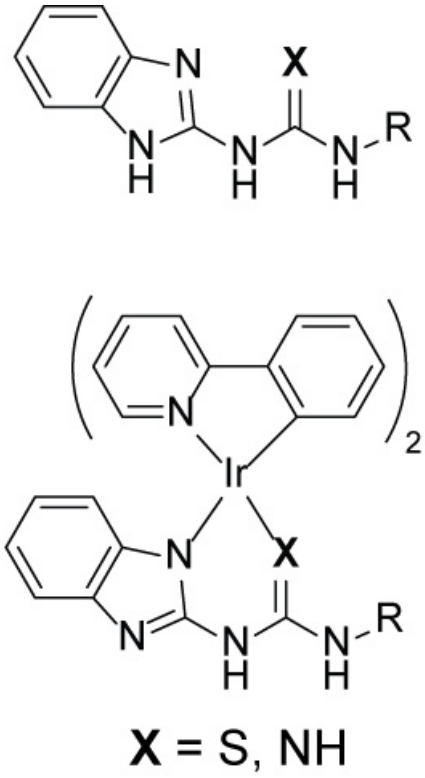
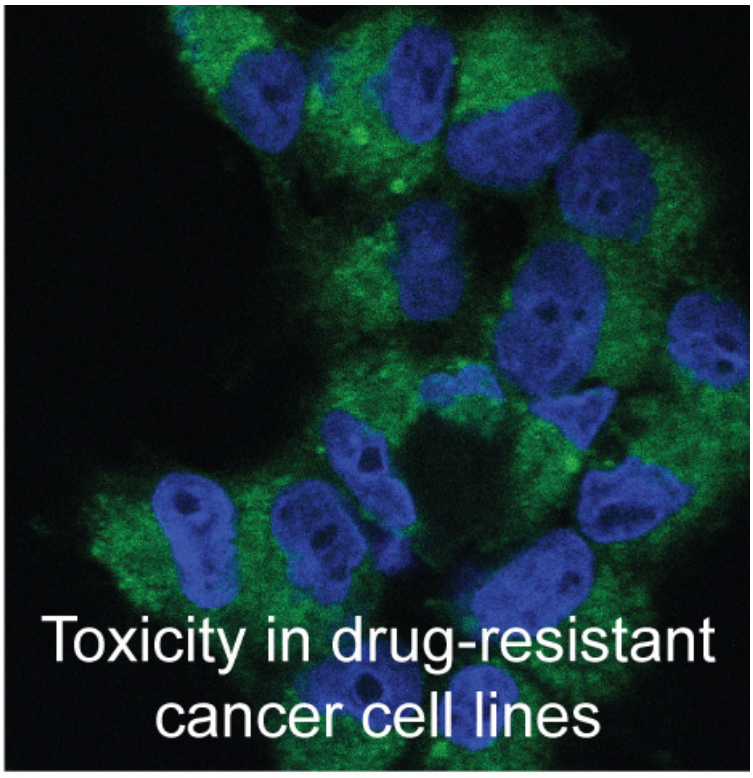
Thiourea and Guanidine Compounds and their Iridium Complexes in Drug-Resistant Cancer Cell Lines: Structure-Activity Relationships and Direct Luminescent Imaging

Samuel J. Thomas,[a] Barbora Balónová,[b] Jindrich Cinatl jr.,[c] Mark N. Wass,[a] Christopher J. Serpell,[d]* Barry A. Blight,[b]* Martin Michaelis[a]*

[a] School of Biosciences, Stacey Building, University of Kent, CT2 7NJ, UK
[b] University of New Brunswick, Department of Chemistry, Fredericton, New Brunswick, E3B 5A3, Canada
[c] Institute of Medical Virology, Goethe University Frankfurt, Paul-Ehrlich-Strasse 40, 60596 Frankfurt am Main, Germany
[d] School of Physical Sciences, Ingram Building, University of Kent, Canterbury, CT2 7NH, UK

