Pre-Treatment with BET Inhibitors Increases the Sensitivity of Cancer Cell Lines to Cisplatin and Oxaliplatin

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Declaration

No part of this thesis has been submitted in support of an application for any degree or other qualification of the University of Kent, or any other University or Institution of learning.

Andrew Wicks Date

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AKT	Protein kinase B
ANOVA	Analysis of variance
ATP7A or B	Copper-transporting P-type adenosine triphosphatase A or B
ATM	Ataxia-telangiectasia mutated serine/threonine kinase
ATR	ATM- and RAD3-related protein
BCL2	B-cell lymphoma 2
BDF1	Bromodomain-containing factor 1
BET	Bromodomain and Extra-Terminal domain
BRCA1	Breast cancer 1
BRCA2	Breast cancer 2
BRD	Bromodomain
BRDT	Bromodomain testis-specific protein
CDDP	Cisplatin
CHEK1	Checkpoint kinase 1
CK2	Casein kinase 2
CTD	C-terminal domain
CTR1	Copper transporter 1
DMF	Dimethylformamide
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
ERCC1	Excision repair complementation group 1 protein
ERK	Extracellular signal-related kinase
ET	Extra-terminal domain
GSH	Glutathione
GST	Glutathione-S-transferase
IC10	Concentration required to give 10% inhibition
IC50	Concentration required to give 50% inhibition

IC90	Concentration required to give 90% inhibition
IMDM	Iscove's Modified Dulbecco's Medium
JAK	Janus kinase
JNK	c-Jun NH2-terminal kinase
MAPK	Mitogen-activated protein kinase
MDR	Multi-drug resistance
MDR1	P-glycoprotein, also known as multi-drug resistance protein 1
MMR	DNA mismatch repair
Milli-Q H_20	Milli-Q water
mRNA	Messenger ribonucleic acid
MRP	Multi-drug resistance-associated protein
MT	Metallothionein
MTT	(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NER	Nuclear excision repair
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NSCLC	Non-small cell lung cancer
OXALI	Oxaliplatin
p53	Tumour protein p53
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PD-L	Programmed cell death receptor ligand
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
p-TEFb	Positive transcription elongation factor b
PUMA	p53 upregulated modulator of apoptosis
RCCL	The Resistant Cancer Cell Line Collection
RNA	Ribonucleic acid
RNAi	RNA interference
SDS	Sodium dodecyl sulphate
siRNA	Silencing RNA
STAT	Signal transducer and activator of transcription
TNBC	triple-negative breast cancer

1. Abstract

Platinum compounds, such as cisplatin, and oxaliplatin are utilised widely in therapeutic strategies against a range of malignancies, for instance Neuroblastoma and Ovarian cancer. These compounds elicit anticancer effects by damaging DNA via the formation of crosslinks with nitrogenous bases, leading to DNA damage responses and the induction of apoptotic signalling. Although these compounds may produce promising initial responses, the emergence and establishment of chemoresistant tumour cells can often result in tumour relapses and treatment failure. The mechanisms driving resistance to platinum compounds are numerous, and act via a diverse range of cellular processes, such as reducing uptake of the drug, increasing DNA repair activity, and reducing apoptotic signalling.

There are also research efforts to elucidate the epigenetic modifications of resistant tumours, and the means in which these changes are recognised and transduced. The Bromodomain and Extra-Terminal domain (BET) family of proteins recognise acetylated histones, and regulate transcription via association with transcriptional co-activators and RNA polymerase II. BET family members, such as BRD4, are able to form 'super-enhancers', promoting oncogenic activity of genes such as MYC. Consequently, BET proteins have been identified as potential anticancer drug targets, prompting the development of BET inhibitors.

This study investigated whether the BET inhibitors, JQ1 and I-BET726, were able to increase the sensitivity of parental and oxaliplatin-resistant Neuroblastoma (UKF-NB-3) and cisplatin-resistant Ovarian cancer cell lines (COLO-704, EFO-21 and EFO-27) to their respective platinum compounds. BET inhibitor pre-treatments, using the IC10 and IC50 concentrations of JQ1 and I-BET726 as single-agents for each cell line, resulted in increased sensitivity to the platinum compounds, as determined by MTT cell viability assays. Increasing the pre-treatment incubation duration from 0hrs to 24hrs and 48hrs improved the response to platinum agents, and the same effect was observed with increasing pre-treatment dosage. In summary, these results suggest that BET inhibitors are able to increase the sensitivity of cancer cells to platinum chemotherapeutics.

2. Introduction

2.1 Overview of Cancer

2.1.1 Cancer Incidence and Survival Statistics

Cancer is a prominent cause of death worldwide, with a rising number of cases as populations grow, with prolonged life expectancies, and the adoption of lifestyle choices which increase cancer risk. In 2012, there were approximately 14.1 milllion new cases of cancer worldwide, and there were 8.2 million cancer deaths globally (Torre et al., 2016). Incidence rates vary widely from country-to-country, with the highest incidences generally seen in Europe, North America and Oceania.

In England in 2015, there were 299,923 registrations of newly diagnosed cases of cancer, and 134,679 deaths from cancer (Kaur & Poole, Office for National Statistics, 2017). The most frequently diagnosed cancer types were lung, colorectal, skin, breast and prostate cancer. In many cancer types there have been improvements in the 5-year survival rates throughout the past 10 years, however for many cancers the prognosis remains poor and there is a need for improved treatment strategies. For instance, data from 2001-2005 found the 5-year survival rate for lung cancer patients in England was 7.4%, improving slightly with a rise to 13.8% in the 2010-2014 report, however this figure is still very low in comparison to tumours such as melanoma (90.7%) (Bannister, Office for National Statistics, 2017).

2.1.2 Neuroblastoma

Neuroblastoma is the most common extracranial solid tumour diagnosed in children (Salim et al., 2011). The disease affects mostly children under 5 years old , and around 100 children are diagnosed with neuroblastoma each year in the UK (Cancer Research UK, 2015).

Neuroblastomas are formed of small, undifferentiated sympathetic neural precursor cells, known as neuroblasts. Over half of neuroblastomas present as tumours in the adrenal medlla, but tumours can often arise in the paraspinal ganglia in the abdomen, chest and pelvis (Brodeur, 2003). Patients with the disease often present with poor appetite, abdominal pain and distension, bruising, nausea and diarrhoea. Over half of the patients with neuroblastoma are diagnosed with metastatic disease, with the disease commonly spreading to the bones, skin, lymph nodes, and liver.

The prognosis for neuroblastoma is variable depending on the age and stage of disease at the time of diagnosis. There are also genetic features which subdivide the disease to either a low-risk neuroblastoma with a better prognosis, or a high-risk neuroblastoma which has a much poorer 5-year survival rate (PDQ® Pediatric Treatment Editorial Board, 2017).

A key feature which has a strong influence on survival is the *MYCN* amplification status of the tumour, which gives a poorer prognosis across all age groups with any stage of disease (Ambros et al., 2009). *MYCN* is a transcription factor required for regulating the proliferation, differentiation and survival of neural cells in the developing neural crest. *MYCN* amplification (more than 10 copies per diploid genome) is seen in 22% of all neuroblastoma cases, and in 44% of high-risk neuroblastoma cases (Brodeur, 2003; Ambros et al., 2009).

2.1.3 Ovarian Cancer

The World Cancer Report 2014, produced by the World Health Organisation (Stewart & Wild, 2014), estimated that there were approximately 239,000 new cases of ovarian cancer worldwide in 2012, making the disease the seventh most common cancer of women globally. In the same year, ovarian cancer was accountable for around 152,000 deaths. In the UK, there were 7,378 new cases of ovarian cancer in 2014, and 4,128 deaths from the disease, making ovarian cancer the sixth most common cancer in females, and the fifth most common cause of female cancer death (Cancer Research UK). In the UK, the lifetime risk of developing ovarian cancer is about 1 in 60 (Jayson et al., 2014).

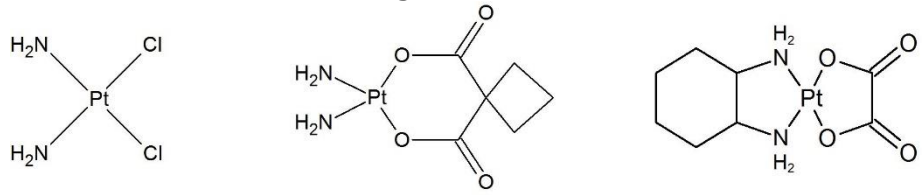
Over 90% of ovarian cancers arise from the epithelial surface (Rosen et al., 2009), with germ cells and stromal cells each accounting for approximately 5% of the remaining cases. Epithelial ovarian cancers can be subdivided using the histopathology of tumour biopsies to identify predominant cell types/lineages. Epithelial tumours are classified (from most to least common) as serous, endometrioid, mucinous, clear cell, or undifferentiated. Each of these sub-types have differing prognoses, with 5-year survival rates varying from 40–69% for mucinous tumours, to 11–29% for undifferentiated ovarian carcinomas (Rosen et al., 2009).

Ovarian cancers can also be graded as low-grade or high-grade, based on the abundance of abnormal nuclei (atypia), and the presence of genetic mutations. Common mutations which are observed in high-grade ovarian carcinomas affect *TP53*,

BRCA1/2, *P21*^{WAF1}, and *C-MYC* (Jayson et al., 2014; Plisiecka-Halasa et al., 2003). The consequences of such mutations are very proliferative tumours with high levels of genetic instability, resulting in increased likelihood of treatment failure and poorer prognoses for patients.

2.2 Platinum Drugs and the Emergence of Resistance

Cisplatin (cis-diamminedichloroplatinum II) is a member of a group of compounds which characteristically feature platinum as the central component of a complex (*Figure 1*). Initially (during the 1960's), it was found that cisplatin was effective in inhibiting the growth of E.coli and, during the 1970's, further studies of experimental tumour cell lines suggested that cisplatin may prove to be an effective anti-cancer agent, and therefore a number of clinical trials were initiated (Prestayko et al., 1979). These studies demonstrated antitumour activity across a range of malignancies, including neuroblastoma and ovarian cancer, as well as other tumours such as head and neck cancer and lung cancer.



Cisplatin Carboplatin Oxaliplatin

Figure 1: *The structures of Cisplatin, Carboplatin and Oxaliplatin, produced using Advanced Chemistry Development, Inc. (ACD/Labs) ChemSketch software.*

As a general overview of its mechanism, cisplatin functions by interacting with DNA, particularly targeting the purine bases, Adenine and Guanine. This interaction forms DNA adducts, such as inter- or intra-strand ApG and GpG crosslinks (Siddik, 2003). These DNA adducts induce cell cycle arrest, inhibited DNA synthesis and transcription of RNA, and the upregulation of pro-apoptotic signalling pathways. Specific features of the cisplatin mechanism of action shall be discussed in the context of chemoresistance to the compound (Section 2.3).

The nature in which cisplatin targets tumours means that the drug has also been shown to cause a number of side effects in the kidneys (nephrotoxicity), nervous system (neurotoxicity) and inner ear (ototoxicity) (Galluzzi et al., 2012). However, the main factor limiting the efficacy of cisplatin therapy is the emergence of chemoresistance. Chemoresistance may be acquired over time as an adaptive response to prolonged exposure to the compound, or cells may display intrinsic resistance.

Consequently, cisplatin resistance is responsible for the high relapse rates and the greatly reduced 5-year patient survival rates seen in ovarian cancers and non-small cell lung cancer (NSCLC) (Siddik, 2003).

In response to the emergence of cisplatin resistance, and in an attempt to mitigate some of the side effects to cisplatin, the compounds carboplatin (cis-diammine (cyclobutane-1,1-dicarboxylate-O,O') platinum(II)) and oxaliplatin ([(1R,2R)-cyclohexane-1,2-diamine](ethanedioato-O,O') platinum(II)) have been developed. Carboplatin was found to induce less severe side effects, however the drug has less potency, and functions using the same mechanism as cisplatin so is susceptible to the same resistance issues (Harrap, 1985). Oxaliplatin was subsequently developed, with a distinct pharmacological and immunological profile to cisplatin. Evidence suggests that cisplatin-resistant tumours may be susceptible to oxaliplatin, however a degree of cross-resistance has been observed (Stordal et al., 2007).

2.3 Mechanisms of Chemoresistance to Platinum Compounds

Due to the significant clinical impact of cisplatin resistance across a wide range of malignancies, a large volume of research has been conducted to characterise the mechanisms driving chemoresistance, and investigate means to negate the issue.

The traits which promote resistance to platinum compounds can be broadly grouped into three categories: those which restrict the cisplatin-DNA interaction, those which give increased repair to the DNA damage caused by the compound, and adaptations which are able to reduce the anti-tumour signalling pathways induced by platinum compounds.

2.3.1 Inhibiting the Cisplatin-DNA Interaction

One way of promoting survival in the presence of cisplatin is to reduce the uptake of the compound. Resistant cell lines have been seen to show a decreased accumulation of cisplatin within the cell, a mechanism of pre-target resistance. One aspect of this reduced accumulation is likely due to reduced influx of cisplatin into the cell. A study by Holzer et al. (2006) found that the uptake of cisplatin into a cell is closely associated with the expression of Copper Transporter 1 (CTR1) (*Figure 2*). Wild type (+/+) CTR1 cells showed normal influx of copper, as well as platinum drugs. Conversely CTR1 depletion (-/-) reduced drug uptake, leading to approximately 3-fold increases in

cisplatin resistance. CTR1 was seen to be downregulated in cisplatin-resistant cell lines.

Additionally, chemoresistance may arise from increased efflux of platinum compounds from the cell. Copper homeostasis transporters have again been implicated in chemoresistance via the increased efflux of platinum compounds. Safaei et al. (2004) observed that the P-type ATPase copper efflux transporters, ATP7A and ATP7B, were expressed at higher levels in tumour cell lines selected for resistance to cisplatin, oxaliplatin, and carboplatin. Cell lines with higher ATP7A and ATP7B expression were also found to have less DNA damage in response to treatment with platinum compounds. In many human cancers, the transport proteins, P-glycoprotein (P-gp, also known as ABCB1) and multidrug-resistant proteins (MRPs, also known as the ABCC family) are commonly seen to be overexpressed leading to resistance to a range of compounds. However there are exceptions, for instance, acquired multi-drug resistance in neuroblastoma cell lines was found to occur independently of P-gp expression (Kotchetkov et al., 2005).

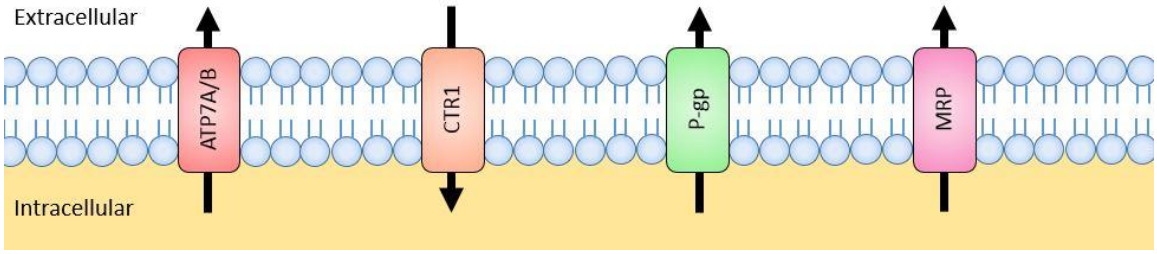


Figure 2: *An overview of the transporters thought to be involved in cisplatin influx or efflux (directionality indicated with arrows).*

Increases in the levels of cytoplasmic thiol-containing compounds such as glutathione or metallothioneins is another means by which cells can detoxify platinum compounds, thus preventing subsequent interactions with DNA (Kelland, 2007). These compounds contain sulphur groups which facilitate the binding of the platinum compounds. This interaction produces inactive conjugates which can then be expelled from the cell. Kasahara et al. (1991) analysed and compared the levels of metallothioneins in non-small cell lung cancer cell lines displaying 6- and 11-fold resistance to cisplatin, finding a positive correlation between metallothionein content and the degree of resistance (determined by changes in the IC_{50}).

2.3.2 Repair of Cisplatin-DNA Adducts

Evidence suggests that a source of cisplatin susceptibility lies in the competency of DNA repair mechanisms within a cell line. For example, in malignancies such as

testicular cancer which seem to show deficiencies in DNA repair, there appears to be reasonable levels of susceptibility to cisplatin. By contrast, less responsive cell lines such as ovarian cancer appear to exhibit more proficient DNA repair (Kelland, 2007).

Nucleotide excision repair (NER) is a vital mechanism in the repair of DNA damage and therefore it was postulated to have a role in platinum resistance. Studies have shown that the excision repair cross-complementation group 1 (ERCC1) enzyme is involved in the repair of cisplatin-induced DNA adducts, using its ability to repair inter-strand crosslinks (Olaussen et al., 2006). ERCC1-positive non-small cell lung cancer cell lines were found to be less responsive to cisplatin adjuvant therapy compared to ERCC1-negative cell lines. This led to the hypothesis that ERCC1 expression could be examined in resected tumours, and used as a predictor of success with subsequent adjuvant therapy.

Another DNA mechanism, mismatch repair (MMR), is required for the recognition and repair of DNA damage, as well as the coupling of this recognition with cell cycle arrest, and pro-apoptotic signalling. Subunits of the MutLα-mismatch repair complex, hMLH1 and hPMS2, were found to show reduced expression in cisplatin-resistant ovarian cancer cell lines (Brown et al., 1997). Cell lines showing deficient mismatch repair were seen to be more tolerant to DNA damage, and lost the ability to induce G1 and G2 cell cycle arrest. In cells with functional hMLH1, cells are able to recognise DNA adducts, and induce cell cycle arrest and apoptosis, which suggests that hMLH1 depletion allows for survival by negating the DNA lesion or failing to induce signalling pathways.

Due to structural differences between cisplatin and oxaliplatin, namely the larger size and presence of the diaminocyclohexane group of oxaliplatin, it is thought that ERCC1 plays a key role in the processing of (and resistance to) oxaliplatin, but changes in MMR do not seem to contribute to oxaliplatin resistance (Seetharam et al., 2009).

2.3.3 Alterations in Signalling

The most prominent pathway linking DNA damage to apoptosis is the activation of the ataxia telangiectasia mutated (ATM)- and RAD3-related protein (ATR), which is able to act as a sensor of DNA damage. Upon activation, ATR is able to phosphorylate the downstream effector, checkpoint kinase 1 (CHEK1), which itself is able to provide activating phosphorylations to p53 (Shieh et al., 2000; Zhao and Piwnica-Worms, 2001). Additionally, ATR is able to phosphorylate the histone H2A variant, H2AX, at

Ser-139 to generate γ-H2AX. This phosphorylation occurs not just within the immediate vicinity of the DNA lesion, but spreads throughout the proximal chromatin to create a γ-H2AX focus which acts as a 'launch-pad' for DNA damage response signalling (Kinner et al., 2008; Pabla et al., 2008). As a consequence, γ-H2AX is a useful biomarker which is frequently used for the study of DNA damage and repair (Mah et al., 2010).

Cisplatin induces p53 to activate a number of downstream genes, leading to cell cycle arrest and apoptosis. In a study of ovarian cancer patients, wild-type p53 status predicted a better clinical outcome than patients expressing mutated p53 (Gadducci et al., 2002). This study also found that paclitaxel induces apoptosis independently of p53, and so could prove to be a useful treatment in such instances. A study of metastatic colorectal cancer cell lines (Yang et al., 2016) found a similar result: cells with wild-type p53 showed sensitivity to oxaliplatin, and those with defective p53 showed resistance. This study observed an accumulation of p53 after treatment with oxaliplatin, as well as upregulation of the cytochrome enzyme, CYP2S1, leading to downregulation of Wnt/β-catenin and PGE2 signalling pathways. Additionally, knockdowns of CYP2S1 resulted in increased proliferation and cell survival after oxaliplatin treatment, illustrating the importance of the cytochrome enzymes in mediating the response to oxaliplatin.

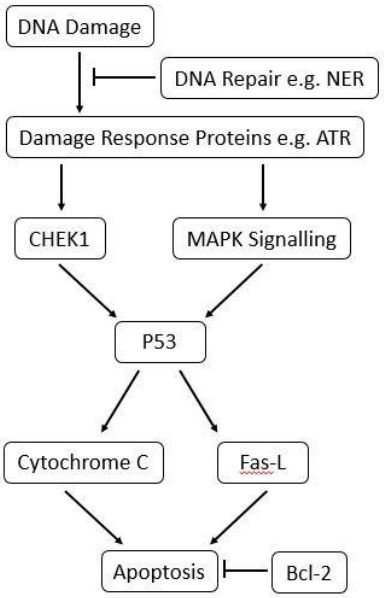


Figure 3: *A simplified depiction outlining some of the mechanisms by which cisplatin-induced DNA damage is coupled to apoptosis. This schematic is focused largely on elements shown to be modified in resistant cell lines. Produced using Microsoft Powerpoint (2016).*

Bcl-2 is an inhibitor of apoptosis, and is commonly overexpressed in a number of malignancies such as leukaemias and neuroblastoma. Neuroblastoma cell lines expressing higher levels of Bcl-2 were found to show resistance to cisplatin (Dole et al., 1994). Bcl-2 expression was observed in approximately a third of pre-therapy neuroblastoma specimens, with this proportion increasing to approximately 80% of specimens provided post-cisplatin therapy. Higher Bcl-2 levels provide greater tolerance to the cytotoxic cisplatin-induced DNA damage by inhibiting apoptosis.

A signalling network which appears to be distorted in cisplatin-resistant cells are the mitogen-activated protein kinase (MAPK) pathways. Research investigating the

cisplatin-induced activation of the JNK, P38 and ERK pathways in ovarian carcinoma cell lines (Mansouri et al., 2003) yielded varying activation patterns between sensitive and resistant cell lines. Sensitive cell lines showed prolonged (8 to 12 hours) activation of the JNK and P38 pathways in response to cisplatin, whereas resistant cells showed only transient (1 to 3 hours) activation. Blocking the activation of JNK and P38 in resistant cell lines inhibited apoptosis in response to cisplatin. Chemoresistance was associated with an inability to upregulate the pro-apoptotic Fas-ligand. Prolonged activation of the JNK pathway is implicated with increased Fas-ligand expression, and therefore the duration of signal activation appears to be key in producing apoptosis.

Wnt/β-catenin signalling is derailed in a number of diseases (including cancers, Alzheimer's disease, and metabolic disease). Wnt signalling regulates a number of critical genes such as cyclin D1, c-Myc, survivin, and the ABC transporters Multidrug Resistance Protein 4 (MRP4) and Breast Cancer Resistance Protein (BCRP). A key regulator of Wnt/β-catenin signalling is dishevelled, which acts downstream of the Frizzled receptor to modulate GSK3 and allow for β-catenin to accumulate in the nucleus. High levels of dishevelled blocks β-catenin destruction and therefore leads to activation of target genes. A study of alveolar adenocarcinoma cell lines found that the *DVL2* gene is overexpressed in cisplatin-resistant cell lines (Luo et al., 2016). Inhibition of *DVL2* was shown to restore sensitivity of these resistant cell lines to cisplatin. Additionally, the genes for BCRP and MRP4 were stronger expressed in resistant cell lines, with reductions seen after *DVL2* inhibition.

Analysis of tumours from rat models found that expression of the c-Myc oncogene was approximately doubled in cells post-cisplatin treatment (Walker et al., 1996). c-Myc plays a key role in increasing cell proliferation, mitogenesis, and reducing differentiation and apoptotic cell death signalling, increasing the tolerance to cisplatin.

Growth factor receptors can also be upregulated in resistant tumours, leading to increased proliferation and a pro-survival response. The epidermal growth factor receptor (EGFR) is frequently expressed in neuroblastoma cell lines, and was found to have increased expression in cell lines showing chemoresistance to cisplatin (Michaelis et al., 2008). Resistant cell lines were found to be susceptible to anti-EGFR toxins, even in cell lines that were insensitive to the anti-EGFR antibody, cetuximab. Resistant cell lines showed increased EGFR expression even after several passages in the absence of cisplatin, suggesting that these upregulations are stable. Combinations of cisplatin and either of the anti-EGFR toxins produced potent anti-cancer effects; significantly

stronger than either treatment alone. EGFR is able to induce signalling via the PI3-K and PKB/Akt pathway. It was found that inhibition of PI3-K resulted in increased sensitivity to cisplatin in pancreatic and breast cancer cell lines, suggesting that increases in PI3-K and PKB/AKT signalling may provide resistance to cisplatin (Winograd-Katz and Levitzki, 2006).

Drug resistant cells show accelerated tumour progression, with a shift towards a more invasive and migratory phenotype, using shifts in the expression of cell adhesion receptors and pro-angiogenic factors, something which may be a product of the widespread alterations in signalling (Blaheta et al., 2006; Michaelis et al., 2009).

2.4 The Bromodomain and Extra-Terminal Domain (BET) Family

2.4.1 Overview of BET Family

As well as studying genetic mutations which may be involved in tumourigenesis, there is an increase in research assessing the role epigenetic regulatory mechanisms in tumourigenesis (such as aberrant methylation patterns, deregulated acetylation/deacetylation, and altered recognition of modified chromatin).

Acetylation of lysine residues at the N-terminus of histone tails is a modification which is generally associated with transcriptionally active euchromatin. The recognition of acetyl-lysine residues is primarily by proteins containing bromodomains (BRD), such as those from the BRD and Extra-Terminal domain (BET) family, consisting of BRD2, BRD3, BRD4, and the testis-specific BRDT (Filippakopoulos and Knapp, 2014).

