 # ligand count

def format_output(run_directory):
    results_file = pd.read_csv(run_directory + "outfiles/results_file.csv", names=['Ligand', 'Binding_Affinity'])
    results_file.sort_values(by=["Binding_Affinity"], inplace=True)
    results_file.to_csv(run_directory + "outfiles/results_file.csv")

def run(run_directory):
```

```

start_time = time.time()
n=1
n_ligands = len([name for name in os.listdir(run_directory) if name.endswith(".pdbqt") and name !=
"protein.pdbqt"])
calls = gen_sys_calls(run_directory)
submitter(calls, start_time, n_ligands)
check_run_ended(5)

run_directory = '/mnt/c/Users/user/Desktop/test_run/'
dir2='/mnt/c/Users/user/Desktop/test_run/outfiles_models/'

start_time2= time.time()  #Set a timer before the experiment starts

run(run_directory)

all_outputs = {}

for filename in os.listdir((run_directory + "outfiles/")):
    if filename.endswith("_vina_out"):
        all_outputs[filename] = []
        with open(run_directory + "outfiles/" + filename) as file:
            for line in file:
                if line.startswith(" 1"):
                    splice = line.split()
                    all_outputs[filename].append(splice[1])

end_time2=time.time() #Get the time at the end of the experiment
final_time=end_time2-start_time2 #Get the difference of time

print(all_outputs)

print('--- Final Run Time: %s seconds ---' % final_time) #Print the time

# Use to replace print when writing to a new file
newf = open(run_directory + "outfiles/results_file.csv", "w")

for k, v in all_outputs.items():
    for affinity in v:
        newf.write(k.replace(".pdbqt_vina_out", "") + "," + affinity + "\n")

newf.close()

format_output(run_directory)

with open('outfiles/time.txt', 'w') as f:
    f.write('Total time: '+str(final_time)+" seconds"+"\\n")

```

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