Abstract





Thiourea and guanidine units are found in nature, medicine, and materials. Their continued exploration in applications as diverse as cancer therapy, sensors, and electronics means that their toxicity is an important consideration. We have systematically synthesised a set of thiourea compounds and their guanidine analogues, and elucidated structure-activity relationships in terms of cellular toxicity in three ovarian cancer cell lines and their cisplatin-resistant sub-lines. We have been able to use the intrinsic luminescence of iridium complexes to visualise the effect of both structure alteration and cellular resistance mechanisms. These findings provide starting points for the development of new drugs and consideration of safety issues for novel thiourea- and guanidine-based materials.

Introduction

Thiourea and guanidine derivatives are versatile compounds that have previously been synthesised for use in a variety of industries from materials manufacturing to medical research. The two functional

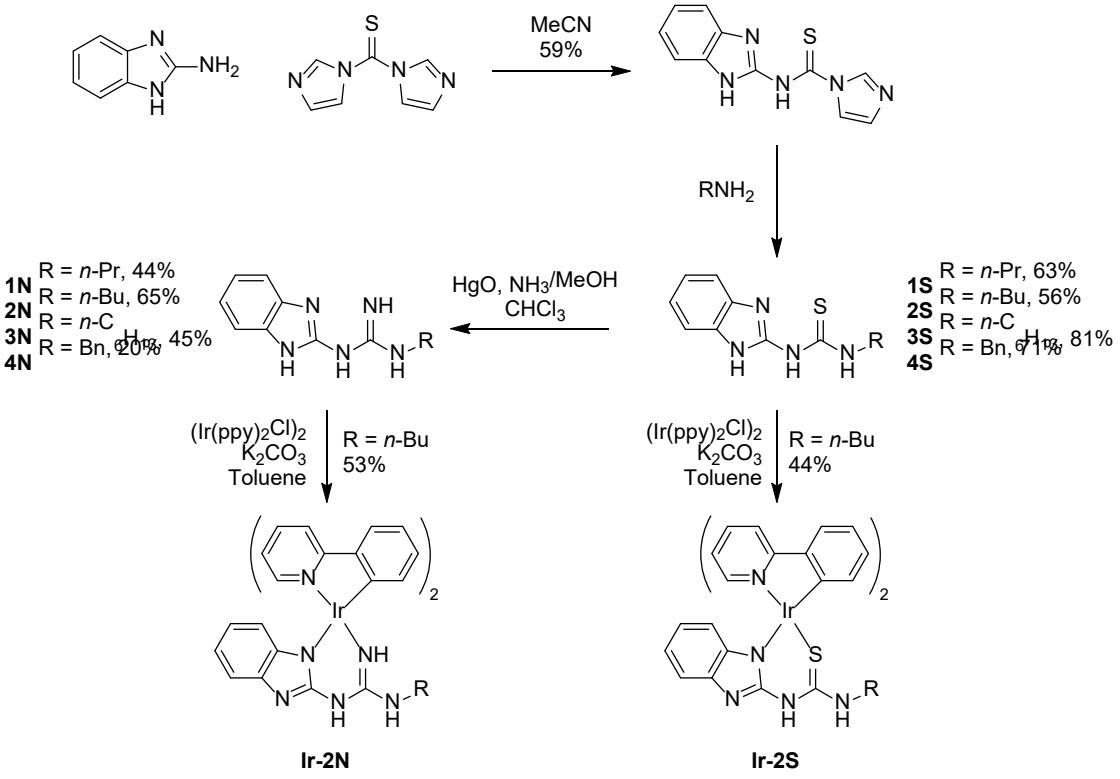
groups represent modifications of urea, with the oxygen being replaced by a sulfur, or by an NH unit, respectively. Compared to urea, thioureas are more acidic and poorer hydrogen bond acceptors. In contrast, guanidines are basic and protonated under most aqueous conditions (pK_a = 13.6 for unsubstituted guanidine). While urea is of low toxicity (oral LD50 in rats of 8471 mg/kg[1]), showing only irritant properties at high concentrations, guanidine is listed as harmful, with an LD50 of 1120 mg/kg for the hydrochloride.[2] Thiourea (LD50 of 1750 mg/kg) is a carcinogen and teratogen and has harmful effects on the thyroid.[3]