BET proteins characteristically feature two N-terminal tandem bromodomains (BRDs), consisting of a four-helix bundle (αZ, αA, αB and αC) linked by diverse ZA and BC loop regions (Wang and Filippakopoulos, 2015). The BC loop contains a conserved asparagine residue which is critical for the interaction of BET proteins with the acetyl-lysine of histone 4. BET proteins also feature well-conserved A, B and SEED (Ser/Glu/Asp) motifs (*Figure 4*). The A motif contains a nuclear localisation sequence

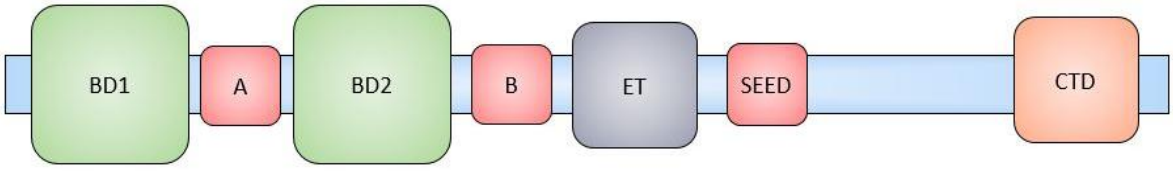


Figure 4: *A generalised overview of the structure of a BET family protein, consisting of two bromodomains (BD - green), A, B and SEED (Ser/Glu/Asp) motifs (red), an extraterminal domain (ET – grey), and a C-terminal domain (CTD – orange).*

of 12 amino acids (KGVKRKADTTTP), and the B motif is thought to be important in facilitating homo- or heterodimerisation of BET proteins. BET proteins are also typified by the presence of a helical extra-terminal (ET) domain, which is preserved between the four BET family members.

2.4.2 Action of BET Family Proteins

Upon binding acetylated histone tails, BET proteins are able to modify transcriptional activity by facilitating the association of a number of transcription factors, co-activators or co-repressors. A key interaction which facilitates this function is the association of BRD4 with the positive transcription elongation factor, P-TEFb, which is comprised of a heterodimer between Cdk9 and cyclin T1 (Yang et al., 2005). P-TEFb is essential for regulating transcriptional elongation by RNA Pol II, and is therefore crucial for ensuring the strict co-ordination of gene expression. P-TEFb is often seen in a sequestered, inactive state, by forming complexes with regulatory 7SK snRNA and the HEXIM1 protein. Yang et al. (2005) demonstrated that P-TEFb associates with the C-terminal domain of BRD4 to become transcriptionally active, recruiting RNA Pol II. BRD4, via its association with acetyl-lysine residues of histones, is therefore able to ensure that P-TEFb (and subsequently RNA Pol II) are localised at transcriptionally active euchromatin sites.

BRD4 has also been found to recruit P-TEFb independently of histones via an interaction with the Mediator complex (Yang et al., 2005; Basheer and Huntly, 2015). Mediator acts as a co-activator, enabling transcription factors to interact with RNA Pol II. BRD4 is able to interact with the MED1 subunit (also known as TRAP220) at transcription start sites, further highlighting an important role of the BRD4-Mediator interaction to regulate transcription.

Studies have shown that BRD4 plays a key role in the progression of the cell cycle. During mitosis a cell must facilitate a transcriptional shutdown, via the dissociation of transcription factors and coiling of chromatin to give gene silencing. Some genes must remain marked, however, to ensure that the transcription of appropriate genes (such as the key M/G1 genes expressed after mitosis) can be inherited in daughter cells. This marking is facilitated by the association of BRD4, which allows for P-TEFb association and subsequent transcription via RNA Pol II (Dey et al., 2009). BRD4 marking was not seen in genes required at later stages of the cell cycle, suggesting that this marking is

purely to ensure the cell cycle progresses post-mitosis, upon which BRD4 can assume its dynamic role in regulating global transcriptional activity.

A large amount of the research into BET family proteins has focused on the interactions of the bromodomain with chromatin, or the interaction of the C-terminal domain with P-TEFb. The extraterminal domain of this protein family is highly conserved, and therefore studies have used proteomics to investigate a role of the extraterminal (ET) domain. Five proteins were found to interact with the ET domain of BRD4: NSD3, CHD4, JMJD6, GLTSCR1, and ATAD5 (Rahman et al., 2011), and these interactions are also conserved with BRD2 and BRD3. NSD3, JMJD6 and GLTSCR1 were found to combine with BRD4 to play a role in transcriptional regulation in a pTEFb-independent manner. The five proteins each have been found to play a role in the regulation of chromatin structure and activity. The association of BRD4/NSD3 was found to be implicated with H3K36 methylation, and so has a role in modifying the epigenetic environment surrounding BRD4 targeted genes.

BET proteins are able to interact directly with a range of transcription factors, including p53, c-Jun and c-Myc/Max (Wu et al., 2013). BRD4 has the ability to recruit these factors to transcriptionally active regions containing acetylated histones, providing a regulatory mechanism for their activity. BRD4 may also regulate the function of these proteins independently of histones, for example the binding of BRD4 to c-Myc appears to also modulate c-Myc protein stability and activity.

2.4.3 Role in Disease

As the BET family of protein are able to function via a number of mechanisms, and can therefore regulate a range of pathways, it has been observed that alterations in BET activity is associated with a number of diseases, such as cancer or inflammatory diseases. For example, high levels of BRD2 have been seen in human leukaemias and B-cell lymphomas, and chromosomal translocations can produce fusions of BRD4 or BRD3 with nuclear protein in testis (NUT), causing NUT midline carcinomas (NMCs) (Wang and Filippakopoulos, 2015; Zhang, Su et al., 2016). A study assessing the effects of BET mutations, particularly mutations commonly observed in cancers, found that mutations were seen to generally cause a decrease in overall stability, and give a flexibility to the tertiary structure (Lori et al., 2016). The mutations occurred in close proximity to the acetyl-lysine binding site which may alter histone binding affinities.

Mutations giving flexibility and loosened tertiary structure may also possibly facilitate alternative interaction networks by exposing new sites.

2.5 BET Inhibitors as Cancer Therapeutics

2.5.1 Rationale Behind BET Inhibitors and Their Mechanism of Action

In addition to changes in the BET proteins themselves, many of the pathways regulated by BET proteins are deregulated in cancer, for example c-Myc and p53 signalling. Therefore, it was postulated that inhibitors of BET proteins could provide therapeutic benefits in these diseases.

A number of studies have used RNA interference by short hairpin RNA (shRNA) or small interfering RNA (siRNA) to inhibit BET proteins. Inhibition was found to result in arrest of the cell cycle and pro-apoptotic affects in NUT midline carcinoma (NMC) and acute myeloid leukaemia cell lines (French et al., 2008; Zuber et al., 2011). These studies provide proof-of-concept that BET inhibition could potentially be a viable strategy in cancer chemotherapy.

A number of compounds have been developed as inhibitors of BET proteins, including JQ1, I-BET151, MS417, and PFI-1 (Zhang, Su et al., 2016; Filippakopoulos et al., 2010). These function by competitively binding the acetyl-lysine recognition motifs, thus inhibiting BET recruitment to chromatin and causing repression of downstream transcription pathways (*Figure 5*).

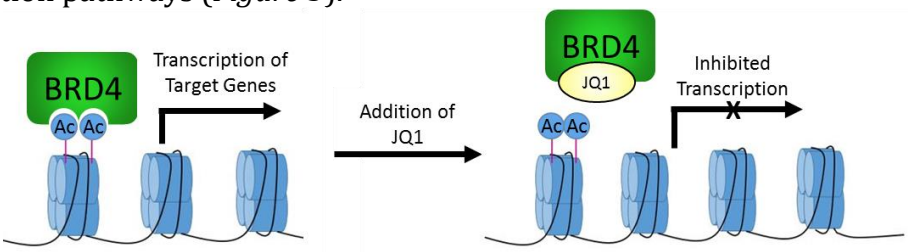


Figure 5: *JQ1 is able to competitively bind the acetyl-lysine recognition domains of BRD4, leading to displacement from histones and inhibited transcription of target genes.*

2.5.2 Uses of BET Inhibitors in Cancer Research

Zhang et al. (2016) observed that JQ1 impaired the malignant transformation of mouse skin epidermal JB6 P+ cells after treatment with the tumour promoter 12-O-tetradecanoylphorbol-13-acetate (TPA). Similarly, the study by Filippakopoulos et al. (2010) noted that JQ1 was able to induce cellular differentiation, phenotypic changes to flattened, squamous shapes, with reduced proliferation and increased apoptosis.

BET proteins are expressed widely across many tissue types, and therefore it might be expected that inhibition could cause widespread toxicity. However, in mouse models, the mice showed tolerance to BET inhibitors at dosages capable of causing tumour inhibition. It has been determined that the disproportionate clustering of BRD4 and mediators at super-enhancer regions is responsible for driving the activity of a number of key cancer genes, such as the MYC oncogene. Super-enhancer sites were found to be more susceptible to JQ1 treatment than typical enhancers, with JQ1 inducing preferential loss of BRD4 at these sites (Loven et al., 2013). Consequently, additional co-factors (such as MED1 and CDK9) were also lost from the super-enhancer as a result of the decrease in BRD4 association.

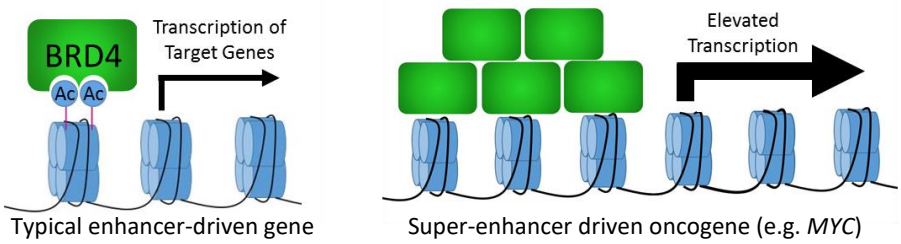


Figure 6: *The interaction between BRD4 with a typical enhancer-driven gene (left), compared with a super-enhancer produced by the aggregation of BRD4, resulting in elevated transcription (right).*

As mentioned previously, BET proteins have been found to interact with and regulate c-Myc activity. *MYC* is one of the most commonly mutated genes observed in human cancers, causing increased cell proliferation, altered metabolism, and preventing terminal differentiation. Genetic studies in mice found that inactivation of MYC transcription can lead to regression of tumours. However, MYC lacks domains which are suitable to give specificity for drug targets e.g. binding sites for competitive or allosteric inhibitors. It was found (Delmore et al., 2011) that BRD4 inhibition is able to inhibit c-Myc signalling, reducing proliferation and inducing cell cycle arrest and apoptosis in multiple myeloma. The compounds I-BET151 and I-BET762 were both also found to downregulate c-Myc in multiple myeloma cell lines, as well as upregulating the HEXIM1 negative regulator of pTEFb (Chaidos et al., 2014).

This effect is similarly seen in neuroblastoma, a malignancy which frequently exhibits overexpression of n-Myc (Ambros et al., 2009; Puissant et al., 2013). BET inhibitors produced downregulation of MYCN transcription and suppression of downstream n-Myc pathways in neuroblastoma, leading to reduced tumour cell proliferation and pro-apoptotic effects. BET inhibitors have been found to downregulate MYC activity in a number of additional tumours, such as non-small cell

lung cancer, prostate cancer and Burkitt's lymphoma (Wang and Filippakopoulos, 2015).

Studies in glioblastoma and B-cell lymphoma have shown that BET inhibitors may also work in a Myc-independent manner. JQ1 treatment of glioblastoma resulted in notable changes in expression of $p21^{WAF1}$, Bcl-2 and Bcl-xL, resulting in cell cycle arrest and apoptosis (Cheng et al., 2013). A study of B-cell lymphoma observed upregulation of the pro-apoptotic Bim, and downregulation of the anti-apoptotic Bcl-2 and Bcl-xL (Hogg et al., 2016). Notably, both studies found that JQ1 was able to induce apoptosis independently of p53 expression, a feature which is commonly derailed in cancer.

The PI3-K pathway is crucial for regulating a range of substrates key to modulating cell growth, metabolism, proliferation and survival, and is commonly disordered in cancers. However, there is emerging resistance to PI3-K inhibiting drugs, particularly to isoform-specific inhibitors of PI3-K. Other PI3-K isoforms have been shown to compensate for each other, and activation of separate, compensatory pathways has also been observed as a means of generating resistance. Recent studies have shown that BET inhibitors are able to counteract the resistance mechanisms to PI3-K inhibitors that would otherwise limit the efficacy of the treatments (Stratikopoulos and Parsons, 2016). In addition, BET inhibitors may cause reductions in signalling via the MAPK, JAK/STAT, and oestrogen receptor pathways, and therefore be useful as part of combinatorial treatment plans.

2.6 Project Aims

A number of the genes targeted by BET inhibitors are seen to be deregulated in tumours exhibiting resistance to cisplatin and other platinum compounds (coding for proteins such as c-Myc, Bcl-2, PI3-K, MAPK). This research project investigated the possibility that treatment with BET inhibitors would prove effective against parental and platinum-resistant neuroblastoma and ovarian cancer cell lines, and whether pre-treating with BET inhibitors would increase the sensitivity to platinum compounds in these cell lines.

3. Materials and Methods

3.1 Compounds

3.1.1 Platinum Compounds

Cisplatin (Sigma, Poole, Dorset, UK, Ref P4394) was dissolved in 0.9% sodium chloride, to produce a stock solution of 1mg/mL (3.33mM). Cisplatin was dissolved in the dark at room temperature for 5 days using a magnetic stirrer. At this point the solution was filter sterilised (0.2 micron), with all filter sterilisation taking place in a Class II biological safety cabinet (BioMAT 2, Contained Air Solutions, Manchester, UK) to ensure sterility before using cisplatin for mammalian cell culture. The solution was aliquoted into sterile tubes and stored at room temperature in the dark, due to the sensitivity of cisplatin to light.

Oxaliplatin (Sigma, Poole, Dorset, UK, Ref O9512) was dissolved using filter-sterilised (0.2micron) 5% (w/v) glucose solution, to produce a 1mg/mL stock solution (2.52mM). This solution was aliquoted in to sterile tubes, stored at -20°C, and was also kept in the dark.

3.1.2 BET inhibitors

JQ1 (Cambridge Bioscience, Cambridge, UK, Ref CAY11187) was dissolved in Dimethyl Sulfoxide (DMSO) (Sigma, Poole, Dorset, UK, Ref D2438), to produce a stock solution of 1mg/mL (2.19mM). I-BET726 (Cambridge Bioscience, Cambridge, UK, Ref CAY16872) was also dissolved in DMSO, to produce a stock solution of 1mg/mL (2.30mM). Both compounds were aliquoted into sterile tubes and stored at -20°C.

3.2 Tissue Culture

3.2.1 Cell Lines and Growth Conditions

The human neuroblastoma cell line, UKF-NB-3, was derived from bone marrow metastases of a patient with MYCN-amplified stage 4 neuroblastoma (Kotchetkov et al., 2005). Drug resistant cell lines, established by continuous exposure to increasing drug concentrations, were obtained from the Resistant Cancer Cell Line (RCCL) collection (www.kent.ac.uk/stms/cmp/RCCL/RCCLabout.html). An oxaliplatin-resistant UKF-NB-3 cell subline had been established by adapting UKF-NB-3 to growth in the presence of oxaliplatin (2000ng/mL) and designated as UKF-NB-3^{r}OXALI^{2000}.

Cisplatin-resistant sub-lines of the ovarian cancer cell lines COLO-704, EFO-21 and EFO-27 had been generated in the same way and were termed COLO-704^{r}CDDP^{1000}, EFO-21^{r}CDDP^{2000}, and EFO-27^{r}CDDP^{2000}. COLO-704 is a cell line of high grade serous adenocarcinoma, established from ascites fluid stemming from the colon metastasis of a 46-year-old woman in 1986. EFO-21 was established in 1979 from the ascitic fluid of a 56-year-old woman with dedifferentiated serous cystadenocarcinoma. EFO-27, also established in 1979, was established from the solid omental metastasis of a 36-year-old woman with mucinous papillary adenocarcinoma.

All cell lines were cultured in Iscove's Modified Dulbecco's Medium (IMDM) (Gibco, as part of Thermo Fisher Scientific, Loughborough, Leicestershire, UK, Ref 12440061) supplemented with 100 U/mL penicillin/100 µg/mL streptomycin (Gibco, as part of Thermo Fisher Scientific, Loughborough, Leicestershire, UK, Ref 15140) and 10% Foetal Bovine Serum (FBS) (Sigma, Poole, Dorset, UK, Ref F7524). The media for the drug-resistant cell lines were additionally supplemented with either oxaliplatin or cisplatin to the required concentrations, using the stocks outlined in Section 3.1.1. The cells were cultured in T25cm^2 flasks (Sarstedt AG & Co, Sarstedtstraße 1, 51588 Nümbrecht, Germany, Ref 83.3910.002) containing 10mL of media, which were kept in an incubator set at 37°C and 5% CO_2 (ThermoForma Series II water jacketed CO_2 incubator, Thermo Fisher Scientific, Loughborough, Leicestershire, UK). The cell lines were always handled in a Class II biological safety cabinet, and only one cell line was used at any one time.

3.2.2 Passaging of Cell Lines

The UKF-NB-3, EFO-21 and EFO-27 sets of cell lines are all adherent, whereas the COLO-704 set of cell lines grow as a suspension in media and required a different passaging procedure.

Adherent Cell Lines:

To passage the adherent cells for maintenance and/or experiments, the following procedure was utilised. The media of the cells was aspirated using a 10mL serological pipette, and then the cells were washed with 2mL of Phosphate Buffered Saline (PBS) (Oxoid™ Dulbecco A solution, without Ca^{2+} or Mg^{2+}) (Fisher Scientific, as part of Thermo Fisher Scientific, Loughborough, Leicestershire, UK, Ref BR0014G).

The UKF-NB-3 cell lines were dissociated from the flask using 1mL of 0.05% Trypsin-EDTA (Gibco, as part of Thermo Fisher Scientific, Loughborough, Leicestershire, UK, Ref 25300054) for 2 minutes in a 37°C/ 5% CO_2 incubator. The EFO-21 and EFO-27 cell lines adhere much stronger, and so were instead dissociated using 1mL of the more concentrated Trypsin-EDTA (1X) (Sigma, Poole, Dorset, UK, Ref 59430C), spending 20 minutes in the 37°C/ 5% CO_2 incubator. Cells were then suspended in 10mL of media to cease the enzymatic reaction and allow for the passaging and/or plating of cells at appropriate densities. Separate media, PBS and Trypsin were used for each cell line to help prevent contamination. Cell lines were passaged after reaching a confluency of approximately 70-80%, and typically the cell lines were split at a ratio of 1 in 50 to give weekly passage intervals.

Suspension Cell Lines:

To passage the COLO-704 set of cell lines for maintenance and/or experiments, the following procedure was utilised. The cells (suspended in media) were aspirated using a 10mL serological pipette, and this suspension was centrifuged (Centaur 2, MSE UK Ltd, London, UK, Ref MSB020.CX1) at 1200rpm for 5 minutes to form cell pellets. The supernatants were removed and the cells were resuspended in 10mL of new media. The appropriate volume of cell suspension could then be added to a new flask for passaging and/or to plates at the appropriate densities for assays. As with the adherent cell lines, the COLO-704 lines were passaged after reaching a confluency of approximately 70-80%, and typically the cell lines were split at a ratio of 1 in 50 to give weekly passage intervals.

3.3 MTT Cell Viability Assay

3.3.1 Assay Premise

This assay uses (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) to measure metabolic activity as a marker of viable cells. Viable cells with active metabolism are able to convert the MTT (with a yellow colouration) into the purple-coloured (E,Z)-5-(4, 5-dimethylthiazol-2-yl)-1,3-diphenylformazan (formazan) via mitochondrial activity (*Figure 7*). This allows for colourimetric assessment of cell viability by measuring the absorbance at 600nm.

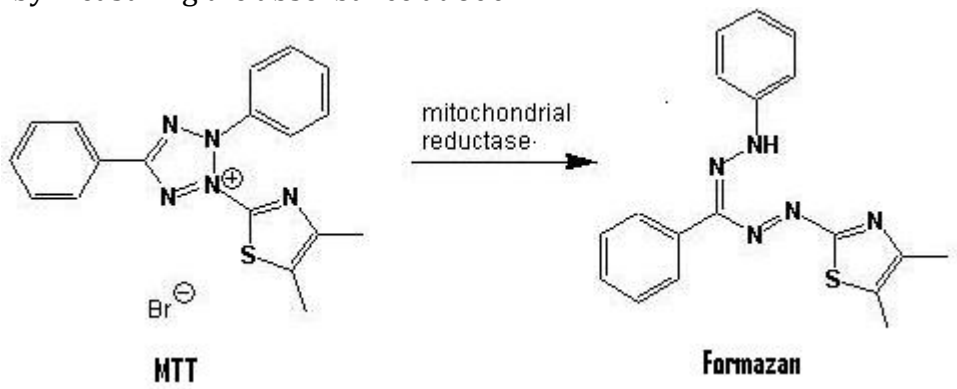


Figure 7: *The reduction of MTT to formazan via mitochondrial metabolism. (Brescia and Banks, 2009) BioTek Instruments, Inc., Winooski, VT*

The MTT assay was used to assess the viability of the cancer cell lines after being challenged with platinum drugs or BET inhibitors as single agents, to establish dose-response profiles, and determine the IC50 and IC90 for each cell line to the drugs. The cell lines would then be pre-treated with the BET inhibitors to determine whether this alters their sensitivities to platinum compounds (detailed further in Section 3.3.5). Concentrations of drug which are able to inhibit cell viability would result in less conversion of MTT to formazan and therefore lower absorbance at 600nm, relative to untreated cells.

3.3.2 MTT Plate Setup

The MTT assays were always set-up in a Class II biological safety cabinet and the assays were arranged using a 96-well plate to give 8-point serial dilutions of drug. Each concentration of a particular drug was plated in triplicate wells, to give 3 technical repeats per plate. The plates also had media-only wells containing no cells (termed MIN – outlined with blue in *Figure 8*), and wells containing cells and media in the absence of drug (termed MAX – outlined with red in *Figure 8*). These allowed for the establishment of relative cell viability, and for the elimination of any 'background'

absorbance values produced from the media. Each well of the plate was made up to a total volume of 100µL. The 96-well assay plates were set up using the following protocol (*Figure 8*A-C).

A – Complete IMDM media added to outer wells, and to MIN and MAX wells

	1	2	3	4	5	6	7	8	9	10	11	12
A	100	100	100	100	100	100	100	100	100	100	100	100
B	100	50									100	100
C	100	50									100	100
D	100	50									100	100
E	100	50									100	100
F	100	50									100	100
G	100	50									100	100
H	100	100	100	100	100	100	100	100	100	100	100	100

B – Cells added (at 5000 cells per well) to sample and MAX wells

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		50	50	50	50	50	50	50	50	50		
C		50	50	50	50	50	50	50	50	50		
D		50	50	50	50	50	50	50	50	50		
E		50	50	50	50	50	50	50	50	50		
F		50	50	50	50	50	50	50	50	50		
G		50	50	50	50	50	50	50	50	50		
H												

C – Drug added (50µL) at 2x concentration to adjust for dilution by cells in media

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B			1	2	3	4	5	6	7	8		
C			1	2	3	4	5	6	7	8		
D			1	2	3	4	5	6	7	8		
E			1	2	3	4	5	6	7	8		
F			1	2	3	4	5	6	7	8		
G			1	2	3	4	5	6	7	8		
H												

***Figure 8** : The protocol for setting up an MTT assay using a 96 well plate. **A)** Initially complete IMDM media was added to the outermost wells to prevent evaporation of sample wells, and also added to the media-only (MIN) wells (blue outline) and to the untreated cell (MAX) wells (red outline). Numbers represent the volume of media (in µL added per well) **B)** At this stage, the suspension of cells in media was added to the MAX wells, and to the experimental wells (B3-G10). Prior to this, the cells had been passaged and counted to ensure that 5000 cells would be present in the 50µL added to each well. **C)** 50µL of drug was added to each well, to give an 8-point serial dilution, which is triplicated for each drug. In this figure, the 2 different drugs are illustrated as pink and green, with the numbers representing the triplicates of each drug concentration.*

The procedure for counting cells is outlined in the subsequent section (3.3.3), and was used after passaging the confluent (70-80%) cell lines to ensure that 5000 cells

were present in the 50µL added to each of the required wells. Serial drug dilutions were prepared using a 96 deep well mixing plate, with the drug being diluted in complete IMDM media. The drugs were prepared at twice the desired concentrations for the assay, to account for dilution once added to the 50µL of cells present in the wells. The plates were then incubated at 37°C/ 5% CO_2 for 120 hours.

After this period of incubation, 25µL of MTT reagent was added to each well of the plate. MTT reagent was made up by diluting 0.5g of MTT (Universal Biologicals Ltd, Cambridge, UK, Ref 20395) in 250mL of PBS, and then filter sterilising this solution, before being stored at 4°C wrapped in foil. The plates were incubated at 37°C/ 5% CO_2 after the addition of MTT for 4 hours, before the addition of 100µL of a solution of 20% (w/v) Sodium Dodecyl Sulphate (SDS) in 1:1 Milli-Q H_20:DMF (Dimethylformamide (DMF), acidified to pH 4.4. The plates were incubated at 37°C/ 5% CO_2 overnight to allow for the lysis of the cells and for the dissolution of formazan. After this, the plates could be read in a plate reader (VICTOR X4, PerkinElmer, Waltham, MA, USA) to measure the absorbance at 600nm.

3.3.3 Cell Counting

After the cells had been disassociated from the flasks using Trypsin and re-suspended in media, a 20µL sample of suspended cells was used to establish cell counts and determine the average number of cells per mL. The 20µL of cell suspension was further diluted with 20µL of PBS, and 40µL of 0.4% Trypan Blue solution. At this point a haemocytometer was used to count the cells, viewed using a microscope (Olympus CKX53 inverted microscope, Olympus Life Sciences). Viable cells (appearing unstained) were counted from the four outer quadrants of the haemocytometer counting grid. In instances where cells were situated on boundary lines, only cells located on the inside boundaries were included.

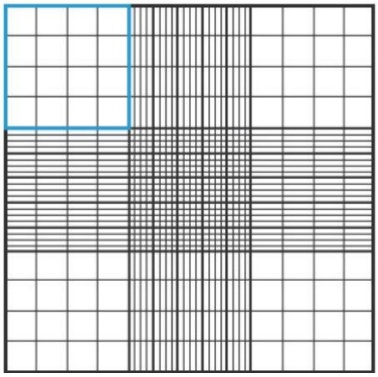


***Figure 9** : The layout of a haemocytometer counting grid. Cells were counted in the four outer quadrants (with one highlighted in blue). Image source: Abcam.*

To establish the average cell count per mL, the counts from each of the 4 quadrants was averaged, multiplied by 10^4 and then multiplied by 4 to account for dilution in PBS and Trypan Blue.

For MTT assays, the cells were seeded in the plate at a density of 5000 cells in 50µL added to each well. Therefore, the cell suspension was diluted such that there would be 100 cells per µL, or 100,000 cells per mL. The cell count (in cells per mL) was divided by 100,000 cells per mL to give the dilution factor needed to establish the appropriate ratio of cells:media.

3.3.4 Analysis of MTT Data

The MTT assay is able to establish the viability of cells relative to their untreated controls (MAX) based on the formation of formazan (and the subsequent absorbance levels at 600nm). In order to do this, the following calculation was used:

$$Relative\ cell\ viability\ (\%) = \frac{Absorbance - MIN}{MAX - MIN} \times 100$$

The mean background absorbance value produced in the media-only (MIN) wells was subtracted from each sample well, and also subtracted from the mean absorbance from the untreated wells (MAX). At this point the percentage viability of each sample relative to the untreated (MAX) cells could be calculated. These data were plotted to produce dose-response curves for each assay, and the IC_{50} and IC_{90} concentrations were calculated using the software, Calcusyn (Version 1.1, Biosoft, 1996).

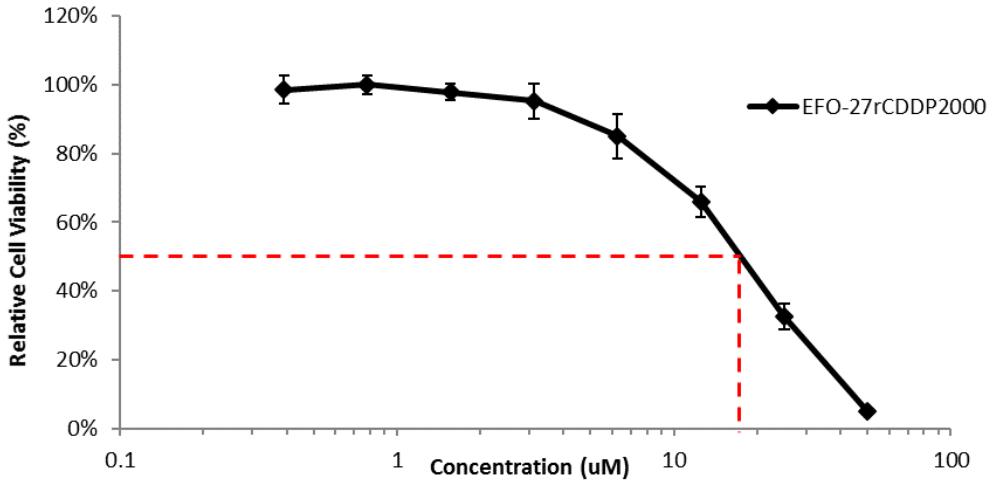


***Figure 10** : A dose-response plot produced from an MTT assay of the EFO-27rCDDP2000 cell line against cisplatin, plotted using Microsoft Excel 2016. The IC50 for this assay (dashed red line) was calculated using Calcusyn as 17.44µM.*

3.3.5 BET Inhibitor Pre-Treatment Assays

Initially, MTT assays were conducted to assess the effect of the compounds as single-agents. This would be used to determine the sensitivities of the cell lines to cisplatin, oxaliplatin, and the BET inhibitors JQ1 and I-BET726. The BET inhibitors were subsequently used to pre-treat the cell lines, determining whether this altered the sensitivities to platinum drugs, and therefore these initial single-agent assays were also used to determine pre-treatment concentrations for JQ1 and I-BET726.

The cells were pre-treated using the IC50 and IC10 concentrations of the BET inhibitors for each cell line. The IC10 was used to act as the minimum effective dose required to elicit a response, and would be used alongside the IC50 to assess the effect of pre-treatment dosage on the sensitivities to platinum drugs.

Additionally, the effect of pre-treatment duration was investigated by incubating the cells with the BET inhibitor pre-treatments over 0hrs, 24hrs or 48hrs prior to the MTT assays against the platinum drugs.

The MTT plates were prepared using the method outlined in Section 3.3.2, with the only modification being the addition of pre-treatment-only wells, which would not be treated with platinum drugs (marked as PTO in *Figure 11*). This reduced the dosing-range of the platinum drugs to 7-point serial dilution across the 96-well plate.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B			PTO	1	2	3	4	5	6	7		
C			PTO	1	2	3	4	5	6	7		
D			PTO	1	2	3	4	5	6	7		
E			PTO	1	2	3	4	5	6	7		
F			PTO	1	2	3	4	5	6	7		
G			PTO	1	2	3	4	5	6	7		
H												

***Figure 11** : The modified arrangement for a 96 well plate MTT assay, used to investigate the effect of BET inhibitor pre-treatment on the sensitivities to platinum drugs. The media-only (MIN) wells are marked with a blue outline, and the untreated cell (MAX) wells with a red outline. The addition of pre-treatment-only control wells (marked PTO) means that the platinum drug would be investigated along a 7-point serial dilution (with triplicate wells labelled 1-7). The different plating conditions (pre-treatment + platinum drug) are represented with pink and green shading.*

3.4 Statistical Analyses

The software, Minitab 17 (version 17.3.1, Minitab Inc., 2016), was used to conduct all of the statistical analyses used in this study. Statistical tests of the IC50 and IC90 data were conducted to assess for differences in the responses of parental and resistant cell lines, or between compounds (e.g. JQ1 and I-BET726).

When a single comparison was being conducted, for example comparing the IC50 of oxaliplatin between the parental UKF-NB-3 and oxaliplatin-resistant cell lines, a two-sample T-test was conducted.

When multiple comparisons would be made, for example when comparing the effects of multiple drugs against both parental and resistant cell lines, the data was instead tested using a two-way Analysis of Variance (ANOVA). This was followed by post-hoc testing by Tukey's pairwise comparison, which compares the means of every experimental treatment, and uses 95% confidence intervals to identify means which differ beyond the boundaries of the expected standard error. Statistically different means are then assigned into groups, which are denoted in my figures with letters. Means which do not share a letter are significantly different.

4. Results

4.1 Characterisation of Platinum Compounds as Single-Agents

4.1.1 Neuroblastoma

The parental UKF-NB-3 and oxaliplatin-resistant UKF-NB-3^{r}OXALI^{2000} cell lines were both tested against oxaliplatin, using the MTT viability assay (*Section 3.3*). These assays were used to characterise their sensitivities to the compound via the generation of dose-response profiles, establish IC50 and IC90 concentrations. Resistance-factors were also calculated, which is the fold-change in the IC50 of a drug in the resistant cell line, compared to the IC50 in the parental cell line. Figure 12 shows these data plotted for the UKF-NB-3 cell lines.

It can be seen from the dose response curves (*Figure 12A*) that the UKF-NB-3^{r}OXALI^{2000} cell line is able to retain viability at substantially higher concentrations than the parental cell line. This is perhaps most evident when the concentration of oxaliplatin approaches 1μM, which would be sufficient to reduce the parental cell line to 0% viability, whereas the oxaliplatin-resistant cell line exhibited approximately 90% viability.

The mean IC50 and IC90 concentrations of oxaliplatin against the UKF-NB-3 cell lines are plotted in Figure 12B. The mean IC50 of oxaliplatin against the UKF-NB-3^{r}OXALI^{2000} cell line (3.95μM) was significantly higher than when the drug was tested against parental UKF-NB-3 cells (0.34μM) ($t(4)= 27.21$, $p<0.001$), as determined by two-sample T-test. Likewise the UKF-NB-3^{r}OXALI^{2000} cell line has a significantly higher IC90 of oxaliplatin (6.58μM) than the parental cell line (0.41μM) ($t(4)= 32.41$, $p<0.001$).

Figure 12C illustrates the fold-changes (relative to the parental cell line) in the IC50 and IC90 of oxaliplatin against the UKF-NB-3 cell lines. A cell line exhibiting a resistance factor equal or above 2 is considered to be resistant to a compound. The oxaliplatin-resistant cell line had a resistance factor of 12.01 when the IC50 was compared, and a factor of 15.95 when the IC90 was compared.

These data would serve as the baseline when investigating the effect of BET inhibitor pre-treatment on the sensitivity of the UKF-NB-3 cell lines to oxaliplatin. Any changes in the dose-response, IC50 or IC90, or resistance factor would be compared against these initial results.

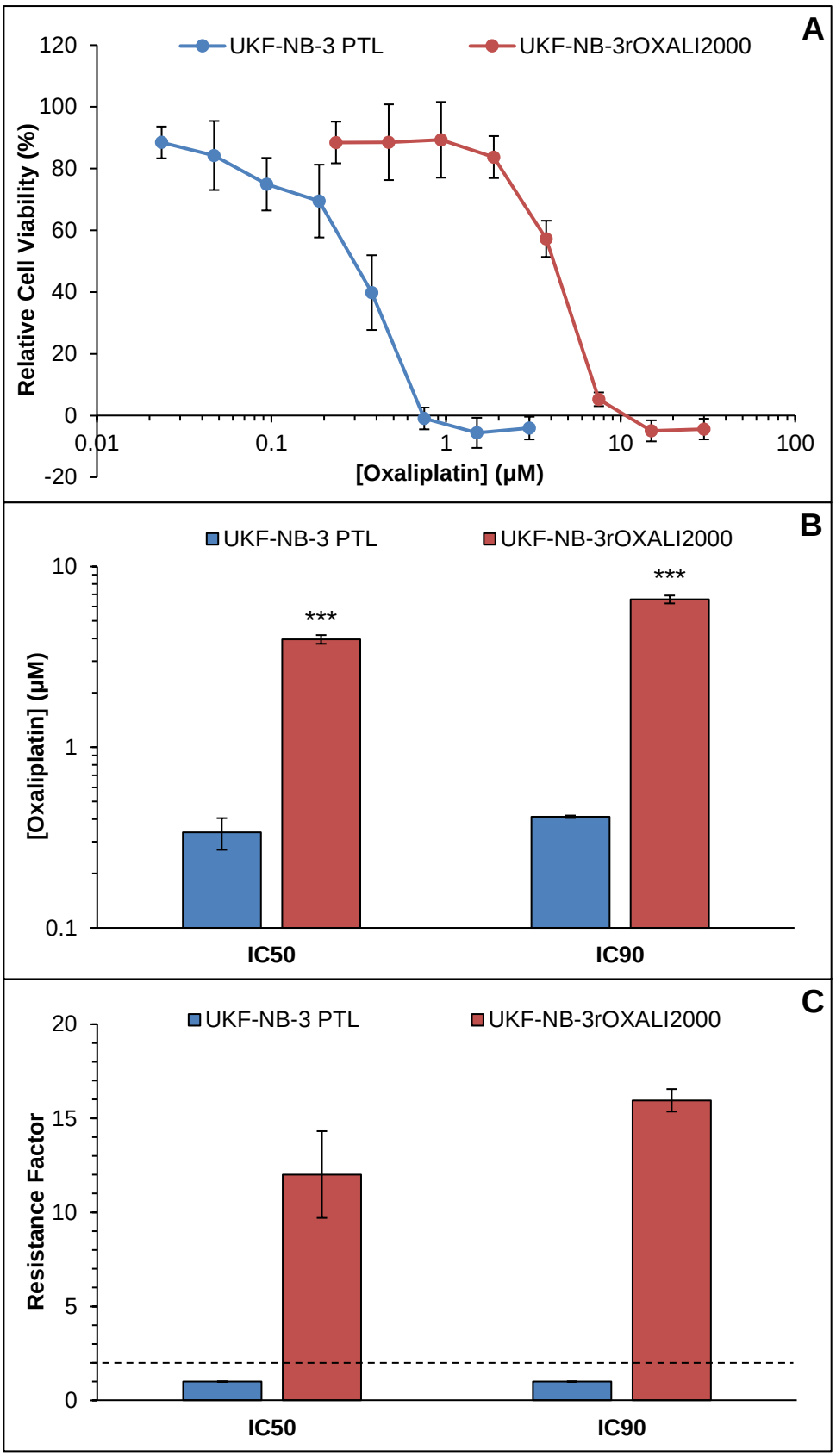


Figure 12 *: Drug sensitivity profiling of the parental neuroblastoma UKF-NB-3 PTL cell line and oxaliplatin-resistant cell line UKF-NB-3^{r}OXALI^{2000} against oxaliplatin, investigated by MTT assay.* ***A)*** *Dose-response curves of oxaliplatin against UKF-NB-3 PTL (blue) and UKF-NB-3^{r}OXALI^{2000} (red)* ***B)*** *Mean IC50 and IC90 of oxaliplatin for each cell line. UKF-NB-3^{r}OXALI^{2000} had a significantly higher IC50 ($t(4)= 27.21$, $p<0.001$) and IC90 ($t(4)= 32.41$, $p<0.001$) than UKF-NB-3 PTL, determined by two-sample T-test.* ***C)*** *The factor of resistance against oxaliplatin seen in the neuroblastoma cell lines. This is calculated as the fold difference between the IC50 or IC90, compared to that seen in the parental cell line. A threshold at 2-fold resistance (dashed line) is used to discriminate whether a cell line is resistant to a compound.*

4.1.2 Ovarian Cancer

As with the neuroblastoma UKF-NB-3 cell lines, the ovarian cancer COLO-704, EFO-21 and EFO-27 cell lines were all tested using the MTT viability assay, against cisplatin rather than oxaliplatin. These assays would again be used to characterise their baseline sensitivities to cisplatin via the generation of dose-response profiles, IC50 and IC90 concentrations, and resistance-factors. These data are plotted successively in Figure 13 (COLO-704), Figure 14 (EFO-21), and Figure 15 (EFO-27).

The dose response curves of cisplatin against the ovarian cancer cell lines are plotted in segment A of each cell line's respective figures. As with the UKF-NB-3^{r}OXALI^{2000} cells, each cisplatin-resistant ovarian cancer cell line was able to remain comparatively viable at concentrations which would be largely inhibitory to their parental counterpart cell line.

The mean IC50 and IC90 concentrations of cisplatin against each ovarian cancer cell line are plotted in segment B. As with the neuroblastoma cell lines, there was a significant difference in the IC50 and IC90 concentrations of cisplatin between the parental and resistant cell lines. The statistical outputs of each two-sample T-test are given in the figure caption for each cell line, with the levels of significance also presented as asterisks on each figure. For 2 of the 3 sets of cell lines (COLO-704 and EFO-21), the difference in IC90 had lower statistical significance than the difference in IC50. This could be explained by the fact that the IC90 of cisplatin appears more variable, with greater standard deviation than the IC50.

This variability in the IC90 of cisplatin can also be seen in segment C, which illustrates the fold-changes (relative to the parental cell line) in the IC50 and IC90 of cisplatin against the three ovarian cancer cell lines. Across all sets of cell lines we see that the resistance factor is above the 2-fold threshold used to determine resistance.

As with the neuroblastoma UKF-NB-3 cell lines, these data would serve as the baseline for investigating the effect of BET inhibitor pre-treatment on the sensitivity of ovarian cancer cell lines to cisplatin.

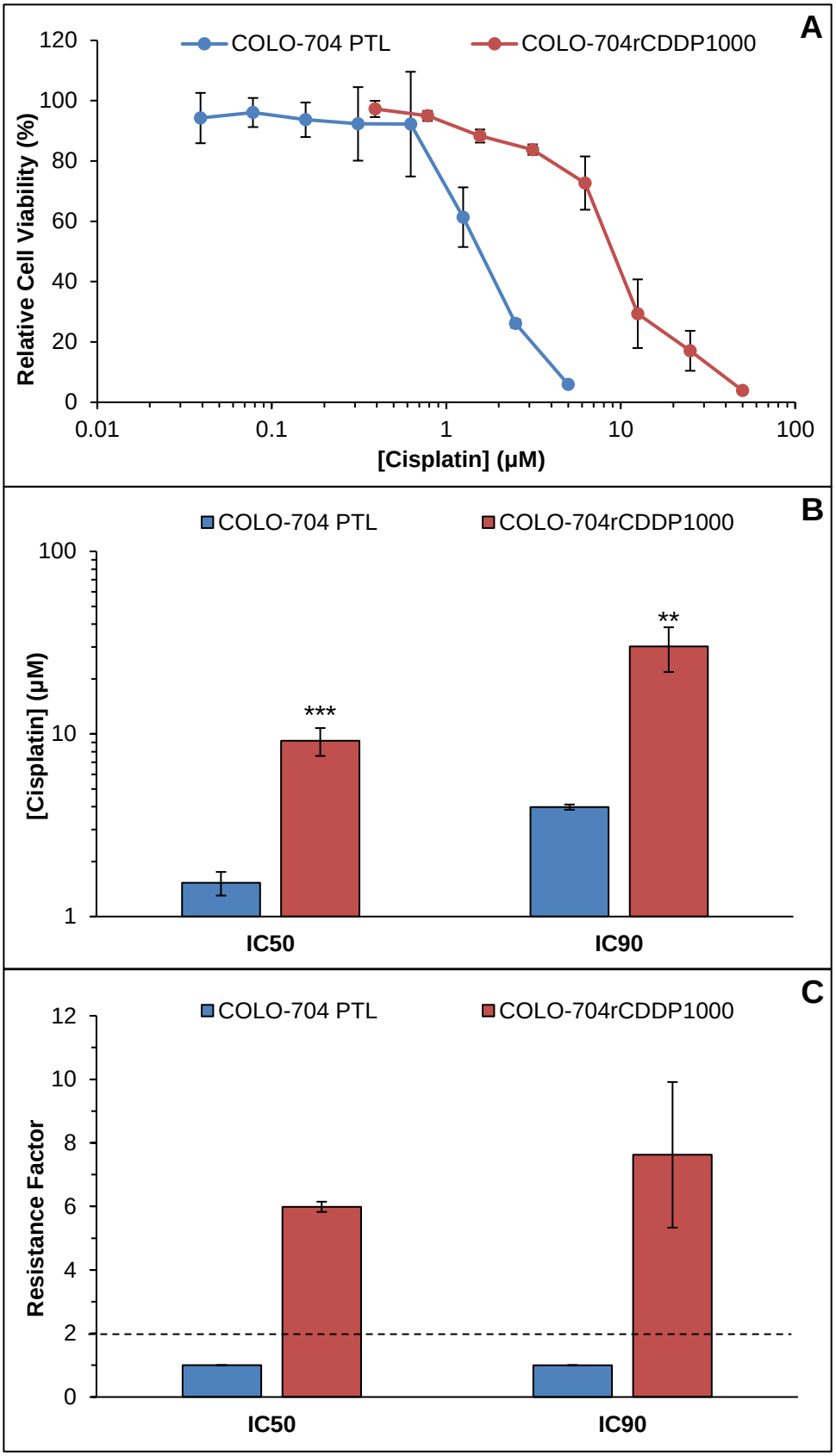


Figure 13 *: Drug sensitivity profiling of the parental ovarian cancer COLO-704 PTL cell line and cisplatin-resistant cell line COLO-704^{r}CDDP^{1000} against cisplatin, investigated by MTT assay.* ***A)*** *Dose-response curves of cisplatin against COLO-704 PTL (blue) and COLO-704^{r}CDDP^{1000} (red)* ***B)*** *Mean IC50 and IC90 of cisplatin for each cell line. COLO-704^{r}CDDP^{1000} had a significantly higher IC50 ($t(4)= 8.21$, $p=0.001$) and IC90 ($t(4)= 5.48$, $p=0.005$) than COLO-704 PTL, as determined by two-sample T-test.* ***C)*** *The factor of resistance against cisplatin seen in the COLO-704 cell lines. This is calculated as the fold difference between the IC50 or IC90, compared to that seen in the parental cell line. A threshold at 2-fold resistance (dashed line) is used to discriminate whether or not a cell line is resistant to a compound.*

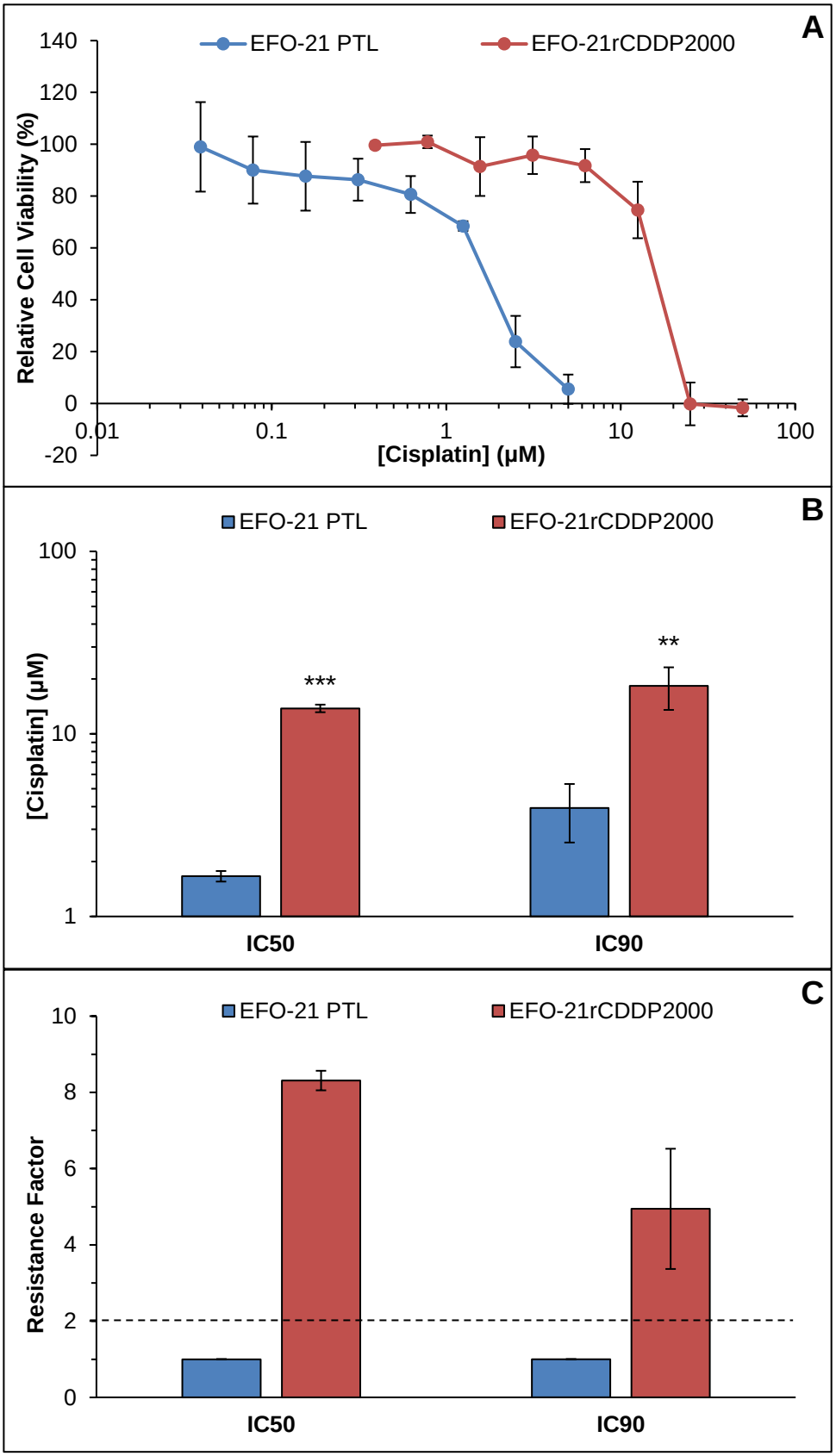


Figure 14 *: Drug sensitivity profiling of the parental ovarian cancer EFO-21 PTL cell line and cisplatin-resistant cell line EFO-21^{r}CDDP^{2000} against cisplatin, investigated by MTT assay.* ***A****) Dose-response curves of cisplatin against EFO-21 PTL (blue) and EFO-21^{r}CDDP^{2000} (red)* ***B****) Mean IC50 and IC90 of cisplatin for each cell line. EFO-21^{r}CDDP^{2000} had a significantly higher IC50 ($t(4)= 31.51$, $p<0.001$) and IC90 ($t(4)= 4.99$, $p=0.008$) than EFO-21 PTL, as determined by two-sample T-test.* ***C****) The factor of resistance against cisplatin seen in the EFO-21 cell lines. This is calculated as the fold difference between the IC50 or IC90, compared to that seen in the parental cell line. A threshold at 2-fold resistance (dashed line) is used to discriminate whether or not a cell line is resistant to a compound.*