Guanidines occur in biology in the form of the nucleobase guanine and the amino acid arginine, and are also found in urine.[4,5] These compounds have also been employed in the catalytic synthesis in green chemistry,[6] as potential antivirals for the treatment of poliovirus,[7] and as adhesive promotors in the materials industry.[8] We are currently investigating guanidine-based molecules as components in advanced organic LED materials.[9] A focal point of guanidine research has been in medicinal chemistry. For example, metformin (*N*,*N*-dimethylbiguanide) is widely used in the treatment of type 2 diabetes.[10] Further research has been conducted on their antimicrobial and anticancer properties.[11–13] In these cases, the compounds studied included chalcones[14] and also platinum centred derivatives.[15] Findings like these support advances in synthesising further derivatives of guanidine compounds to target new areas of cancer therapeutics, and further illustrate the biological importance of these simple and easily manipulatable structures.

Compared to guanidines, thioureas are found more rarely, for example in 2-thiouridine in tRNAs.[16] Within chemistry, interest has centred on their hydrogen bonding capability which has enabled sensing of anions,[17] self-assembly,[18] and organocatalysis,[19] for example. The thiourea functional group has been used widely in medicine, with 2-thiouracil itself used to treat thyroid disorders for some time.[20] Thioureas are investigated as antiviral agents,[21] including non-nucleoside HIV-1 reverse transcriptase inhibitors,[22] insecticidal growth regulators,[23] anti-inflammatory,[23] and anticancer drugs.[21] Thioureas show promise as anticancer drug candidates due to their sulfur atoms,[24] with sulfur itself being a versatile and biologically important element to all living organisms.[25] Thiourea derivatives have been synthesised with an assortment of partnering organic structures and demonstrated to exert anti-cancer effects in human cancer cell lines from different entities including breast and lung cancer as well as leukaemia[26–28] [29]

Given the broad application of guanidines and thioureas in upcoming drugs and materials, their toxicity is of interest both from the standpoint of potentially beneficial or detrimental effects. In this light, we decided to perform a systematic screen of a series of thiourea and guanidine compounds for toxicity in ovarian cancer cell lines, including those which display resistance to cisplatin, the mainstay of ovarian cancer therapies and one of the most commonly used anticancer drugs.[30,31] These studies identify trends which could be used as first pointers for initial toxicity predictions of similar compounds. We have also been able to make use of the intrinsic luminescence of iridium complexes to observe extent and distribution of uptake, and its relationship to guanidine or thiourea function.

Results and Discussion



Scheme 1. Synthesis of guanidine and thiourea compounds and their iridium complexes.

A series of guanidine and thiourea compounds was synthesised based upon a 2-aminobenzimidazole unit; this was selected since it is a 'drug-like' unit which also has capacity to form the type of metal complexes which could be used in OLED materials. Monosubstitution of 1,1'-thiocarbonyldiimidazole was achieved by reaction with substoichiometric 2-aminobenzimidazole in acetonitrile. The second imidzoyl unit was displaced by a selection of amines (n-propylamine, n-butylamine,[32] n-hexylamine, and benzylamine) in the presence of dimethylaminopyridine (DMAP) in dimethylformamide (DMF) to give compounds **1S** to **4S** in 63 - 81 % yield after purification.[22] Portions of these compounds were converted to the corresponding guanidines by reaction with mercury(II) oxide and methanolic ammonia in chloroform to give **1N** to **4N**[33,34] in 40 – 65% yield.[35] Two iridium complexes[9,36] based on these systems were prepared by reacting **2S** and **2N** separately with $[Ir(ppy)_2Cl]_2$ in toluene in the presence of potassium carbonate, giving bright yellow powders **Ir-2S** and **Ir-2N** in 53 % and 44 % yield respectively. All compounds were characterised by ^1H and ^{13}C NMR, EI-MS, and elemental analysis.

To examine the biological activity of the compounds synthesised, they were tested for effects on the viability of the human ovarian cancer cell lines EFO-21, EFO-27 and COLO-704 and their cisplatin-resistant sublines EFO-21^{r}CDDP^{2000}, EFO-27^{r}CDDP^{2000} (both adapted to cisplatin 2µg/mL), and COLO-704^{r}CDDP^{1000} (adapted to cisplatin 1µg/mL) using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphennyltetrazolium bromide) (MTT) assay modified after Mosmann[37] as previously described.[38] Cisplatin is one of the most commonly used anticancer drugs, and resistance formation to cisplatin represents a major obstacle to the development of improved anticancer therapies.[30,39,40]. The assay principle is based on metabolization of the yellow MTT reagent into a purple insoluble formazan compound within the mitochondria of viable cells.[37] This colour change from yellow to purple enables for the collection of rapid and coherent cell proliferation data.[41] Concentrations that reduce cell

viability by 50% relative to an untreated control (IC50) were calculated by treating the cell lines with sequential dilutions of the compounds.

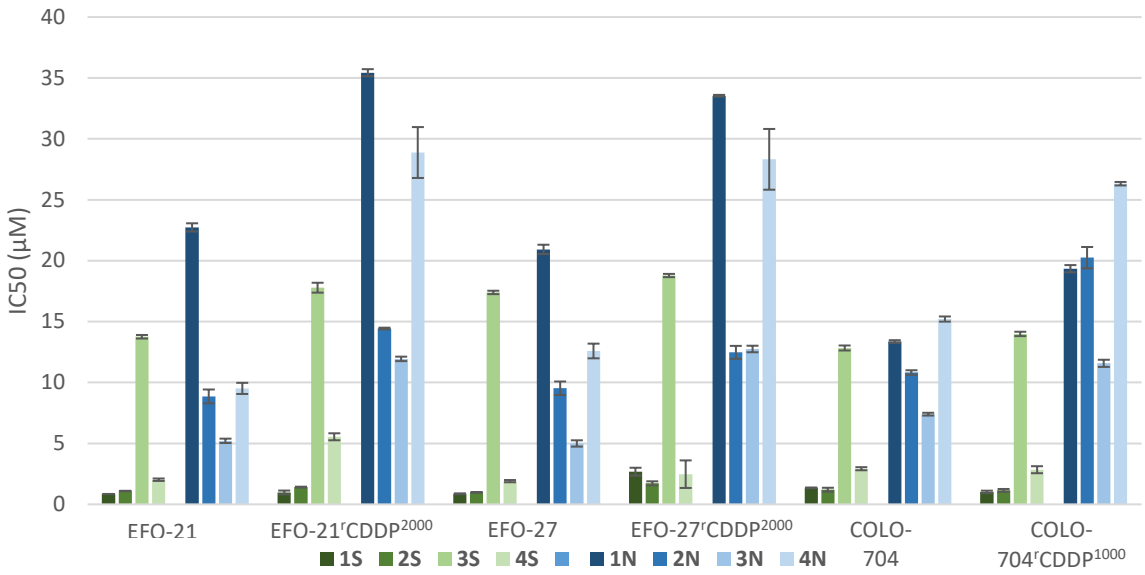


Figure 1. IC50 values for compounds **1S-4S** and **1N-4N** against six cancer cell lines. Error bars represent one standard deviation, over three repeats.