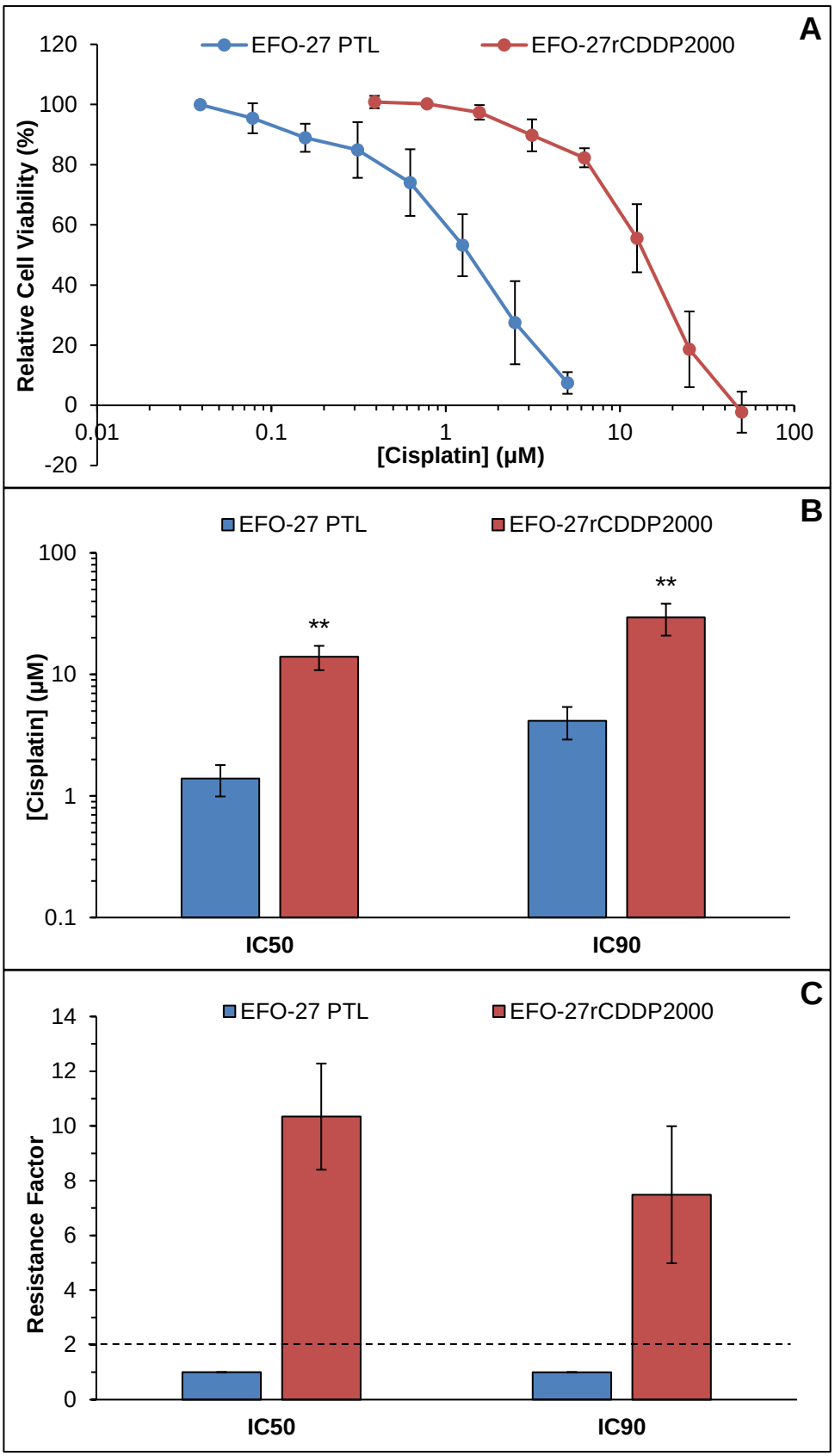


Figure 15 *: Drug sensitivity profiling of the parental ovarian cancer EFO-27 PTL cell line and cisplatin-resistant cell line EFO-27^{r}CDDP^{2000} against cisplatin, investigated by MTT assay.* ***A****) Dose-response curves of cisplatin against EFO-27 PTL (blue) and EFO-27^{r}CDDP^{2000} (red)* ***B****) Mean IC50 and IC90 of cisplatin for each cell line. EFO-27^{r}CDDP^{2000} had a significantly higher IC50 (t(4)= 6.82, p=0.002) and IC90 (t(4)= 5.02, p=0.007) than EFO-27 PTL, as determined by two-sample T-test.* ***C****) The factor of resistance against cisplatin seen in the EFO-27 cell lines. This is calculated as the fold difference between the IC50 or IC90, compared to that seen in the parental cell line. A threshold at 2-fold resistance (dashed line) is used to discriminate whether or not a cell line is resistant to a compound.*

4.2 Characterisation of BET Inhibitors as Single-Agents

4.2.1 Neuroblastoma

MTT assays were used to determine the sensitivities of the neuroblastoma cell lines to the BET inhibitors, JQ1 and I-BET726, as single-agents. These assays were also used to determine the IC10 and IC50 concentrations of each drug, which would later be used as pre-treatment dosages.

The dose-response curves for the BET inhibitors against the UKF-NB-3 parental cell line are plotted in Figure 16A, with the dose-responses of the UKF-NB-3^{r}OXALI^{2000} cell line are plotted in Figure 16B. The parental and oxaliplatin-resistant cell line both appear to respond very similarly to the BET inhibitors, and additionally there does not seem to be a notable difference in the profiles of the two compounds compared to each other.

The mean IC50 and IC90 concentrations of the BET inhibitors against each cell line are plotted in Figure 16C. These data were tested using a two-way Analysis of Variance (ANOVA) with post-hoc Tukey's pairwise comparison, using individual 95% confidence intervals. These tests yielded no significant difference in the IC50 of the BET inhibitors against either cell line, and only the IC90's of UKF-NB-3 treated with I-BET726 and UKF-NB-3^{r}OXALI^{2000} treated with JQ1 were found to be significantly different. However, analysis using pooled cell line data found significant differences between JQ1 and I-BET726 at both IC50 ($F(1,16)=5.78$, $p=0.029$) and IC90 ($F(1,16)=5.45$, $p=0.033$).

As described in Section 3.3.5, JQ1 and I-BET726 would be used as pre-treatments to investigate whether BET inhibition would alter the sensitivity of these cell lines to oxaliplatin. The mean IC10 and IC50 concentrations of the BET inhibitors, which would be used to pre-treat each neuroblastoma cell line, are presented (in nM) in Table 1.

***Table 1** : The mean IC10 and IC90 concentrations (in nM) of the BET inhibitors, JQ1 and I-BET726, against the UKF-NB-3 parental and oxaliplatin-resistant cell lines. These concentrations would be used as pre-treatment doses, prior to conducting MTT assays against oxaliplatin, to determine whether BET inhibition alters the sensitivity of these cell lines to oxaliplatin.*

	JQ1		I-BET726	
	IC10 (nM)	IC50 (nM)	IC10 (nM)	IC50 (nM)
UKF-NB-3 PTL	1.52	69.98	1.25	114.42
UKF-NB-3^{r}OXALI^{2000}	4.55	85.67	7.20	98.19

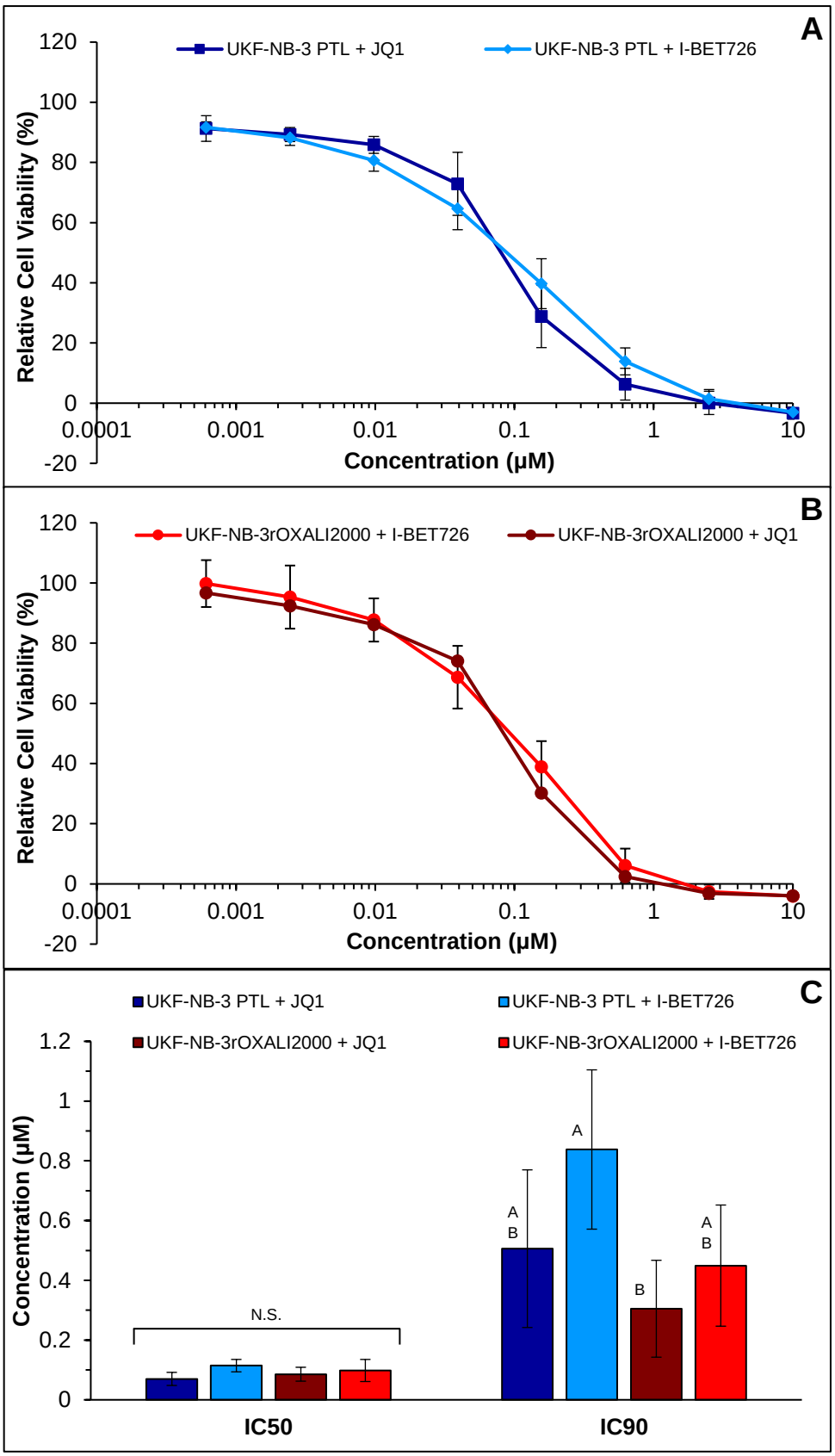


Figure 16 *: Characterisation of the BET inhibitors, JQ1 and I-BET726, against the UKF-NB-3 PTL and UKF-NB-3^{r}OXALI^{2000} cell lines , investigated by MTT assay.* ***A****) Dose-response curves of the UKF-NB-3 PTL cell line after being treated with JQ1 (darker blue) and I-BET726 (lighter blue)* ***B****) Dose-response curves of the UKF-NB-3^{r}OXALI^{2000} cell line after being treated with JQ1 (darker red) and I-BET726 (lighter red)* ***C****) The mean IC50 and IC90s of the BET inhibitors against the parental and oxaliplatin-resistant cell lines. Statistical testing using two-way ANOVA with post-hoc Tukey's pairwise comparison, using individual 95% confidence intervals, yielded no significant difference in the IC50 of the BET inhibitors against either cell line. The groupings of the IC90 comparison are presented as letters (A and B). Means that do not share a letter are significantly different.*

4.2.2 Ovarian Cancer

The sensitivities of the ovarian cancer cell lines to JQ1 and I-BET726 as single-agents were tested using MTT assays. The data for these assays are plotted successively in Figure 17 (COLO-704), Figure 18 (EFO-21), and Figure 19 (EFO-27).

The dose-response curves for the BET inhibitors against the parental cell line are plotted in segment A of each figure, with the dose-responses of the cisplatin-resistant cell line plotted in segment B. The parental and cisplatin-resistant counterpart cell lines appear to respond very similarly to the two BET inhibitors. It would seem that JQ1 is slightly more effective than I-BET726 in these cell lines, however the difference is reasonably small, and the responses seem much more variable than the UKF-NB-3 lines.

The mean IC50 and IC90 concentrations of the BET inhibitors against each cell line are plotted in segment C. Two-way Analysis of Variance (ANOVA) with post-hoc Tukey's pairwise comparison yielded no significant difference in the IC50 or IC90 between the BET inhibitors and between the parental and cisplatin-resistant cell lines. Analyses of each pair of cell lines only found a significant difference at the IC50s of EFO-27, with the pooled response to BET inhibition significantly differing between the parental and cisplatin-resistant cell lines ($F(1,8)=6.44$, $p=0.035$).

The mean IC10 and IC50 concentrations of JQ1 and I-BET726 against each ovarian cancer cell line are presented (in nM) in Table 2. As with the neuroblastoma cell lines, these would be used as pre-treatments before conducting MTT assays against cisplatin.

***Table 2** : The mean IC10 and IC90 concentrations (in nM) of the BET inhibitors, JQ1 and I-BET726, against the parental and cisplatin-resistant ovarian cancer cell lines. These concentrations would be used as pre-treatment doses, prior to conducting MTT assays against cisplatin, to determine whether BET inhibition alters the sensitivity of these cell lines to cisplatin.*

	JQ1		I-BET726	
	IC10 (nM)	IC50 (nM)	IC10 (nM)	IC50 (nM)
COLO-704 PTL	3.49	193.31	6.04	545.41
COLO-704^{r}CDDP^{1000}	2.43	116.95	6.68	351.62
EFO-21 PTL	1.96	513.54	5.48	336.85
EFO-21^{r}CDDP^{2000}	1.18	326.01	3.26	689.60
EFO-27 PTL	3.26	515.84	16.66	597.77
EFO-27^{r}CDDP^{2000}	1.85	165.59	9.62	371.90

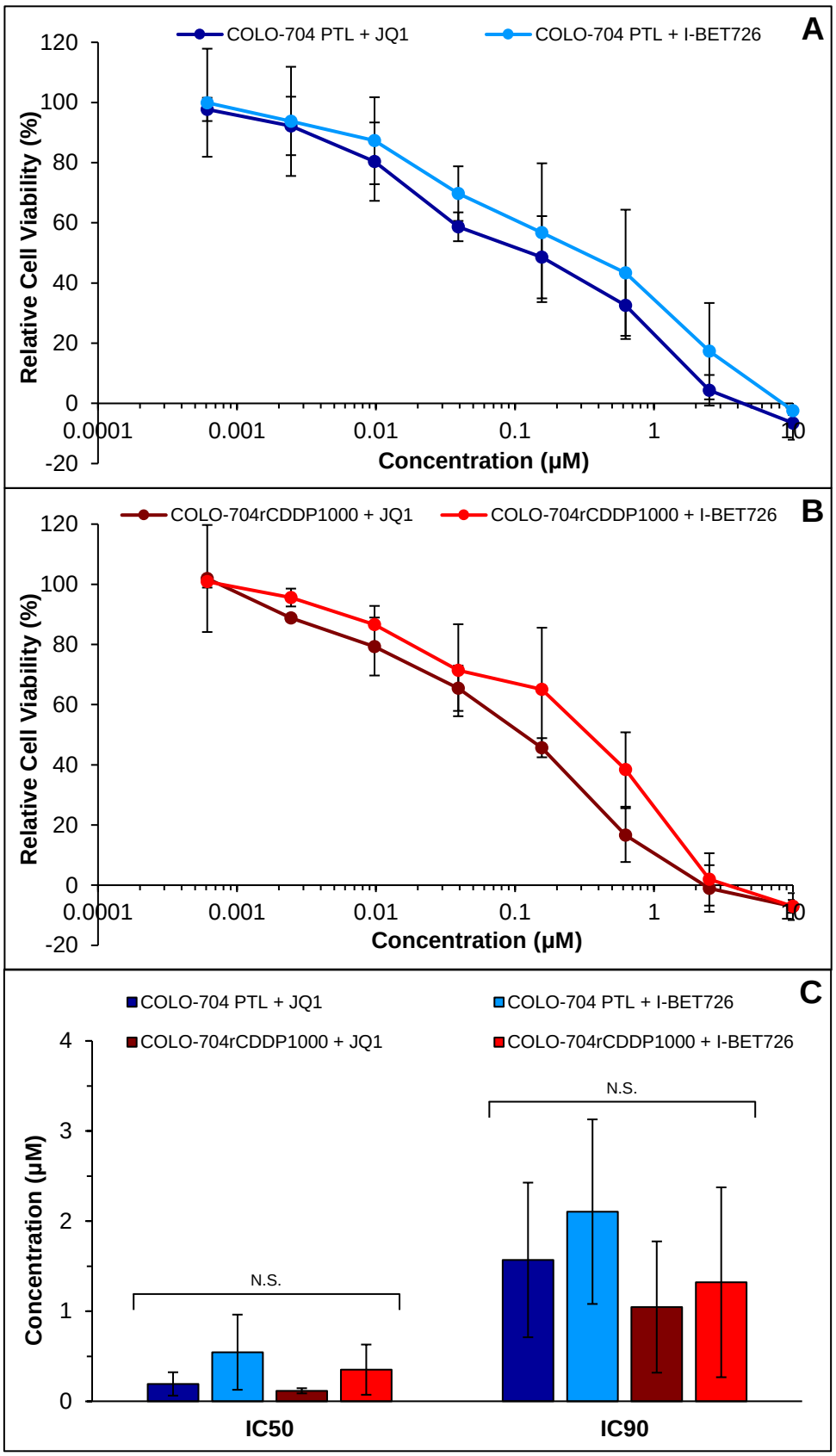


Figure 17 *: Characterisation of the BET inhibitors, JQ1 and I-BET726, against the COLO-704 PTL and COLO-704^{r}CDDP^{1000} cell lines , investigated by MTT assay.* ***A)*** *Dose-response curves of the COLO-704 PTL cell line after being treated with JQ1 (darker blue) and I-BET726 (lighter blue)* ***B)*** *Dose-response curves of the COLO-704^{r}CDDP^{1000} cell line after being treated with JQ1 (darker red) and I-BET726 (lighter red)* ***C)*** *The mean IC50 and IC90s of the BET inhibitors against the parental and cisplatin-resistant cell lines. Statistical testing using two-way ANOVA with post-hoc Tukey's pairwise comparison, using individual 95% confidence intervals, yielded no significant difference in the IC50 or IC90 of the BET inhibitors against either cell line.*

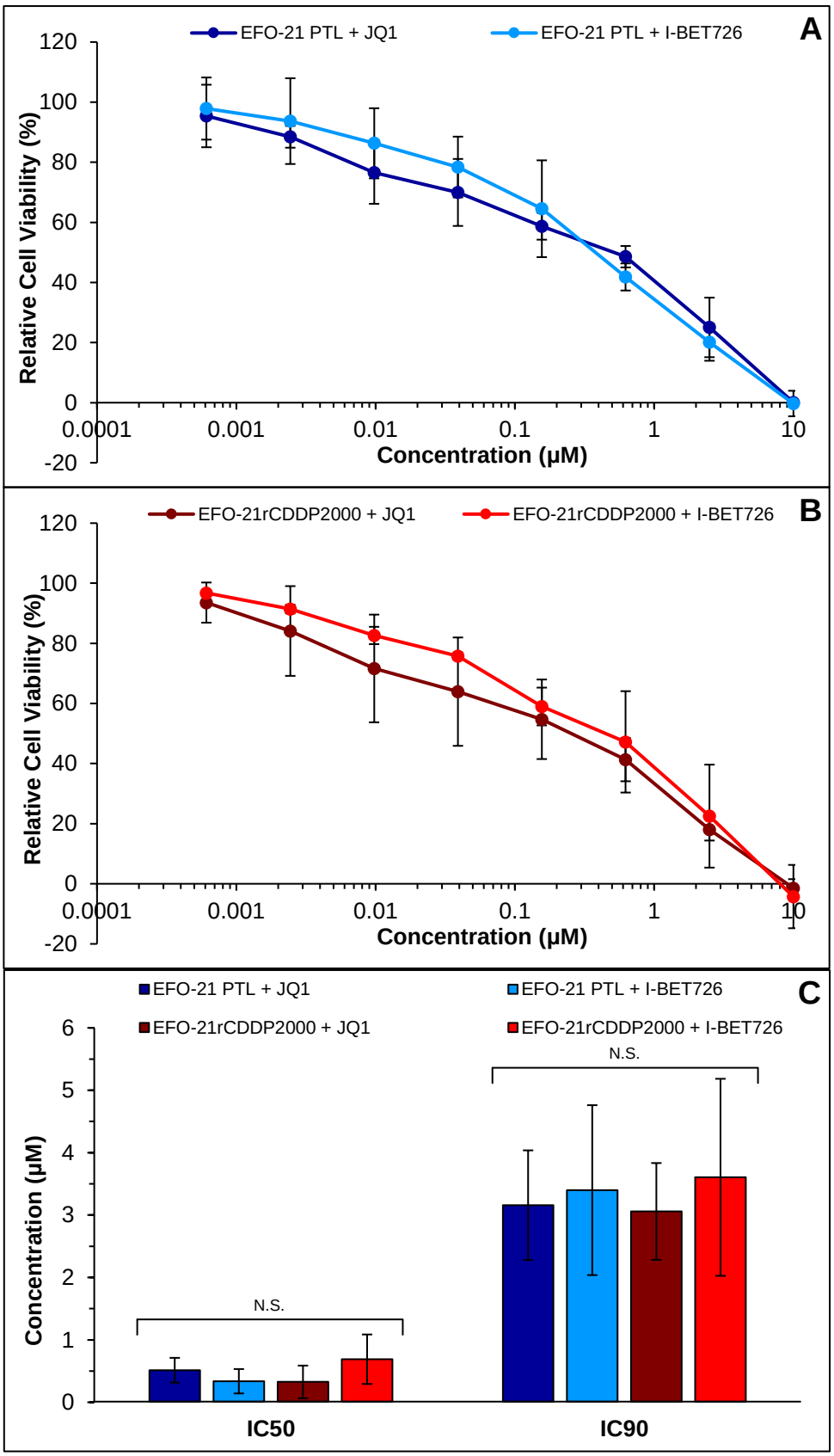


Figure 18 *: Characterisation of the BET inhibitors, JQ1 and I-BET726, against the EFO-21 PTL and EFO-21^{r}CDDP^{2000} cell lines , investigated by MTT assay.* ***A)*** *Dose-response curves of the EFO-21 PTL cell line after being treated with JQ1 (darker blue) and I-BET726 (lighter blue)* ***B)*** *Dose-response curves of the EFO-21^{r}CDDP^{2000} cell line after being treated with JQ1 (darker red) and I-BET726 (lighter red)* ***C)*** *The mean IC50 and IC90s of the BET inhibitors against the parental and cisplatin-resistant cell lines. Statistical testing using two-way ANOVA with post-hoc Tukey's pairwise comparison, using individual 95% confidence intervals, yielded no significant difference in the IC50 or IC90 of the BET inhibitors against either cell line.*

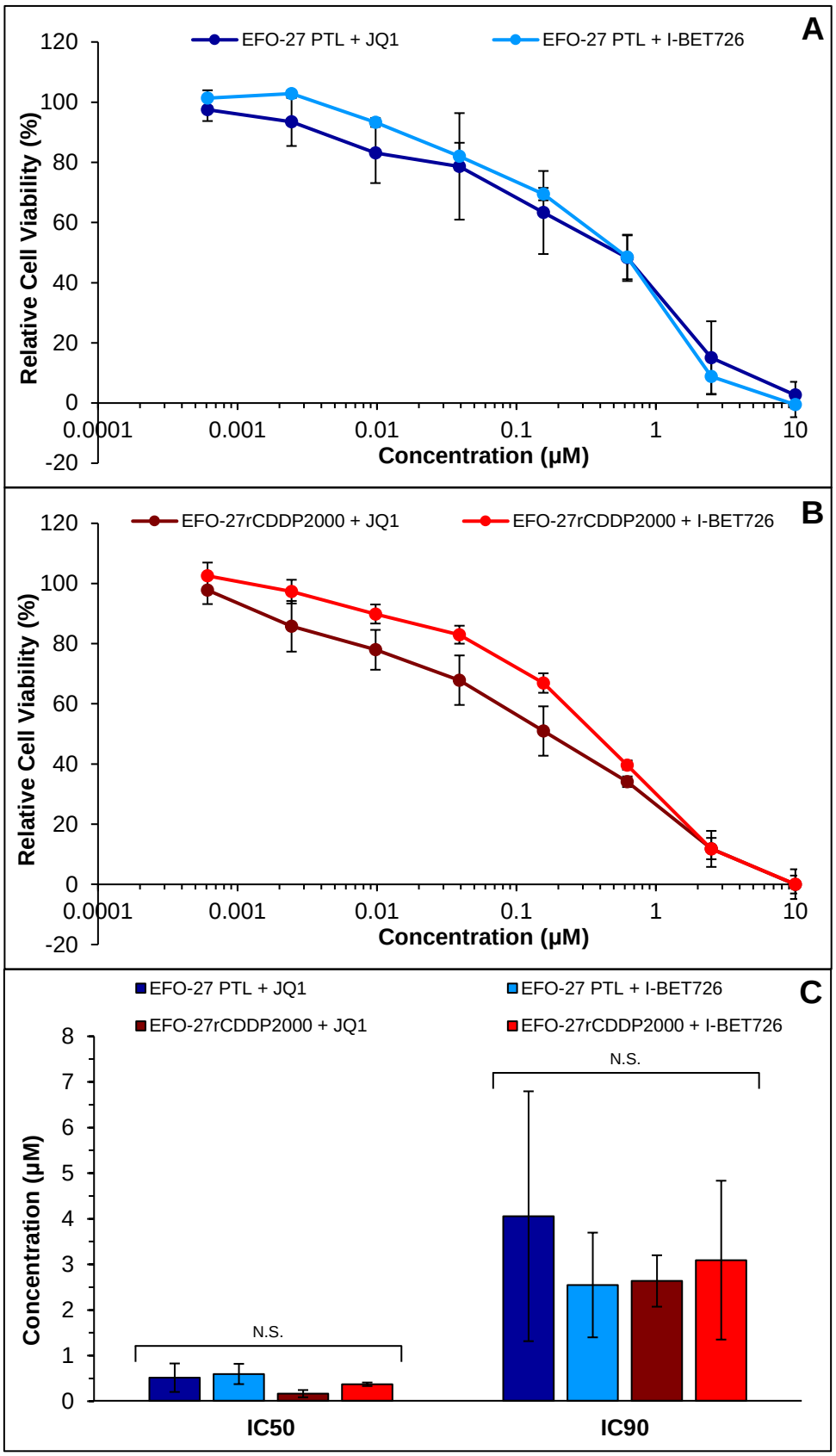


***Figure 19**: Characterisation of the BET inhibitors, JQ1 and I-BET726, against the EFO-27 PTL and EFO-27^{r}CDDP^{2000} cell lines , investigated by MTT assay. **A)** Dose-response curves of the EFO-27 PTL cell line after being treated with JQ1 (darker blue) and I-BET726 (lighter blue) **B)** Dose-response curves of the EFO-27^{r}CDDP^{2000} cell line after being treated with JQ1 (darker red) and I-BET726 (lighter red) **C)** The mean IC50 and IC90s of the BET inhibitors against the parental and cisplatin-resistant cell lines. Statistical testing using two-way ANOVA with post-hoc Tukey's pairwise comparison, using individual 95% confidence intervals, yielded no significant difference in the IC50 or IC90 of the BET inhibitors against either cell line.*

4.3 Pre-Treatment MTT Assays

4.3.1 Neuroblastoma

4.3.1.1 UKF-NB-3 Pre-Treated Using the IC10 of the BET Inhibitors

The UKF-NB-3 cell lines were pre-treated using the IC10 of the BET inhibitors (established in Section 4.2.1), and were incubated with this pre-treatment dosage over 0, 24, or 48 hours prior to conducting an MTT assay against oxaliplatin.

The dose-response curves from these assays are plotted in Figure 20A-C, with the pre-treatment time increasing from A to C. It can be seen from these plots that the IC10, when administered immediately prior to oxaliplatin (0 hours), does not seem to dramatically alter the sensitivity to oxaliplatin. This is particularly evident in the parental cell line, but there is only a small change seen in the resistant, UKF-NB-3^{r}OXALI^{2000}, cell line. However, as the pre-treatment duration increases, the dose-response curves shift to the left, becoming more sensitive to oxaliplatin, and this effect is most apparent in the UKF-NB-3^{r}OXALI^{2000} cell line.

The IC50 and resistance factor values produced in these assays are plotted in Figure 21A-C, again with the pre-treatment duration increasing from A to C. After a 0-hour pre-treatment, there is no significant difference in the IC50 of oxaliplatin between pre-treated cells and their initial responses to oxaliplatin without pre-treatment, with the only difference seen between UKF-NB-3 and UKF-NB-3^{r}OXALI^{2000} cell lines.

As the pre-treatment time increased to 24 and 48 hours, the IC50 of oxaliplatin against the UKF-NB-3^{r}OXALI^{2000} cell line is significantly reduced compared to the baseline IC50. In this resistant cell line, a significant difference in IC50 was also seen between JQ1- and I-BET726-pre-treated cells.

In the parental cell line, even as the pre-treatment duration increased to 48 hours, the mean IC50 of oxaliplatin did not differ sufficiently from the initial IC50 (without pre-treatment) to achieve statistical significance, and no difference was observed between the BET inhibitors.

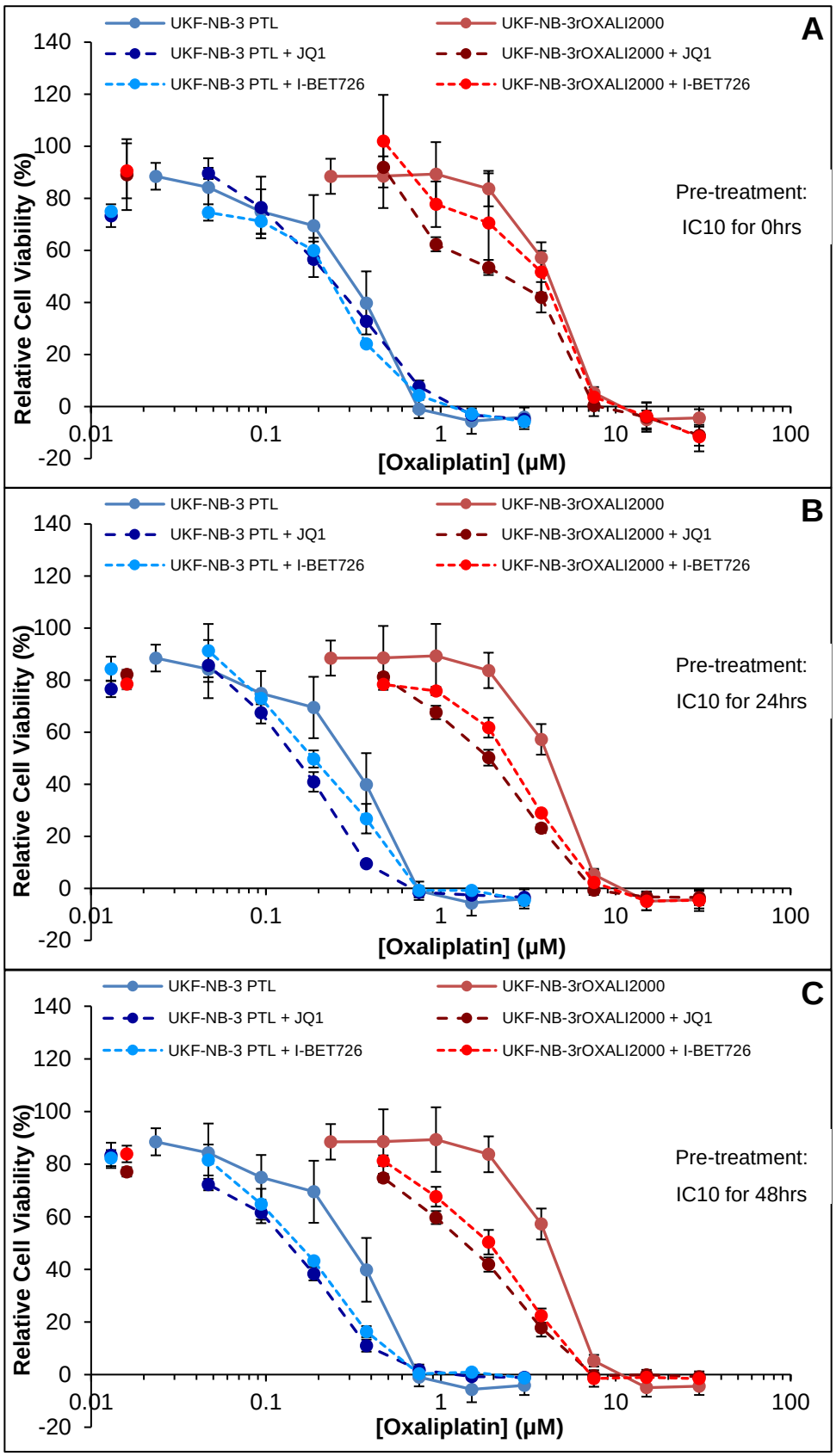


***Figure 20**: Dose-response curves of the UKF-NB-3 PTL (blue) and UKF-NB-3^{r}OXALI^{2000} (red) cell lines to oxaliplatin after pre-treatment using the IC10 of the BET inhibitors over 0 hours (**A**), 24 hours (**B**), or 48 hours (**C**). The dose-response curves from the initial characterisation assays are included as solid lines, with the JQ1-pre-treated assays depicted as the darker dashed lines, and I-BET726-pre-treated depicted with lighter dashed lines. The unattached points at the left of each graph represent the viabilities of the pre-treatment-only samples, which were not exposed to oxaliplatin during the MTT assay.*

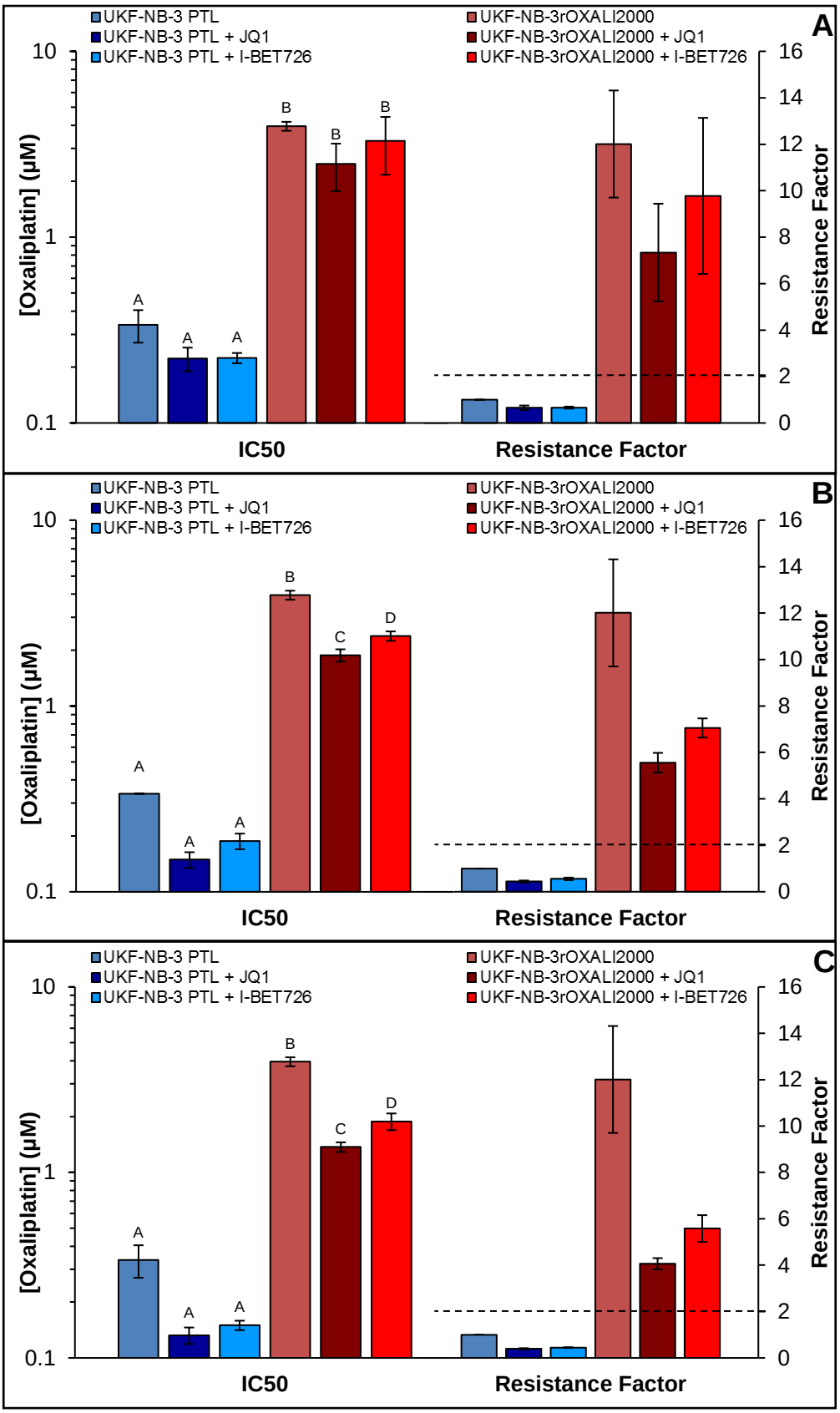


***Figure 21**: Mean IC50 and Resistance Factors of the UKF-NB-3 PTL (blue) and UKF-NB-3^{r}OXALI^{2000} (red) cell lines to oxaliplatin after pre-treatment using the IC10 of the BET inhibitors over 0 hours (**A**), 24 hours (**B**), or 48 hours (**C**). Statistical testing by two-way ANOVA with post-hoc Tukey's pairwise comparison, using individual 95% confidence intervals, yielded no significant difference in the IC50 of oxaliplatin when cells were pre-treated for 0 hours, however after 24 and 48 hours, significant differences were seen in the UKF-NB-3^{r}OXALI^{2000} cell line, but not in UKF-NB-3 PTL. The groupings of the IC50 comparison are presented as letters (A-D). Means that do not share a letter are significantly different.*

4.3.1.2 UKF-NB-3 Pre-Treated Using the IC50 of the BET Inhibitors

The pre-treatment dose of the BET inhibitors was increased to their IC50 (established in Section 4.2.1), and again the cells were incubated with this pre-treatment dosage over 0, 24, or 48 hours prior to conducting an MTT assay against oxaliplatin.

The dose-response curves from these assays are plotted in Figure 22A-C, with the pre-treatment time increasing from A to C. The IC50 of the BET inhibitors, when administered immediately prior to oxaliplatin (0 hours), was able to induce a change in the sensitivity of the UKF-NB-3^{r}OXALI^{2000} cell line to oxaliplatin. The parental cell line did not seem to respond the same, with very little change after the 0 hour pre-treatment. Similarly to when the IC10 was used, as the pre-treatment duration increases, the dose-response curves of both cell lines shift to the left, becoming more sensitive to oxaliplatin, and again this effect is most apparent in the UKF-NB-3^{r}OXALI^{2000} cell line.

The IC50 and resistance factor values produced in these assays are plotted in Figure 23A-C, again with the pre-treatment duration increasing from A to C. The 0-hour pre-treatment was able to induce a significant difference in the IC50 of oxaliplatin in the UKF-NB-3^{r}OXALI^{2000} cells. The IC50s and resistance factors of UKF-NB-3^{r}OXALI^{2000} in these conditions were lower than those produced when the cells had been pre-treated for 48 hours using the IC10 of the BET inhibitors.

As the pre-treatment time increased to 24 and 48 hours, the IC50 of oxaliplatin against the UKF-NB-3^{r}OXALI^{2000} cell line continues to decrease. When the cells had been pre-treated for 48 hours prior to the MTT assay, the resistance factors of pre-treated cells are reduced to the extent that they are very close to the threshold used to discriminate drug-resistance. Statistical testing using two-way ANOVA with post-hoc Tukey's pairwise comparison found no significant difference between the baseline IC50 of the parental cell line and UKF-NB-3^{r}OXALI^{2000} cells pre-treated for 48 hours with JQ1.

Again in the parental cell line, as the pre-treatment duration increased to 48 hours, the mean IC50 of oxaliplatin did not differ sufficiently from the initial IC50 (without pre-treatment) to achieve statistical significance. No significant difference was observed between the BET inhibitors, and this was seen in both cell lines and across all pre-treatment durations.

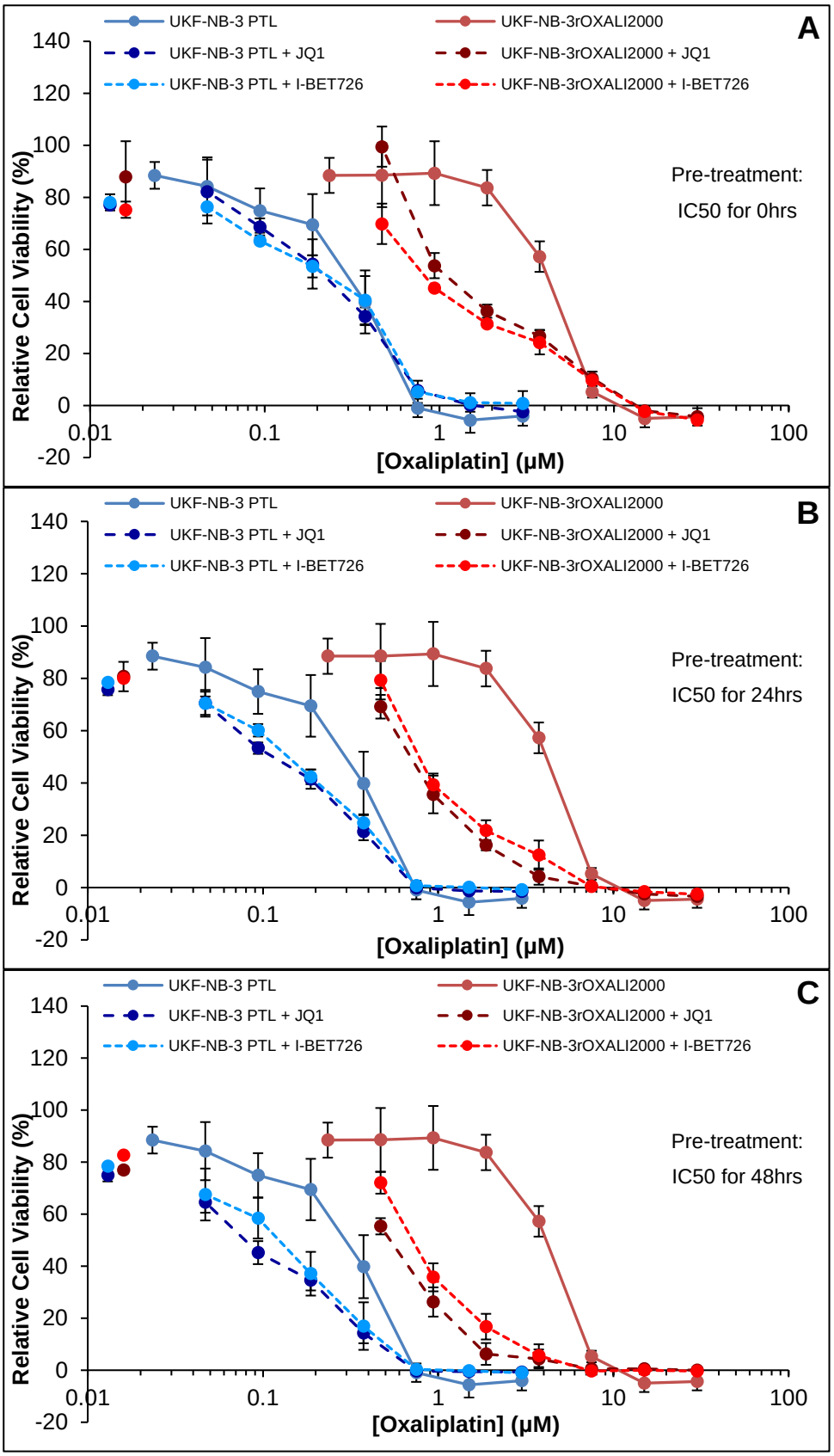


***Figure 22**: Dose-response curves of the UKF-NB-3 PTL (blue) and UKF-NB-3rOXALI2000 (red) cell lines to oxaliplatin after pre-treatment using the IC50 of the BET inhibitors over 0 hours (**A**), 24 hours (**B**), or 48 hours (**C**). The dose-response curves from the initial characterisation assays are included as solid lines, with the JQ1-pre-treated assays depicted as the darker dashed lines, and I-BET726-pre-treated depicted with lighter dashed lines. The unattached points at the left of each graph represent the viabilities of the pre-treatment-only samples, which were not exposed to oxaliplatin during the MTT assay.*

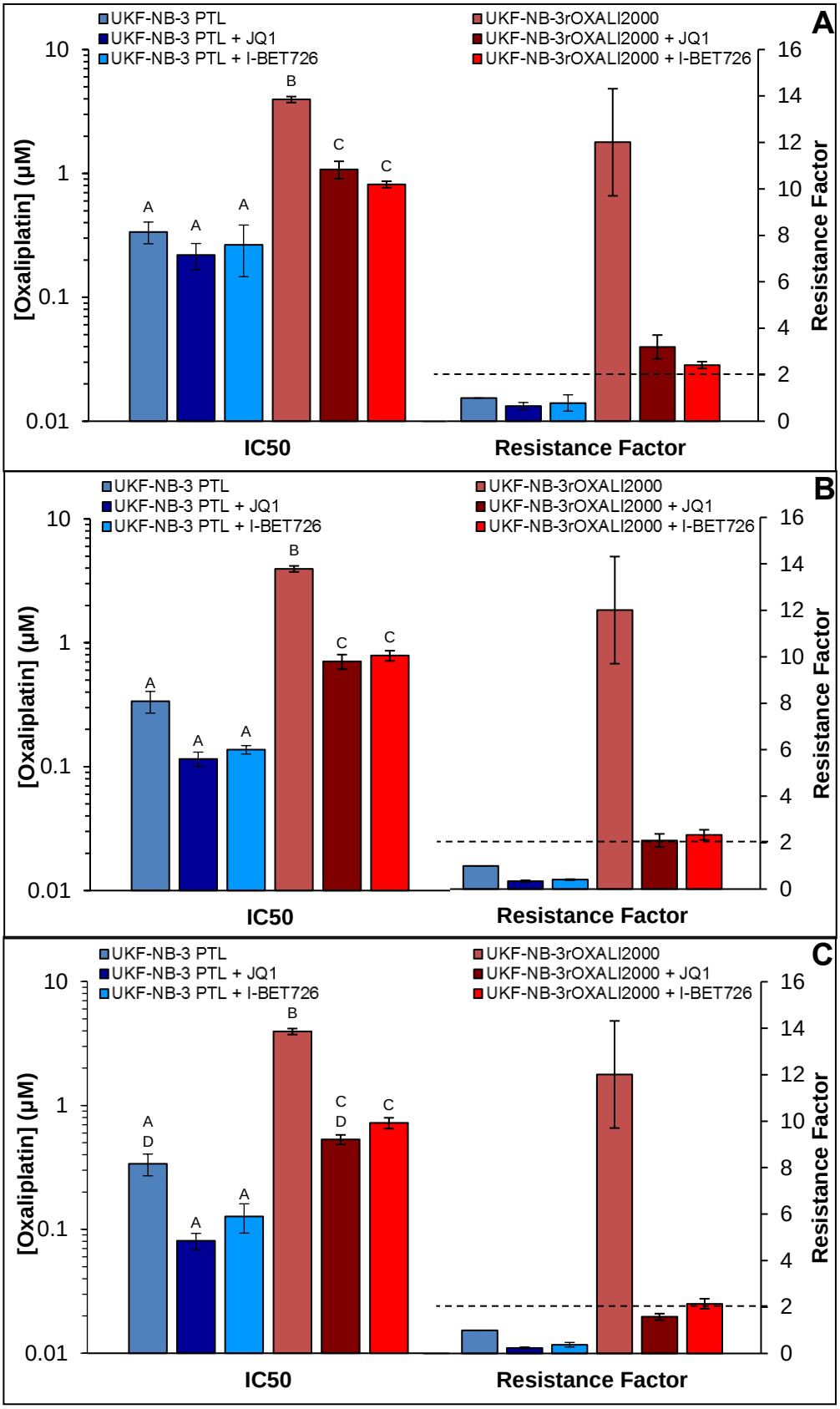


***Figure 23**: Mean IC50 and Resistance Factors of the UKF-NB-3 PTL (blue) and UKF-NB-3^{r}OXALI^{2000} (red) cell lines to oxaliplatin after pre-treatment using the IC50 of the BET inhibitors over 0 hours (**A**), 24 hours (**B**), or 48 hours (**C**). Statistical testing by two-way ANOVA with post-hoc Tukey's pairwise comparison, using individual 95% confidence intervals, yielded significant differences in the IC50 of oxaliplatin when UKF-NB-3^{r}OXALI^{2000} cells were pre-treated over 0, 24 and 48 hours, but no significant differences were seen in the UKF-NB-3 PTL cell line. The groupings of the IC50 comparison are presented as letters (A-D). Means that do not share a letter are significantly different. It can be seen from these groupings that, after 48 hour pre-treatment with JQ1, the IC50 of oxaliplatin against UKF-NB-3^{r}OXALI^{2000} is not significantly different from the untreated UKF-NB-3 PTL cell line.*

4.3.2 Ovarian Cancer

4.3.2.1 COLO-704 Pre-Treated Using the IC10 of the BET Inhibitors

The COLO-704 ovarian cancer cell lines were pre-treated using the IC10 of the BET inhibitors (Section 4.2.2) over 0, 24, or 48 hours prior to conducting an MTT assay against cisplatin.

The dose-response curves from these assays are plotted in Figure 24A-C, with the pre-treatment time increasing from A to C. The IC10 of the BET inhibitors, when administered immediately prior to cisplatin (0 hours), produced a minimal effect in both the parental and cisplatin-resistant cell line, with the data only slightly deviating from the initial sensitivities. As the pre-treatment duration increases, the sensitivity of both cell lines increases, which is particularly noticeable at the higher concentrations of cisplatin. The two BET inhibitors produced very similar results, with largely overlapping dose-response curves.

The IC50 and resistance factor values produced in these assays are plotted in Figure 25A-C, again with the pre-treatment duration increasing from A to C. The 0-hour pre-treatment was able to induce a significant difference in the IC50 of cisplatin in the COLO-704^{r}CDDP^{1000} cells, and was able to decrease the resistance factor markedly. As the pre-treatment time increased to 24 and 48 hours, the IC50 of cisplatin against the cisplatin-resistant cell line continues to decrease. After a 48-hour pre-treatment, the resistance factor of COLO-704^{r}CDDP^{1000} cells had decreased from an initial near 6-fold resistance, down to approximately 4-fold. No significant difference was observed between the IC50s produced after either JQ1 or I-BET726, across all pre-treatment durations.

The IC50s of the COLO-704 PTL cell line did not significantly differ after pre-treatment with either BET-inhibitor, over any duration of pre-treatment.