All of the unmetallated compounds examined showed toxicity in all cell lines, with IC50 values in the low micromolar range (Fig. 1). While thioureas **1S**, **2S**, and **4S** (IC50s across all cell lines averaging 1.29, 1.26, and 2.96 μM, respectively) were notably more toxic than their guanidine counterparts **1N**, **2N**, and **4N** (18.7, 10.1, 6.8 μM respectively), the hexyl-appended **3S** (15.8 μM) was less effective at inhibiting cell proliferation than **3N** (9.0 μM). We propose that these differences relate to the impact that the longer chain makes on the hydrophobicity balance of the molecule. The guanidine compounds are expected to be protonated under these conditions, and addition of a long chain will produce a cationic surfactant-type molecule which could disrupt biological membranes.[42] With other side chains, the charged guanidiniums may not be sufficiently lipophilic to enter the biological membranes when compared to the neutral thioureas. This is supported by ClogP value calculation (Table S1, supplementary information) – only **3N** (ClogP = 2.75) is as lipophilic as any of the S series (lowest ClogP is for **1S**, at 2.58). In the case of **3S**, the lipophilicity may be so high that it prevents the compound leaving the membrane and entering the cells. Despite the variation in activity of the individual compounds, the average sensitivity of the cell lines was remarkably consistent, with the parental EFO-21, EFO-27, and COLO-704 lines have average IC50s of 8.0, 8.7, and 8.1 μM, and their cisplatin-resistant sublines having average IC50s of 14.5, 14.1, and 12.1 μM, respectively. Hence overall, the cisplatin resistant sublines were also more resistant to our compounds, however this effect was larger with **1N-4N** than with **1S-4S** (average ratio of resistant/parental IC50s = 1.81 versus 1.23).

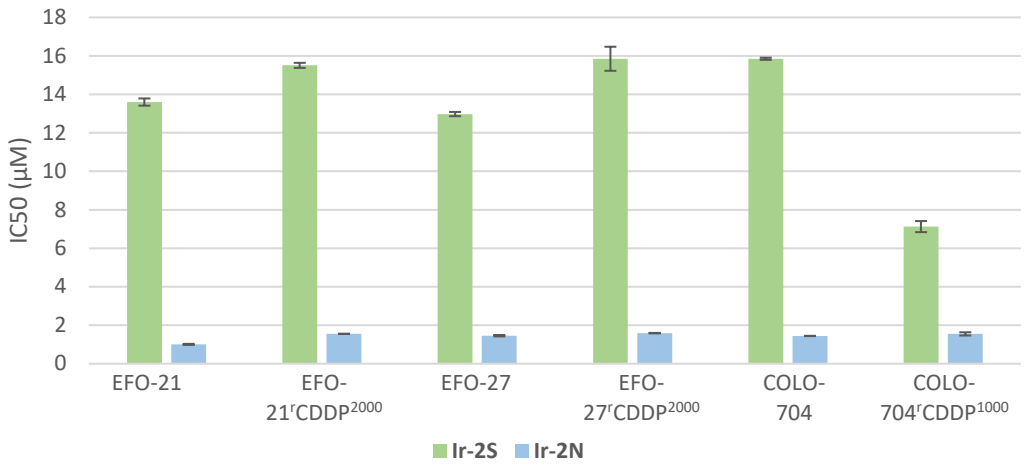


Figure 2. IC50 values for compounds **Ir-2S a**nd **Ir-2N** against six cancer cell lines. Error bars represent one standard deviation over three repeats.

The same studies were then conducted with the iridium complexes **Ir-2S** and **Ir-2N** (Fig. 2). Here, the guanidine compound (average IC50 = 1.4 μM) was approximately ten times more toxic across the board than **Ir-2S** (average IC50 = 13.5 μM). This is a reverse of what was seen for the unmetallated compounds **2S** and **2N**. For both EFO-21 and EFO-27 lines, the cisplatin-resistant sublines were slightly more tolerant of all compounds (IC50s on average 25% higher), but when COLO-704^{r}CDDP^{1000} cells were treated with **Ir-2S** the IC50 was reduced to 45% of the value of the parental cell line, suggesting that a different mechanism of resistance is in place here. In this case, both complexes are uncharged at pH 7, and since the chemical difference is on the interior of the molecule, they could be expected to have similar cell penetration properties, with the difference in toxicity being due to processing within the cell.