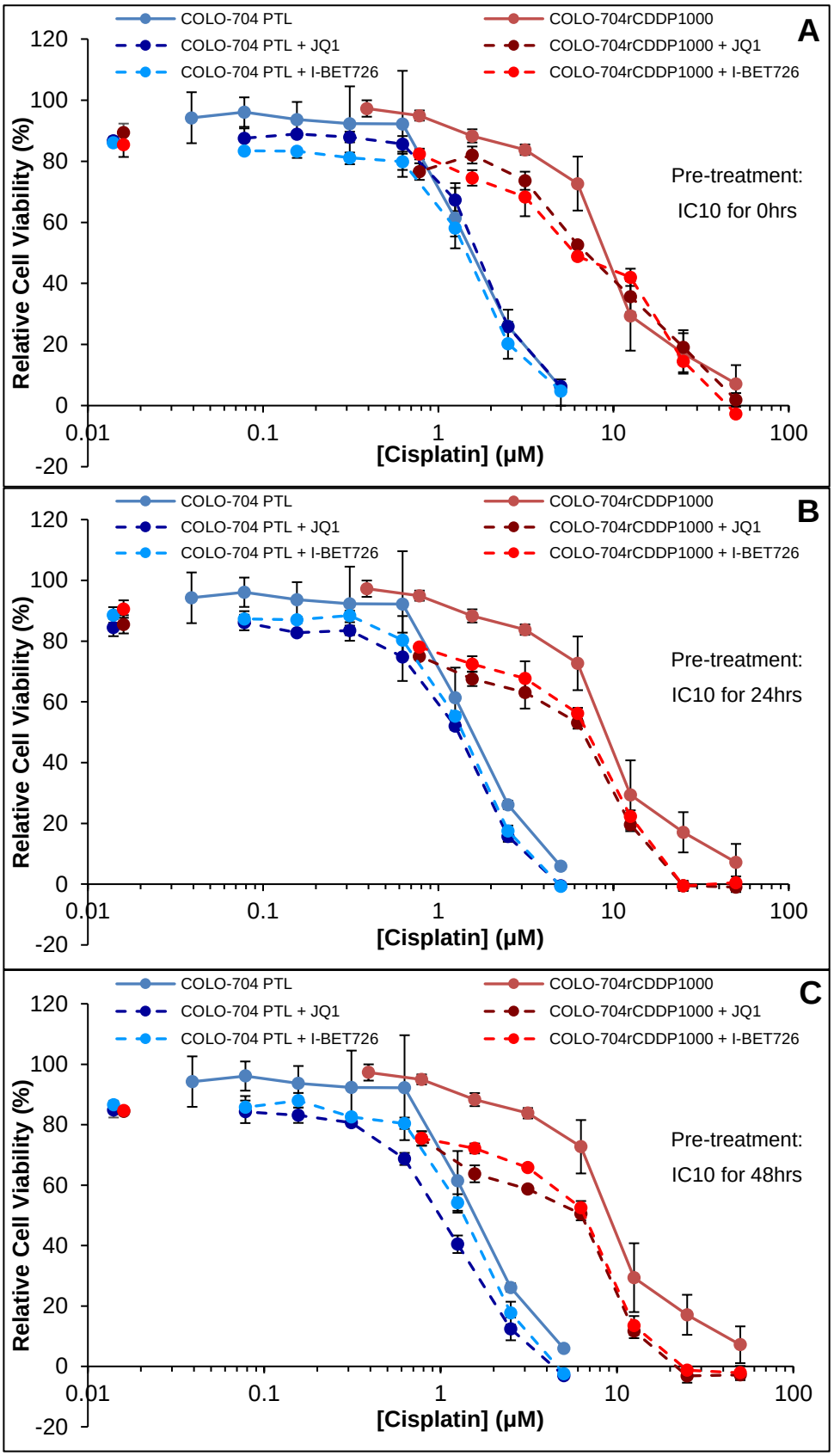


***Figure 24**: Dose-response curves of the COLO-704 PTL (blue) and COLO-704rCDDP1000 (red) cell lines to cisplatin after pre-treatment using the IC10 of the BET inhibitors over 0 hours (**A**), 24 hours (**B**), or 48 hours (**C**). The dose-response curves from the initial characterisation assays are included as solid lines, with the JQ1-pre-treated assays depicted as the darker dashed lines, and I-BET726-pre-treated depicted with lighter dashed lines. The unattached points at the left of each graph represent the viabilities of the pre-treatment-only samples, which were not exposed to cisplatin during the MTT assay.*

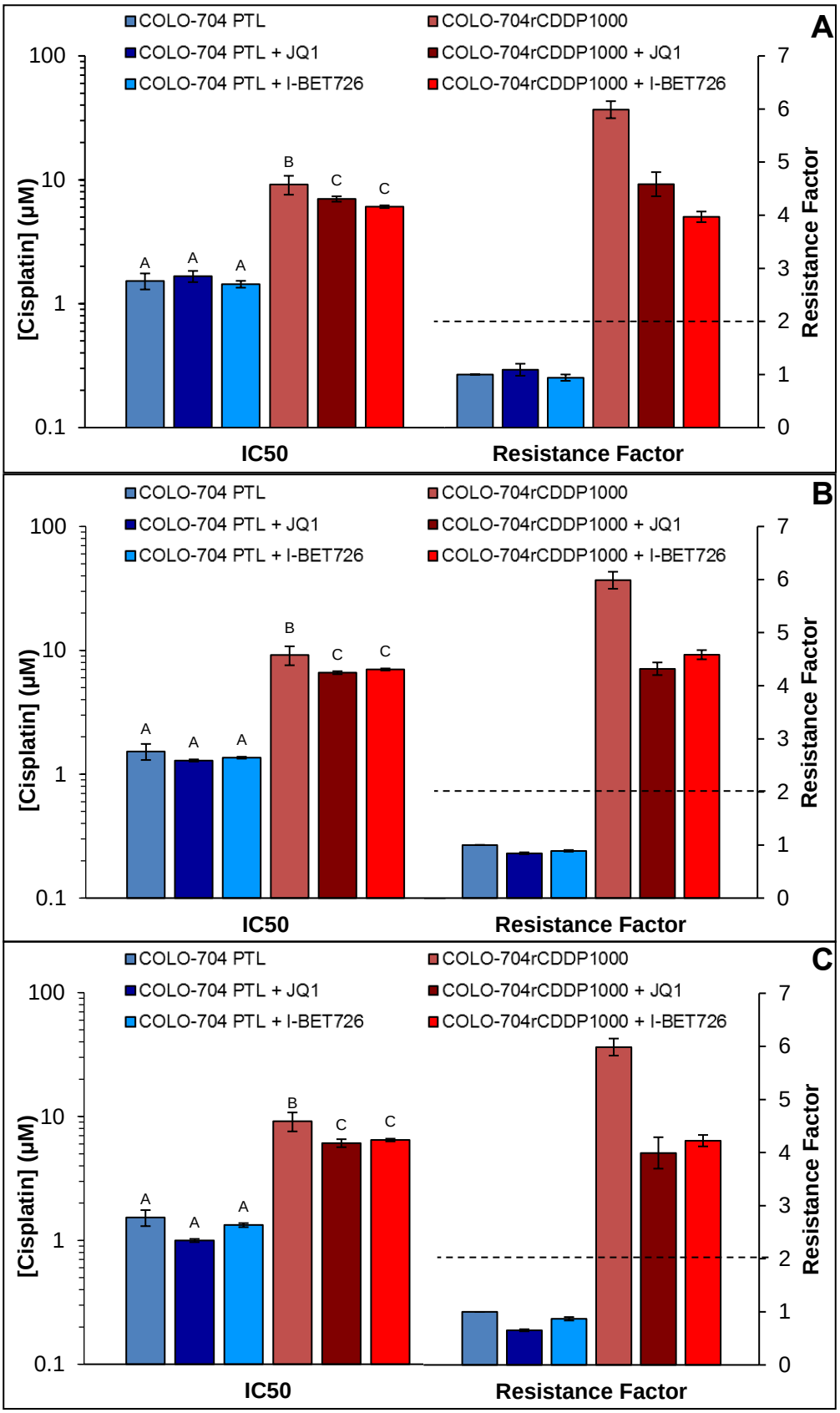


***Figure 25**: Mean IC50 and Resistance Factors of the COLO-704 PTL (blue) and COLO-704^{r}CDDP^{1000} (red) cell lines to cisplatin after pre-treatment using the IC10 of the BET inhibitors over 0 hours (**A**), 24 hours (**B**), or 48 hours (**C**). Statistical testing by two-way ANOVA with post-hoc Tukey's pairwise comparison, using individual 95% confidence intervals, yielded no significant difference in the IC50 of cisplatin against the parental cells across all pre-treatment durations. Conversely, significant differences were seen in the COLO-704^{r}CDDP^{1000} cell line across all pre-treatment durations. The groupings of the IC50 comparison are presented as letters (A-D). Means that do not share a letter are significantly different.*

4.3.2.2 COLO-704 Pre-Treated Using the IC50 of the BET Inhibitors

The dose-response curves of cisplatin against the COLO-704 cell lines after pre-treatment with the IC50 of the BET inhibitors are plotted in Figure 26A-C. When the BET inhibitors were administered immediately prior to cisplatin (0 hours), we again only notice small changes in the sensitivities of both the parental and cisplatin-resistant cell lines. As the pre-treatment duration increases, both cell lines showed increased sensitivity, with a noticeable change between the 0- and 24-hour assays. Another common feature is that the two BET inhibitors produced very similar results. JQ1 appears to increase sensitivity to a slightly greater extent than I-BET726, but there is a large degree of overlap between the datasets.

The IC50 and resistance factor values produced in these assays are plotted in Figure 27A-C. The 0-hour pre-treatment was able to induce a significant difference in the IC50 of cisplatin in the COLO-704^{r}CDDP^{1000} cells, and was able to decrease the resistance factor to a similar degree to that seen when the COLO-704^{r}CDDP^{1000} cells were pre-treated with the IC10 over 24 hours. As the pre-treatment time increased to 24 and 48 hours, the IC50 of cisplatin against the COLO-704^{r}CDDP^{1000} cell line continues to decrease. After a 48-hour pre-treatment, the COLO-704^{r}CDDP^{1000} cells demonstrated marked reductions in their IC50 and resistance factor values. When JQ1 was used as a pre-treatment, no significant difference was calculated between the IC50 of COLO-704^{r}CDDP^{1000} and the baseline COLO-704 PTL sensitivity..

As was seen previously, no significant difference was observed between the IC50s produced from the individual BET inhibitor pre-treatments, across all durations. Analyses of the mean IC50s of the parental cell line calculated no significant difference (relative to the baseline IC50 of cisplatin) after pre-treatment with either BET-inhibitor, over any duration of pre-treatment. It can be seen, however, that as the pre-treatment time increases, there is a decrease in the mean IC50 of cisplatin against the parental cell line, decreasing from approximately 1.3μM (0 hours) to 0.7μM (48 hours).

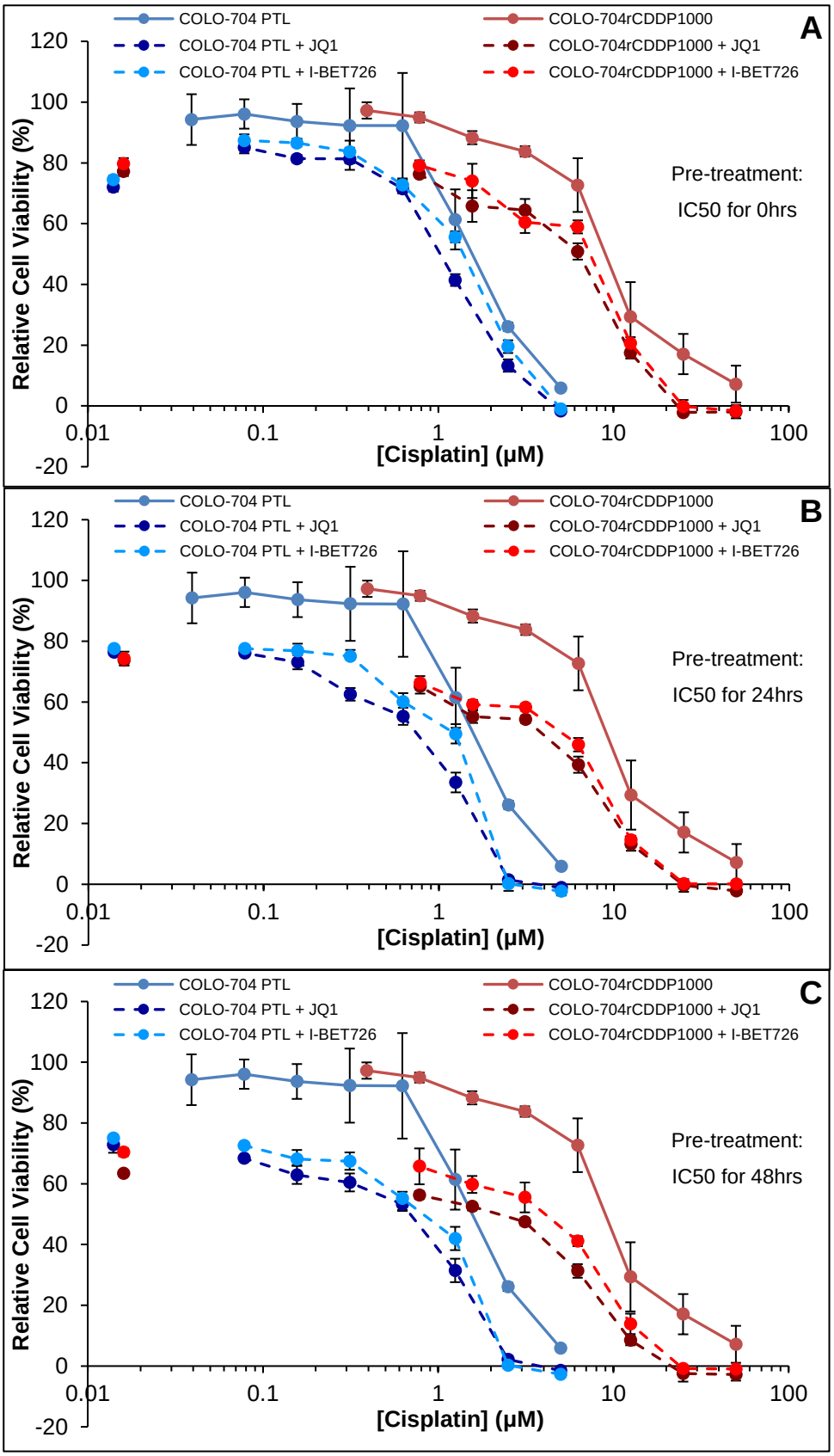


***Figure 26**: Dose-response curves of the COLO-704 PTL (blue) and COLO-704rCDDP1000 (red) cell lines to cisplatin after pre-treatment using the IC50 of the BET inhibitors over 0 hours (**A**), 24 hours (**B**), or 48 hours (**C**). The dose-response curves from the initial characterisation assays are included as solid lines, with the JQ1-pre-treated assays depicted as the darker dashed lines, and I-BET726-pre-treated depicted with lighter dashed lines. The unattached points at the left of each graph represent the viabilities of the pre-treatment-only samples, which were not exposed to cisplatin during the MTT assay.*

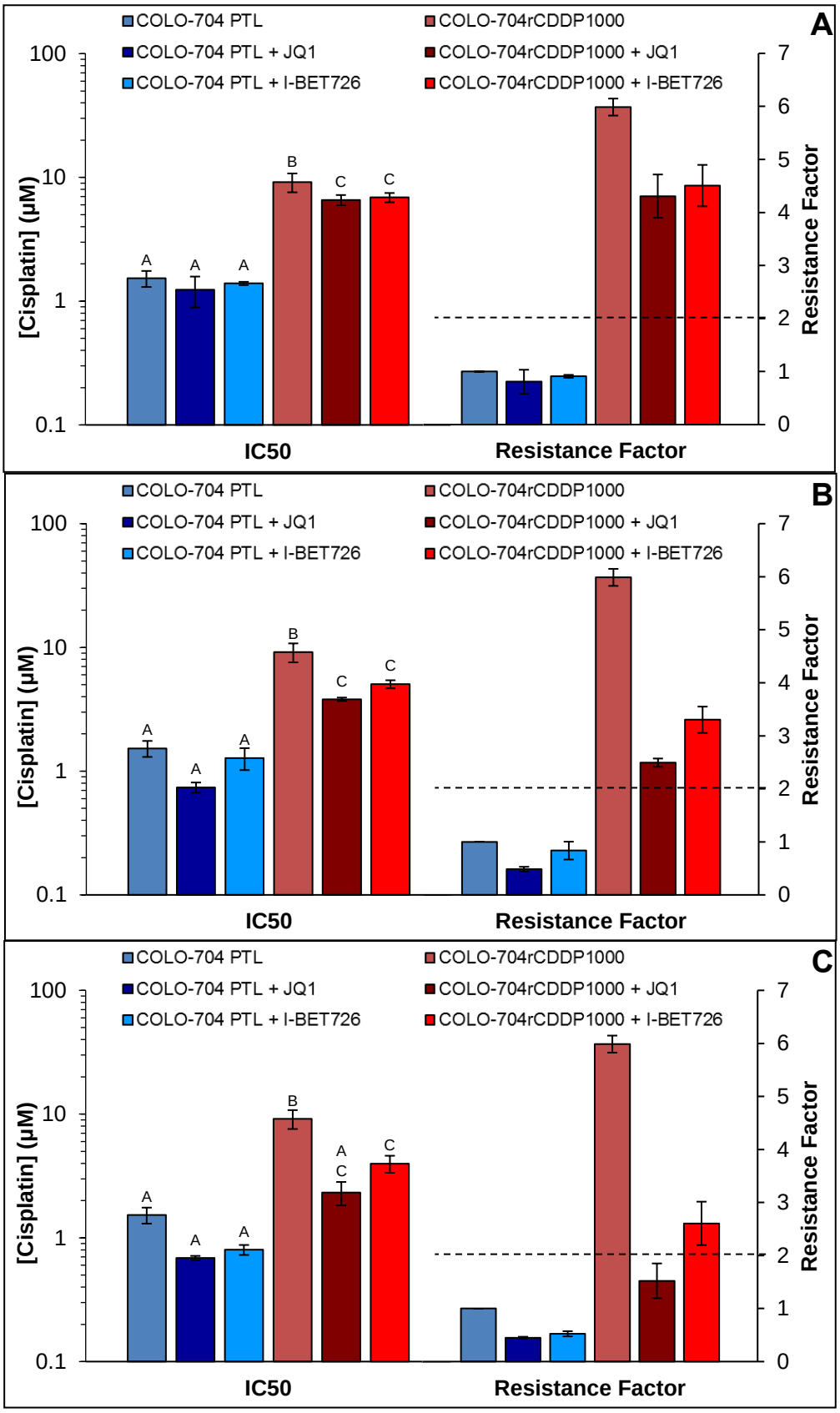


***Figure 27**: Mean IC50 and Resistance Factors of the COLO-704 PTL (blue) and COLO-704^{r}CDDP^{1000} (red) cell lines to cisplatin after pre-treatment using the IC50 of the BET inhibitors over 0 hours (**A**), 24 hours (**B**), or 48 hours (**C**). Statistical testing by two-way ANOVA with post-hoc Tukey's pairwise comparison, using individual 95% confidence intervals, yielded no significant difference in the IC50 of cisplatin against the parental cells across all pre-treatment durations. Conversely, significant differences were seen in the COLO-704^{r}CDDP^{1000} cell line across all pre-treatment durations. The groupings of the IC50 comparison are presented as letters (A-D). Means that do not share a letter are significantly different. After a 48 hour pre-treatment of JQ1, the IC50 of cisplatin against COLO-704^{r}CDDP^{1000} is not significantly different from the untreated parental cell line, and has a resistance factor below the two-fold threshold.*

4.3.2.3 EFO-21 Pre-Treated Using the IC10 of the BET Inhibitors

The EFO-21 cell lines were pre-treated using the IC10 of JQ1 and I-BET726, over 0, 24, or 48 hours, producing the dose-response curves plotted in Figure 28A-C. When the BET inhibitors were administered immediately prior to cisplatin (0 hours), there was little-to-no effect seen in the EFO-21 PTL cell line. As seen in the previous cell lines, there appears to be an improved response to cisplatin as the pre-treatment duration increases, and the responses to each BET inhibitor are very similar.

The IC50 and resistance factor values produced in these assays are plotted in Figure 29A-C. The 0-hour pre-treatment was not able to induce a significant difference in the IC50 of cisplatin against either of the cell lines. As the pre-treatment time increased to 24 and 48 hours, the IC50 of cisplatin against the EFO-21^{r}CDDP^{2000} cell line decreased to become significantly different from the baseline IC50. In these assays, a significant difference was calculated between JQ1 and I-BET726 pre-treated cells. As the pre-treatment duration increases we see a decrease in the resistance factor of the EFO-21^{r}CDDP^{2000} cell line, particularly so when the cells were pre-treated with JQ1.

Another result which appears to be consistent between the cell lines is that the mean IC50 of the parental cell line was not statistically different after pre-treatment using the IC10 of either BET-inhibitor, over any duration of pre-treatment.

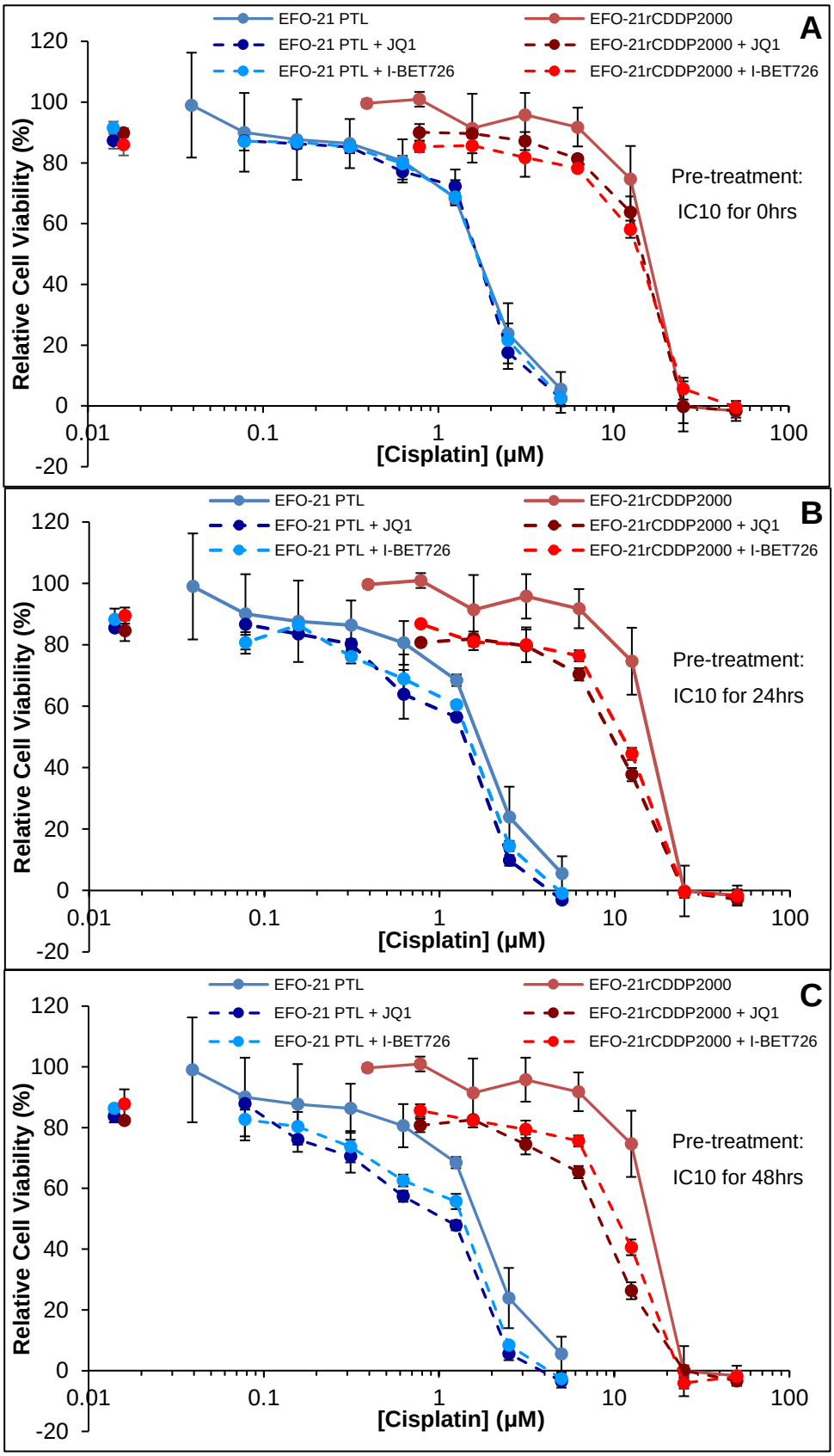


***Figure 28**: Dose-response curves of the EFO-21 PTL (blue) and EFO21^{r}CDDP^{2000} (red) cell lines to cisplatin after pre-treatment using the IC10 of the BET inhibitors over 0 hours (**A**), 24 hours (**B**), or 48 hours (**C**). The dose-response curves from the initial characterisation assays are included as solid lines, with the JQ1-pre-treated assays depicted as the darker dashed lines, and I-BET726-pre-treated depicted with lighter dashed lines. The unattached points at the left of each graph represent the viabilities of the pre-treatment-only samples, which were not exposed to cisplatin during the MTT assay.*

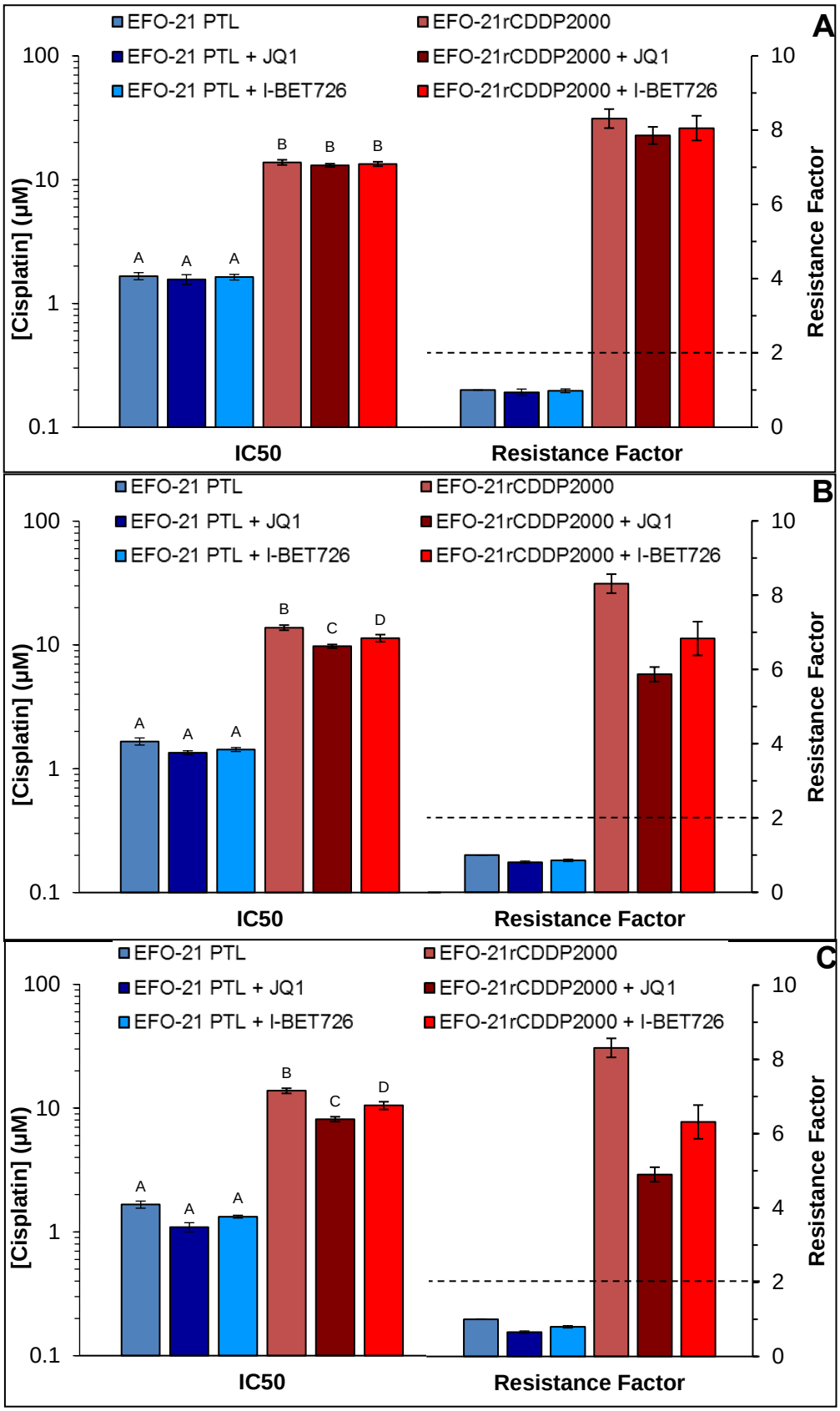


***Figure 29**: Mean IC50 and Resistance Factors of the EFO-21 PTL (blue) and EFO-21^{r}CDDP^{2000} (red) cell lines to cisplatin after pre-treatment using the IC10 of the BET inhibitors over 0 hours (**A**), 24 hours (**B**), or 48 hours (**C**). Statistical testing by two-way ANOVA with post-hoc Tukey's pairwise comparison, using individual 95% confidence intervals, yielded no significant difference in the IC50 of cisplatin against the parental cells across all pre-treatment durations. Significant differences were seen in the EFO-21^{r}CDDP^{2000} cell line across all pre-treatment durations and, after 24- and 48-hour pre-treatments, between the two BET inhibitors. The groupings of the IC50 comparison are presented as letters (A-D). Means that do not share a letter are significantly different.*

4.3.2.4 EFO-21 Pre-Treated Using the IC50 of the BET Inhibitors

The dose-response curves plotted in Figure 30A-C show the EFO-21 cell lines response to cisplatin after being pre-treated using the IC50 of the BET inhibitors. These results seem to show more notable changes in the parental cell line, particularly as the pre-treatment duration increases. For example, a cisplatin concentration of 2.5μM produced a mean viability of 23.9% in the initial baseline assays. After pre-treatment with the BET inhibitors for 24 hours, this cisplatin concentration was sufficient to reduce the mean viability to zero. Similarly, the sensitivity of the EFO-21^{r}CDDP^{2000} was improved when the IC50 was used as a pre-treatment, with this effect improving as pre-treatment durations increased.

Figure 31A-C depicts the IC50 and resistance factor values produced in these assays. As has been observed previously, the EFO-21^{r}CDDP^{2000} cell line exhibits a steady decrease in its IC50 and resistance factor to cisplatin as the duration of pre-treatment with BET inhibitors increases. With a 48-hour pre-treatment using the IC50 of JQ1, the cisplatin-resistant cell line shows no significant difference to the baseline IC50 of the parental EFO-21 cell line. This 48-hour pre-treatment was able to reduce the resistance factor of the EFO-21^{r}CDDP^{2000} cell line to the vicinity of the 2-fold threshold which is used to distinguish resistance.

Interestingly, these assays may seem to show a decrease in the mean IC50 of the parental cell line as a consequence of pre-treatment with BET inhibitors. In the 24-hour pre-treatment assay, a statistically significant difference was observed between the JQ1-pre-treated cells and the initial baseline IC50. The mean IC50 of cisplatin does continue to decrease further (from 0.71μM to 0.44μM) as the pre-treatment duration increases to 48 hours. However, the responses from the 48-hour pre-treatment assays were more variable, and thus had a broader range of their 95% confidence intervals, and the resulting statistical test found no significant difference in these assays.

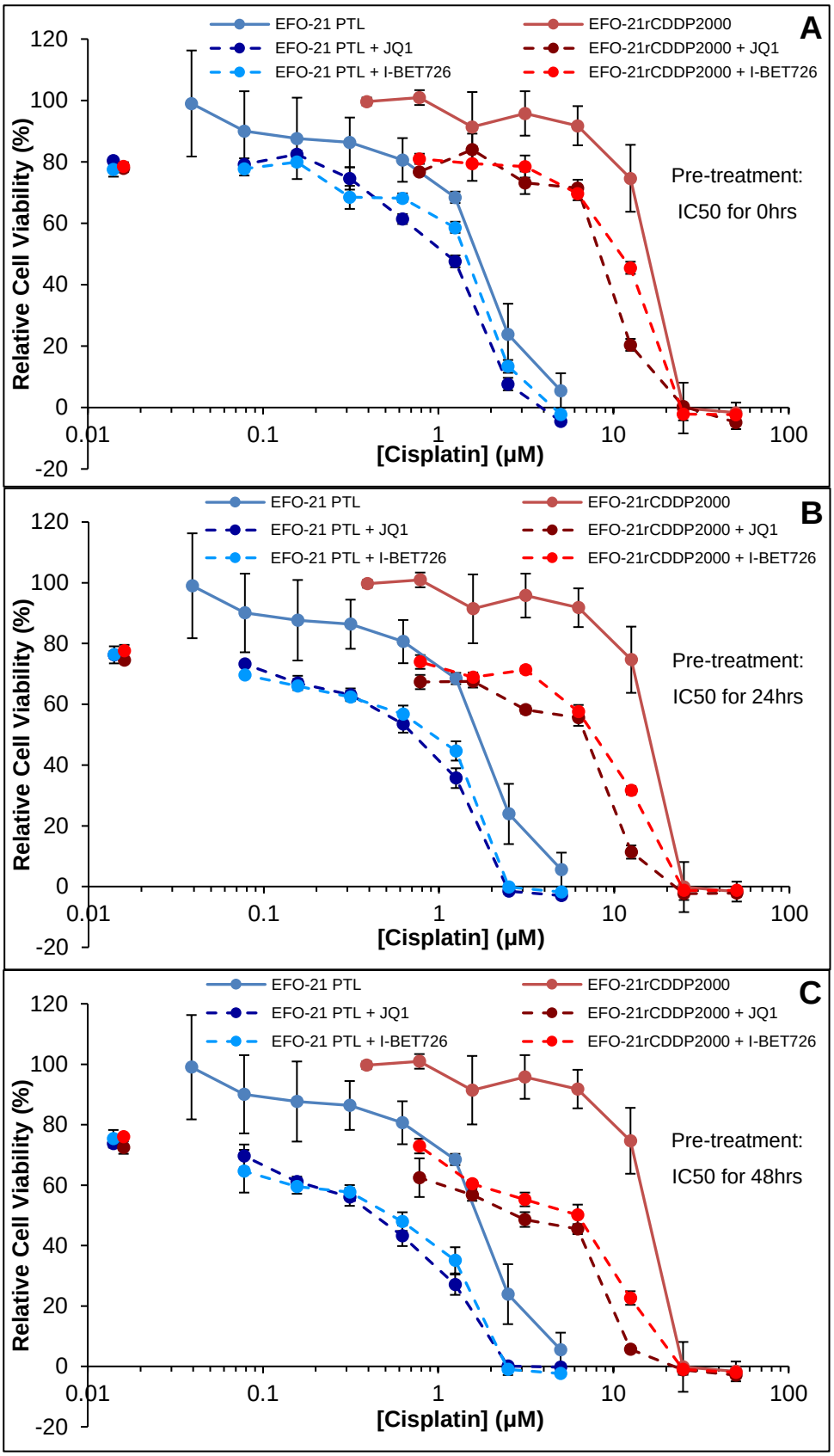


***Figure 30**: Dose-response curves of the EFO-21 PTL (blue) and EFO21^{r}CDDP^{2000} (red) cell lines to cisplatin after pre-treatment using the IC50 of the BET inhibitors over 0 hours (**A**), 24 hours (**B**), or 48 hours (**C**). The dose-response curves from the initial characterisation assays are included as solid lines, with the JQ1-pre-treated assays depicted as the darker dashed lines, and I-BET726-pre-treated depicted with lighter dashed lines. The unattached points at the left of each graph represent the viabilities of the pre-treatment-only samples, which were not exposed to cisplatin during the MTT assay.*

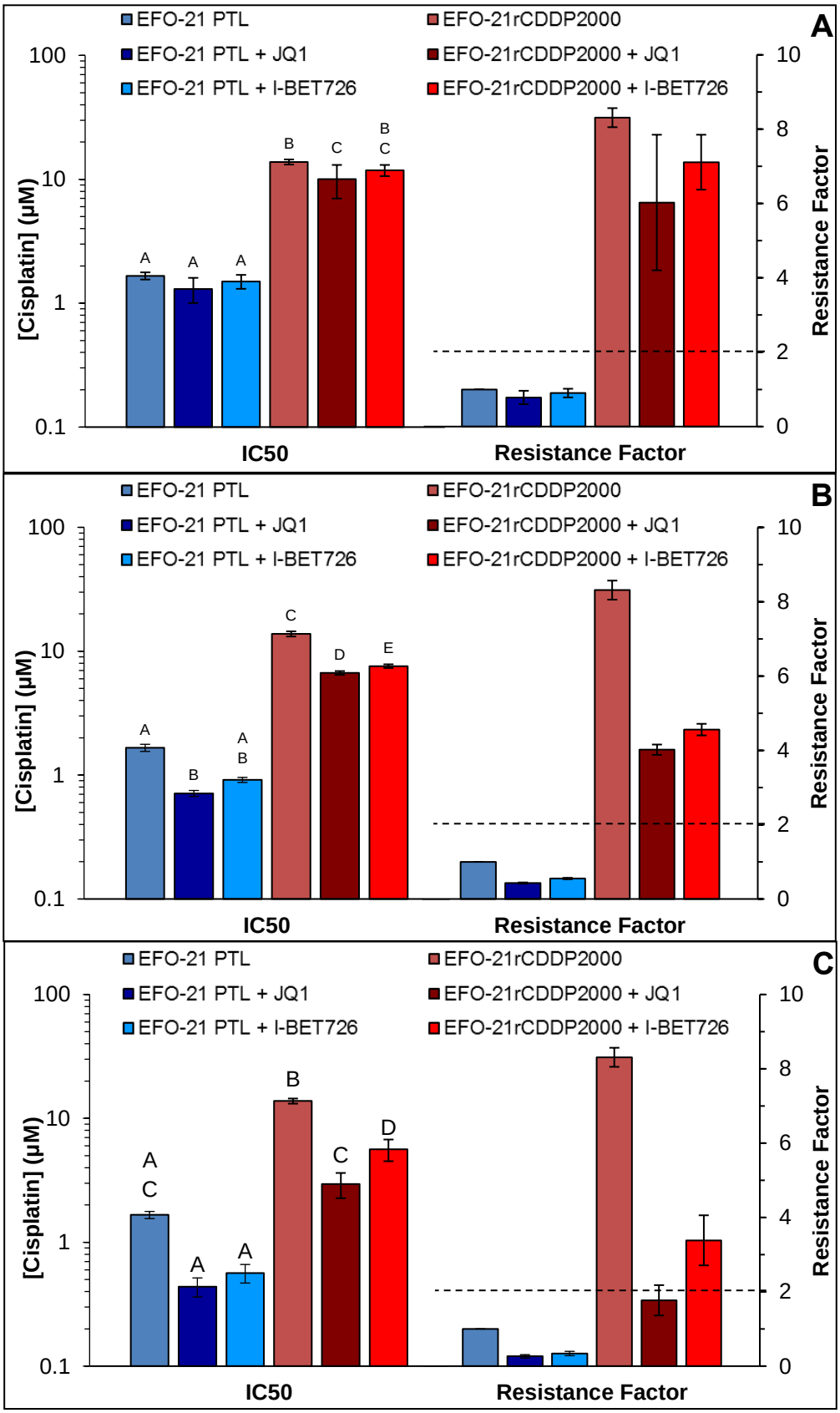


Figure 31*: Mean IC50 and Resistance Factors of the EFO-21 PTL (blue) and EFO-21^{r}CDDP^{2000} (red) cell lines to cisplatin after pre-treatment using the IC50 of the BET inhibitors over 0 hours (**A**), 24 hours (**B**), or 48 hours (**C**). Statistical testing was conducted by two-way ANOVA with post-hoc Tukey's pairwise comparison, using individual 95% confidence intervals. The groupings of the IC50 comparison are presented as letters (A-D). Means that do not share a letter are significantly different. Only when pre-treated for 24 hours did the IC50 of cisplatin against the parental cell show significant differences. Significant differences were seen in the EFO-21^{r}CDDP^{2000} cell line across nearly all pre-treatment conditions, with exception to those treated with I-BET726 for 0 hours.*

4.3.2.5 EFO-27 Pre-Treated Using the IC10 of the BET Inhibitors

The final set of ovarian cancer cell lines used in this study, EFO-27, was pre-treated using the IC10 of the BET inhibitors, producing the dose-response curves to cisplatin which are depicted in Figure 32A-C. We again see this relationship between pre-treatment duration and the changes in sensitivity which has been seen in the other cell lines: a 0-hour pre-treatment using the IC10 produces a minimal change, through to more tangible improvements after 48 hours.

The IC50 and resistance factor values produced in these assays are plotted in Figure 33A-C. The 0-hour pre-treatment was not able to induce a significant difference in the IC50 of cisplatin against either of the cell lines. As the pre-treatment duration increased to 24 and 48 hours, the IC50 of cisplatin against the EFO-27^{r}CDDP^{2000} cell line decreases, becoming significantly different from the baseline IC50. After a 48-hour pre-treatment using the IC10 of the BET inhibitors, we see that the resistance factor of the EFO-27^{r}CDDP^{2000} cell line nearly halves, decreasing from 10.34 to 5.18 (after JQ1) or 6.58 (after I-BET726).

Another result which has been consistent between the cell lines is that, while there may be small reductions in the mean IC50 of the parental cell line after pre-treatment using the IC10 of the BET-inhibitor, these changes were not substantial enough to be deemed statistically different from the baseline IC50 of cisplatin, over any duration of pre-treatment.

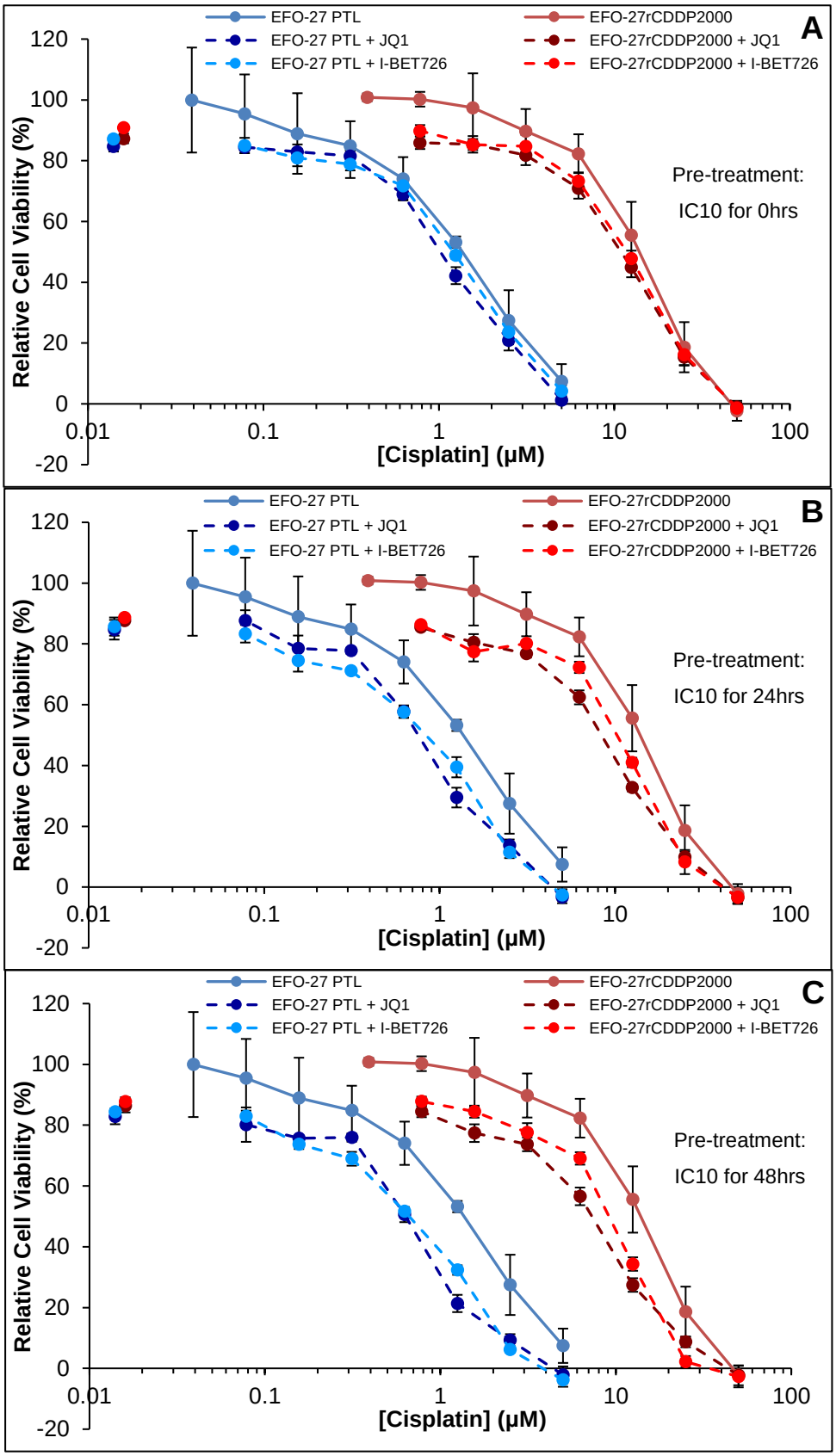


***Figure 32**: Dose-response curves of the EFO-27 PTL (blue) and EFO27^{r}CDDP^{2000} (red) cell lines to cisplatin after pre-treatment using the IC10 of the BET inhibitors over 0 hours (**A**), 24 hours (**B**), or 48 hours (**C**). The dose-response curves from the initial characterisation assays are included as solid lines, with the JQ1-pre-treated assays depicted as the darker dashed lines, and I-BET726-pre-treated depicted with lighter dashed lines. The unattached points at the left of each graph represent the viabilities of the pre-treatment-only samples, which were not exposed to cisplatin during the MTT assay.*

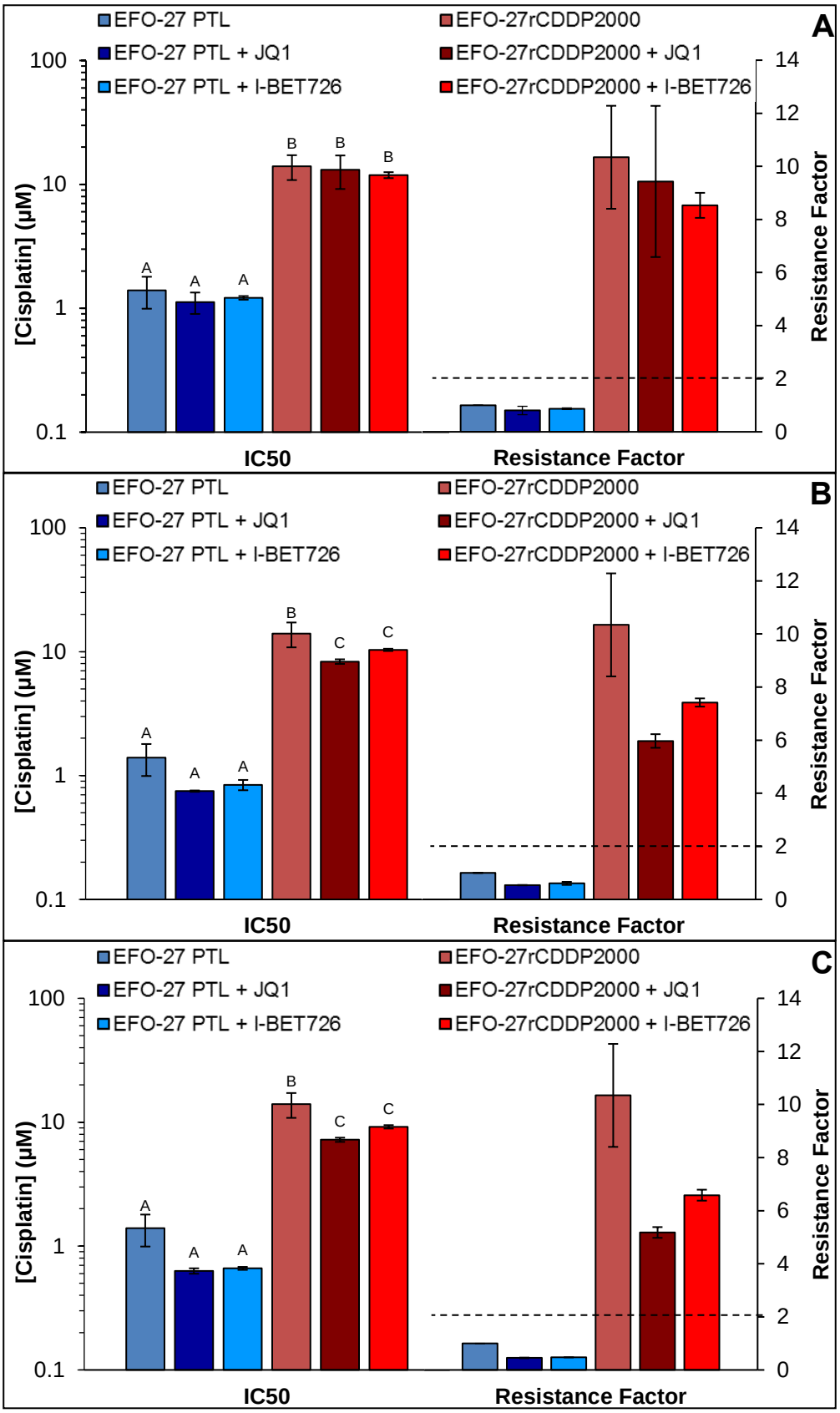


***Figure 33**: Mean IC50 and Resistance Factors of the EFO-27 PTL (blue) and EFO-27^{r}CDDP^{2000} (red) cell lines to cisplatin after pre-treatment using the IC10 of the BET inhibitors over 0 hours (**A**), 24 hours (**B**), or 48 hours (**C**). Statistical testing by two-way ANOVA with post-hoc Tukey's pairwise comparison, using individual 95% confidence intervals, yielded no significant difference in the IC50 of cisplatin against the parental cells across all pre-treatment durations. Significant differences were seen in the EFO-21^{r}CDDP^{2000} cell line after 24- and 48-hour pre-treatments, , but no difference was found between the two BET inhibitors. The groupings of the IC50 comparison are presented as letters (A-D). Means that do not share a letter are significantly different.*

4.3.2.6 EFO-27 Pre-Treated Using the IC50 of the BET Inhibitors

The dose-response curves plotted in Figure 34A-C show the viability of the EFO-27 cell lines in response to cisplatin after being pre-treated using the IC50 of the BET inhibitors. Similarly to the EFO-21 cell lines, these results seem to show more prominent changes in the sensitivity of the parental cell line, in addition to the cisplatin-resistant lines. It seems that the most notable shifts in the dose-response curves occur from the 0- to 24-hour samples, with further changes seen between 24- and 48 hours, but to a lesser extent.

Figure 35A-C depicts the IC50 and resistance factor values produced in these assays. When the EFO-27^{r}CDDP^{2000} cells had been pre-treated for 0 hours, only JQ1 was found to induce a significant change in the IC50 of cisplatin, however as the pre-treatment time increases both BET inhibitors induced significant changes. After a 48-hour pre-treatment using the IC50 of BET inhibitors, the cisplatin-resistant cell line shows no significant difference to the baseline IC50 of the parental EFO-21 cell line. This 48-hour pre-treatment was able to reduce the resistance factor of the EFO-27^{r}CDDP^{2000} cell line from 10.34, to 3.06 (JQ1) or 3.61-fold resistance (I-BET726).

Unlike the EFO-21 cell lines, there were no pre-treatment conditions which yielded statistically significant reductions in the IC50 of cisplatin against the EFO-27 PTL cell line. When we assess the general trend of the IC50 data across the pre-treatment durations, there does seem to be a reduction in the IC50 of cisplatin. For example, when JQ1 was used for 0-hours, this yielded a mean IC50 of 0.86μM, and this approximately halves to 0.45μM when the cells were incubated for 48 hours.

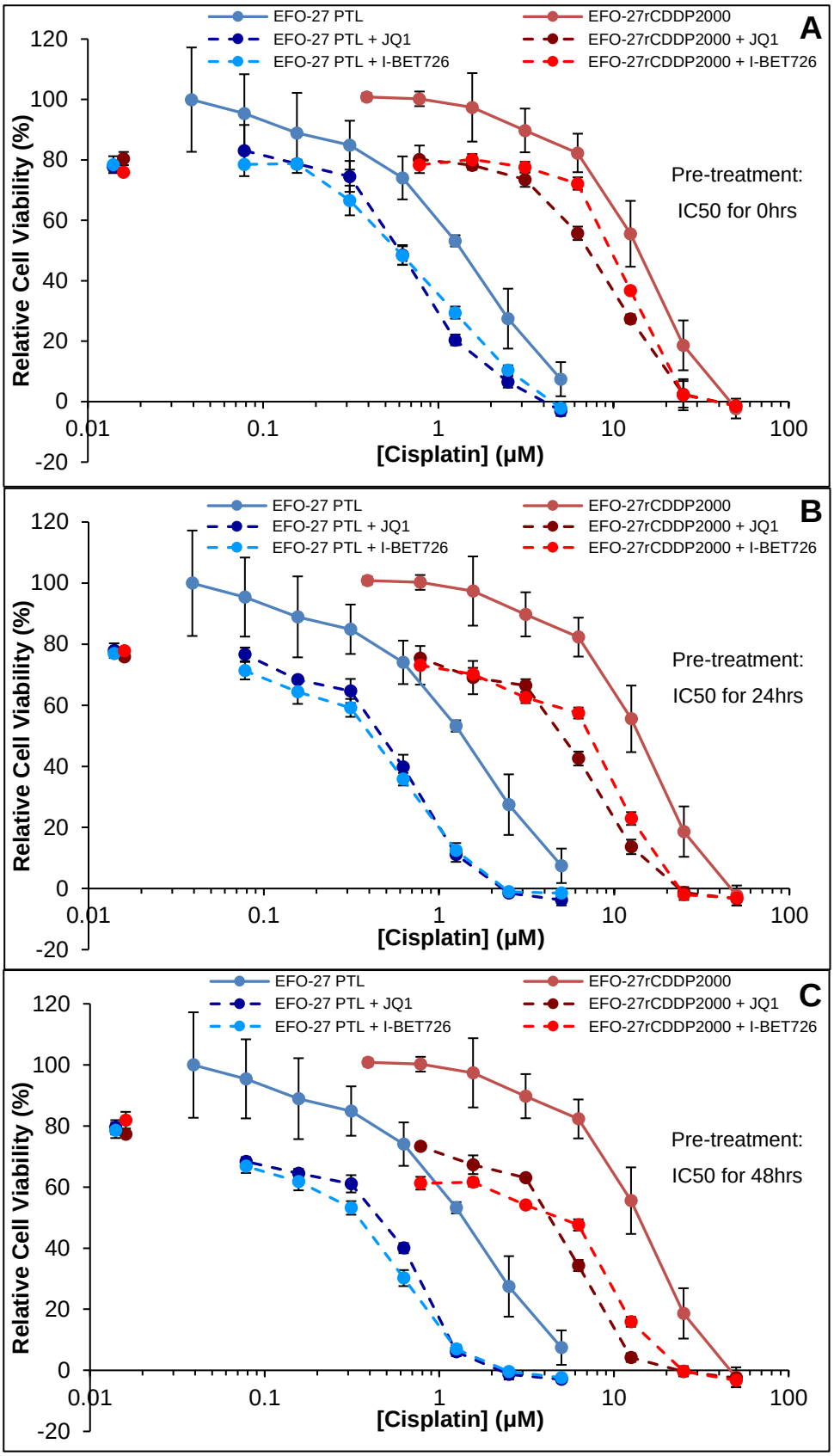


***Figure 34**: Dose-response curves of the EFO-27 PTL (blue) and EFO27^{rCDDP2000} (red) cell lines to cisplatin after pre-treatment using the IC50 of the BET inhibitors over 0 hours (**A**), 24 hours (**B**), or 48 hours (**C**). The dose-response curves from the initial characterisation assays are included as solid lines, with the JQ1-pre-treated assays depicted as the darker dashed lines, and I-BET726-pre-treated depicted with lighter dashed lines. The unattached points at the left of each graph represent the viabilities of the pre-treatment-only samples, which were not exposed to cisplatin during the MTT assay.*

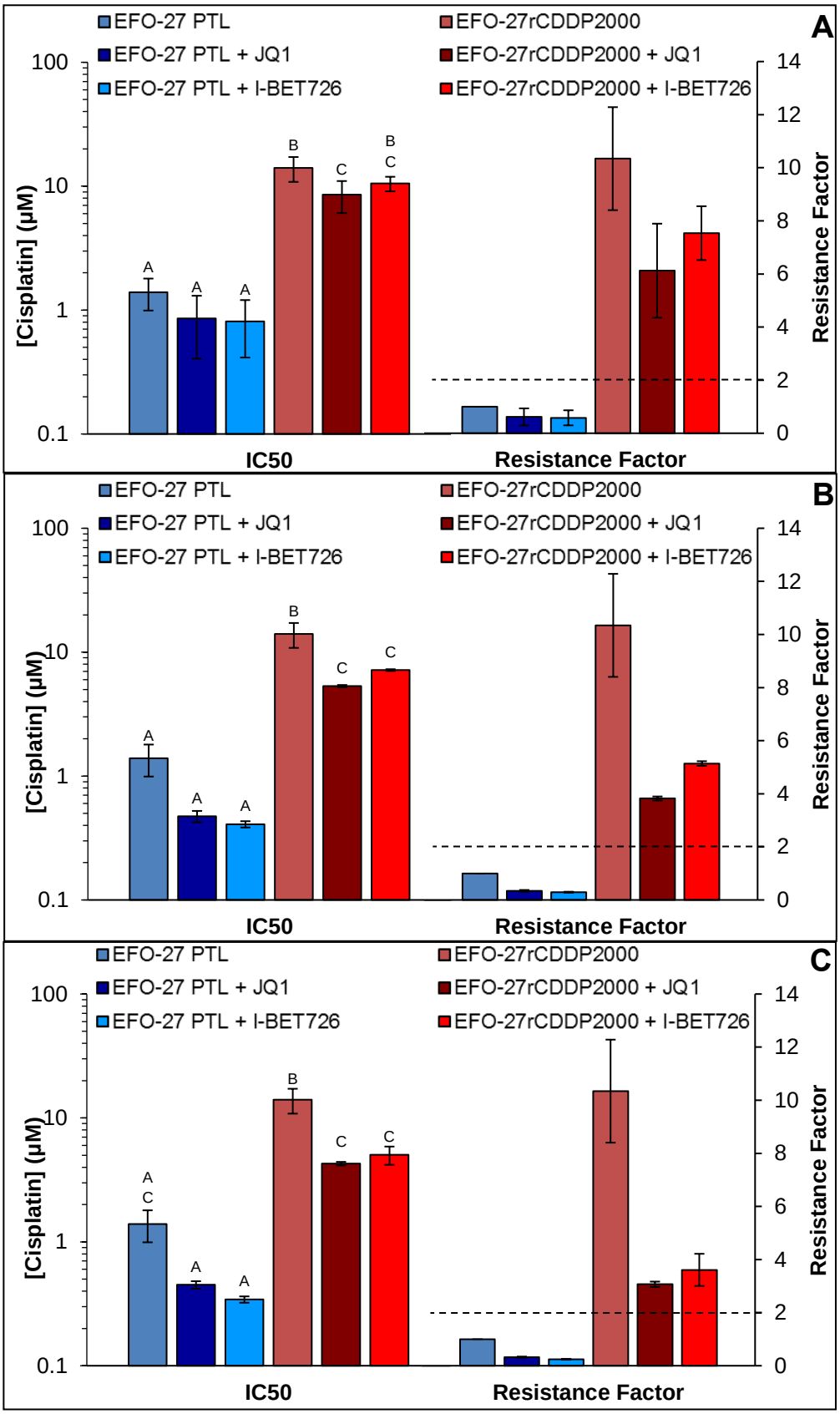


***Figure 35**: Mean IC50 and Resistance Factors of the EFO-27 PTL (blue) and EFO-27^{r}CDDP^{2000} (red) cell lines to cisplatin after pre-treatment using the IC50 of the BET inhibitors over 0 hours (**A**), 24 hours (**B**), or 48 hours (**C**). Statistical testing by two-way ANOVA with post-hoc Tukey's pairwise comparison, using individual 95% confidence intervals, yielded no significant difference in the IC50 of cisplatin against the parental cells across all pre-treatment durations. Significant differences were seen in the EFO-27^{r}CDDP^{2000} cell line across nearly all pre-treatment durations, except for the 0-hour I-BET726 pre-treatment. No difference was found between the two BET inhibitors. The groupings of the IC50 comparison are presented as letters (A-D). Means that do not share a letter are significantly different.*

5. Discussion

As discussed in my Introduction, resistance to platinum compounds is a major cause of treatment failure and reduced survival rates across a range of different cancers. The mechanisms of resistance are complex, affecting a hugely diverse array of cellular processes; and this range of mechanisms is only partly understood (Holohan et al., 2013; Shahzad et al., 2009). There is consequently an apparent need for further insight into how platinum resistance emerges and is facilitated in tumours, and also a need for ways to inhibit this resistance formation and/or reverse this resistance in tumours.

There is evidence to suggest that BET proteins have a role to play in oncogenesis. Overexpression of BET proteins seen in leukaemias and B-cell lymphomas, NUT midline carcinomas, and lung cancers (Florence & Faller, 2001; Wang & Filippakopoulos, 2015; Zhang et al., 2016), and BET proteins have also been seen to regulate many of the pathways which are implicated in platinum resistance.

The aims of this study were to investigate whether BET inhibitors were able to inhibit the viability of parental and platinum-resistant neuroblastoma and ovarian cancer cell lines, and to see whether BET inhibitors would alter the sensitivities of these cell lines to platinum agents.

5.1 Responses of Cell Lines to BET Inhibition

5.1.1 Summary of Results

When MTT assays were conducted against JQ1 and I-BET726 as single agents (Section 4.2), there were no substantial differences between the responses of the parental and platinum-resistant counterpart cell lines. This could suggest that the mechanisms able to drive considerable levels of resistance to platinum compounds in these cell lines do not appear to confer some degree of cross-resistance against the two BET inhibitors.

There did, however, appear to be variation in the sensitivity of the different cancer cell lines to BET inhibition, particularly so when the response of the neuroblastoma UKF-NB-3 cell lines are compared to the ovarian cancer cell lines. For instance, the IC50s of JQ1 against UKF-NB-3 cell lines were found to be approximately

three times lower than those of the ovarian cell lines, which could suggest that there may be specific features of each tumour cell line which determine sensitivity.

Despite the differences in the initial sensitivities of the cancer cell lines to BET inhibition, this investigation found that pre-treatment with BET inhibitors was able to increase the sensitivity of all of the platinum-resistant cell lines to their respective platinum drug. There were improvements (although only small) in the responses to platinum drugs even when the IC10 of the BET inhibitors, which was chosen to represent a minimal effective dose, was used as a pre-treatment. Each of the cell lines appeared to show improved responses to platinum compounds as the pre-treatment dosage was elevated, and as the pre-treatment duration was increased. It was also noticeable that, while BET inhibition was able to alter the dose-response and cause small decreases in the mean IC50 of the parental cell lines to platinum compounds, the most notable changes were observed in the platinum-resistant cell lines.

5.1.2 Potential Mechanisms of Action

It is not known precisely what is happening mechanistically as a result of BET inhibition in these cell lines, and I feel that will be an important area for future study (detailed further in Section 5.3). It is possible that, if BET proteins are influential in maintaining the anti-apoptotic, pro-survival phenotype seen in resistant cells via transcriptional regulation, the action of BET inhibitors could be able to nullify this response by inhibiting the transcription of these pro-survival genes.

Of the possible explanations for my results, one of the most studied mechanisms is the ability of BET inhibitors to inhibit the *MYC* oncogene. Amplification of *MYCN* is frequently seen in neuroblastoma, particularly so in high-risk cases, and is considered to convey a poor prognosis for patients (Ambros et al., 2009). Dysregulation of *C-MYC* is also a feature which is prevalent amongst high-grade ovarian carcinomas, which also show poorer patient survival rates (Plisiecka-Halasa et al., 2003).

Of the cell lines used in this study, UKF-NB-3, EFO-21 have been found in studies to show elevated expression of *MYC* (Kotchetkov et al., 2005; Wiedemeyer et al., 2013). Interestingly, the Wiedemeyer (2013) study found that JQ1 was able to inhibit expression of *C-MYC* in the EFO-21 cell line, and further postulated that this could result in downstream inhibition of *BRCA* gene expression. *BRCA1* and *BRCA2* are both implicated as means of platinum resistance in ovarian cancers due to their role in DNA damage responses and repair (Sakai et al., 2008; Swisher et al., 2008). A study

investigating I-BET726 against a panel of neuroblastoma similarly found that BET inhibition reduced the expression of *MYCN*, as well as the anti-apoptotic *BCL2* (Wyce et al., 2013). This study also used microarrays and qPCR to monitor the effects this would have on expression of downstream *MYCN* targets, finding downregulation of genes such as *NME2*, an inhibitor of differentiation which is prone to amplification in aggressive neuroblastomas.

Beneficial effects of JQ1 paired with cisplatin have also been seen in the ovarian cancer cell line, A2780, with the combination resulting in increased sensitivity of the cisplatin resistant cell line (Khabele et al., 2013). In this study, however, there was no correlation between the inhibitory effects of JQ1 and a downregulation of *MYC* suggesting that there are other oncogenic targets of JQ1 which require identifying.

In addition to their ability to downregulate *MYC*, BET inhibitors have also been found to elicit anti-tumour effects via alternative mechanisms. A study of lung adenocarcinoma cell lines (Lockwood et al., 2012) found that JQ1 was able inhibit these cell lines with high potency, giving an increased number of cells arrested at G0/G1 or undergoing apoptosis. JQ1 was not found to cause downregulation of *MYC* in these cell lines. JQ1 was able to inhibit expression of *FOSL1*, preventing the formation of the heterodimeric AP-1 transcription factor (by association with Jun) which regulates cellular processes such as differentiation, growth and proliferation, and apoptosis. It was found that JQ1 inhibited the recruitment of BRD4 to the enhancer site of *FOSL1*, where it would usually recruit p-TEFb and RNA Pol II for transcriptional elongation.

Another example of a transcription factor which has been downregulated as a result of BET inhibition in tumour cells is *FOXM1*, which was seen to be inhibited in a panel of 28 ovarian cancer cell lines (Zhang, Ma et al., 2016), including the EFO-27 cell line which was used in my study. BET inhibition was found to induce cell cycle arrest and apoptosis across these cell lines without notable discrepancies, despite the fact that these cell lines were obtained from a diverse range of tumour types from different tissues.

An interesting study by Klingbeil et al. (2016), which could possibly shed some light onto my own results, investigated the combination of JQ1 and cisplatin in a panel of KRAS-mutated non-small cell lung cancer cell lines as well as in mouse models. This research determined that expression of the apoptosis regulators c-FLIP and XIAP were largely dependent on BET activity. The study also found dose-dependent responses to BET inhibition, as well as changes associated with treatment length. At shorter

durations, cells showed predominantly cell cycle arrest, with increases in apoptosis after longer durations. Finally, the combination of JQ1 with cisplatin was seen to have beneficial effects, with cisplatin more effectively able to induce apoptosis in the absence of the downregulated negative regulators.

Prior work conducted in the Michaelis research group, based at the University of Kent, used yeast (*Saccharomyces cerevisae*) models to assess the contribution of *BDF1* (an equivalent BET protein found in yeast) on the resistance to platinum compounds (Sanders, 2016 – unpublished). This research found that *ΔBdf1* deletion strains showed inhibited growth and viability in the presence of platinum compounds, and this viability could be improved by reintroducing a BDF1 plasmid into the deletion strain. It was thought that by inhibiting the transcriptional activity of the deletion strain, *BDF1* inhibition would reduce the capacity of the cells to detect and mount a repair response to these DNA lesions. It was also postulated that, if the BDF proteins are able to modify the chromatin integrity, that inhibition may alter the accessibility of the DNA to the compounds, or the accessibility of the DNA to the repair machinery (Chua & Roeder, 1995; Mymryk et al., 1995).

As mentioned in my Introduction, BRD4 in humans is able to interact with a number of chromatin regulators (such as NSD3, JMJD6, CHD4, and ATAD5) via their association with the extra-terminal (ET) domain (Rahman et al., 2011). One example of the role of these proteins is their ability to modify the methylation status of H3K36, a modification which is typically associated with transcriptionally active regions. Inhibition of BRD4 or NSD3 resulted in reduced methylation of H3K36 in the proximity of regulated genes.

Considering the role BET proteins have in regulating the structure of chromatin, it is possible that JQ1 or I-BET726 are able to inhibit these changes in chromatin structure, and produce altered methylation patterns in treated cells. Resistant cells might not able to recruit the necessary chromatin modifiers in response to cellular cues and stresses (such as platinum induced DNA damage), resulting in a lack of necessary gene activation for survival. As mentioned previously it is also possible that the chromatin, without such regulation of its structure, is more accessible for the platinum agents, or less accessible for DNA repair complexes.

5.2 Implications for Wider BET Inhibitor Research

Currently, the BET inhibitors which are widely used in research, such as JQ1, are pan-BET inhibitors, showing high affinity for members across the BET family (BRD2, BRD3, BRD4, and the testis-specific BRDT). These may also exhibit affinity for other bromodomain-containing proteins (such as BRD8) due to their ability to competitively bind the acetyl-lysine recognition sites of the BD1 and BD2 bromodomains. While both JQ1 and I-BET726 are both pan-BET inhibitors, the two compounds have different affinities for the individual BET proteins, and I-BET726 demonstrates a high affinity for BET-family members, but has a low affinity for the other BRD-containing proteins (Filippakopoulos & Knapp, 2014; Wyce et al., 2013). It could perhaps be these subtle affinity divergences which account for the minor differences in the effects induced by the two BET inhibitors as pre-treatments.

Due to the promising pre-clinical research emerging regarding BET inhibition, there are a number of BET inhibitors currently in early phases of clinical trials (www.clinicaltrials.gov; Theodoulou et al., 2016). JQ1 is not currently being used in clinical trials because it was found to have a very short half-life in the body (a matter of hours), however a number of analogous compounds are currently in development which have been modified to improve bioavailability (Gallenkamp et al., 2014). JQ1 still remains a useful tool for pre-clinical research due to its high affinity for BET proteins, and it has a relatively large bank of pre-clinical research characterising its function across a range of tissue and disease types.

Compounds are also in development which are specific to individual members of the BET family in the hope that these may be useful in characterising the individual contributions of these proteins. These might later provide a more targeted treatment strategy if there are disease states where an individual BET member exhibits pathogenic activity (Ferri et al., 2016). In addition, inhibitors have been developed which are specific to the individual bromodomains, such as olinone which is BD1-specific (Ntranos & Casaccia, 2015) and RVX-208, which is BD2-specific (Johansson et al., 2014).

Due to the fact that BET inhibitor research is, in a sense, still in its infancy, without large volumes of data produced in clinical trials of humans, the results and conclusions from this research must be drawn with caution. It is hoped that more stable compounds will yield more prolonged half lives in the body, and therefore offer a more useful

depiction of BET inhibitor action in humans. Although these compounds appear to be well tolerated in the mouse models of pre-clinical research, it is possible that BET inhibition could produce dose-limiting side-effects when used in human patients. The results of this study have demonstrated that BET inhibitors are able to induce notable changes in platinum-resistant cells, but as of yet these changes are not characterised. It might be possible that these changes could make the patient's healthy cells more susceptible to the cytotoxicity side-effects of platinum agents, again meaning that dose-limiting toxicity might become an impeding factor. This is particularly the case when considering that platinum drugs can produce side-effects such as nephrotoxicity in their current treatment protocols.

Another factor which would require consideration, based on the bioavailability of the BET inhibitors, is the timing of pre-treatments prior to administration of platinum compounds. Research by Johnsson et al. (1995) assessed the pharmacokinetics and tissue distribution of cisplatin in nude mice after injection with 7.5 mg/kg cisplatin. This found that the maximal concentration of cisplatin would vary between different organs, blood, and the tumour itself. This study also recorded different lengths of time before the peak concentration was achieved varied in each of these sites, from 15 minutes in the kidneys and liver, through to a gradual accumulation over 1 week in the brain. While these results may seem obvious, this could be an important factor if you were to consider the therapeutic usage of BET inhibitors as pre-treatments before treating with platinum drugs. The two doses would have to aligned such that the most effective pre-treatment duration is timed to coincide with the peak uptake of platinum at the particular site. The results of my study indicated that a longer pre-treatment time would induce the best response to the platinum agent, but the compound will need to be bioavailable sufficiently to provide an effective pre-treatment prior to degradation and clearance from the tissues.

Finally, there is also the possibility of chemoresistance to BET inhibitors such as JQ1 and I-BET726. Because the exact mechanisms of BET inhibitor action have yet to be fully elucidated, there is a lack of predictive biomarkers which may be indicative of sensitivity or resistance to BET inhibition (Helin & Dhanak, 2013). Nevertheless there have been studies which have observed resistance to BET inhibitors and characterised some of the potential mechanisms (Settleman, 2016). For instance, increased activation of the Wnt signalling pathway was observed in leukaemia cells lines, leading to proliferation and increased survival in the presence of JQ1 (Fong et al., 2015; Rathert

et al., 2015). Another study of triple-negative breast cancer cell lines found that, in paired cell lines which had been selected for resistance to JQ1, proteomic investigation observed that the transcriptional regulator MED1 bound more tightly with BRD4 in resistant cells than in sensitive cells (Shu et al., 2016) and so was not displaced by JQ1. This study implicated hyper-phosphorylation of BRD4 for the increased strength of binding, and this is attributable to decreased activity of a principal BRD4 serine phosphatase, PP2A, and the elevated activity of casein kinase 2 (CK2).

5.3 Suggestions for Future Research

As mentioned previously, the exact mechanism by which JQ1 and I-BET726 were able to induce the increases in sensitivity of the platinum-resistant cell lines are not yet known. Transcriptomics, for instance RNA-Seq or microarray techniques, could be used to ascertain the changes in the transcriptional profiles of these cell lines, to identify genes which are up- or downregulated before and after BET inhibition in these cell lines. These potential 'hits' could subsequently be validated using techniques such as quantitative PCR.

It is also possible that BET inhibition is able to alter the accessibility of DNA to the platinum compounds. This could potentially be investigated by measuring the levels of DNA damage in these cell lines with and without pre-treatment using BET inhibitors. As mentioned in my Introduction (Section 2.3.3), the phosphorylation of the histone H2A variant, H2AX, at Ser-139 to generate γ-H2AX is a response to DNA lesions which creates a focus for DNA damage response signalling. (Kinner et al., 2008; Pabla et al., 2008). Consequently, γ-H2AX is a useful biomarker which is frequently used for the study of DNA damage and repair (Mah et al., 2010). Using western blotting, or immunocytochemisty with fluorescence microscopy, it could be possible to investigate whether BET inhibitor pre-treatment increases the presence of platinum-induced DNA damage foci by screening for the Ser-139 phosphorylation of H2AX (γ-H2AX).

Finally, it may be possible that BET inhibitors are able to alter the sensitivity of cancer cell lines exhibiting resistance to other drug classes. Multidrug resistance is a major obstacle precluding successful treatment across many cancer types (Wu et al. 2014). BET inhibitors may be able to alter the sensitivity of cell lines to compounds with distinct mechanisms of action (such as vincristine, which inhibits mitotic spindle

assembly), thus potentially improving patient responses to multiple compounds within their treatment protocols.

6. Concluding Remarks

In this study we found that BET inhibitors are able to increase the sensitivity of platinum resistant neuroblastoma and ovarian cancer cell lines. The responses to platinum compounds were improved as pre-treatment concentration was increased, and as the pre-treatment duration was prolonged. The resistance levels of these cell lines were decreased to the extent that they approached threshold values used to distinguish resistance of a cell line to a compound.

The mechanisms by which tumours can exhibit resistance to platinum compounds are very diverse, and regulate a number of cellular processes which are crucial for cell survival and proliferation. It is not known exactly which pathways and processes are altered as a result of BET inhibition, and so this would seem to be a useful topic for further research.

These results provide support for the concept that BET inhibition may potentially be a useful strategy for improving treatment efficacy of neuroblastoma and ovarian cancer.

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