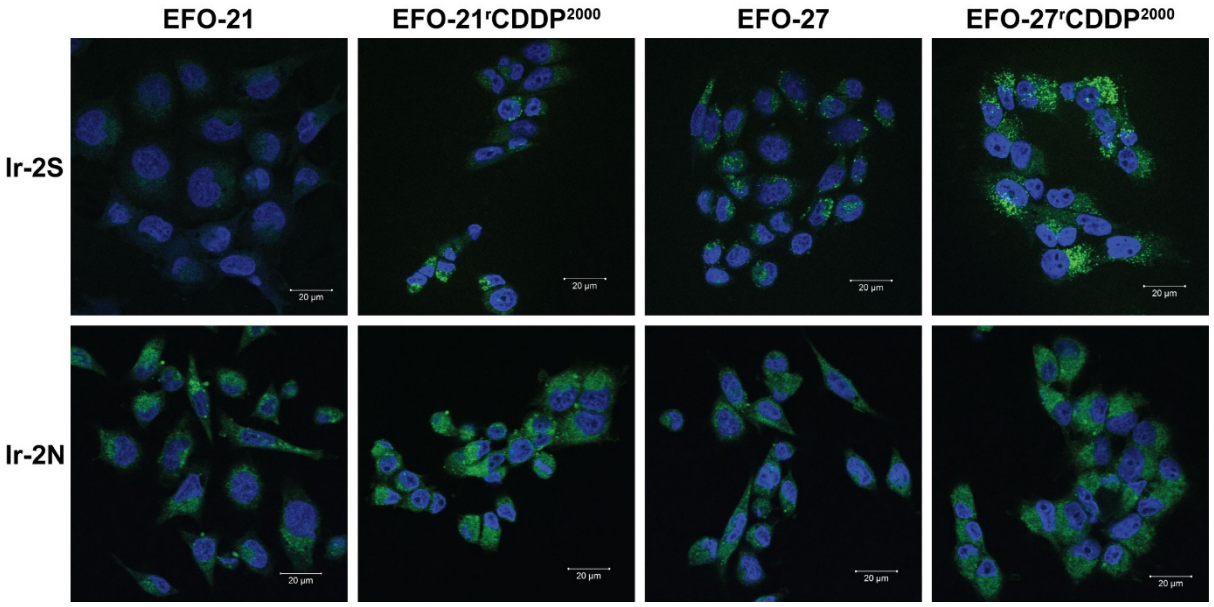


Figure 3. Confocal images of cells treated with **Ir-2S** and **Ir-2N**. Emission from metal complexes are green, and nuclei are shown in blue (stained with DAPI). All cells were treated with compounds at their IC50 concentrations. The brightness, gain and excitation of the confocal microscopes laser for compound expression was kept consistent throughout the assays, although slight adjustments were made to the brightness of the DAPI stain to achieve a clear nuclei mapping image.

Taking advantage of the intrinsic luminescence of the iridium complexes, we were able to study whether the difference in toxicity related to cell uptake or internal processing using confocal microscopy (Fig. 3). Using EFO-21, EFO-21^{r}CDDP^{2000}, EFO-27, and EFO-27^{r}CDDP^{2000} cells, which were amenable to this technique due to good adhesion properties, cell lines were dosed with **Ir-2S** and **Ir-2N** at their IC50 concentrations. **Ir-2S** and **Ir-2N** have similar excitation and emission spectra in terms of peak position and shape, but the quantum yield of **Ir-2N** is roughly ten times that of **Ir-2S** (Fig. S1, supplementary information), since the IC50s of **Ir-2S** are approximately ten times higher than those of **Ir-2N** (Table S2, supplementary information), the total brightness for full uptake of either molecule should be similar, enabling comparison of uptake efficiency. Cell nuclei were stained with DAPI. In all cases, the compounds were observed to be primarily located in the cytoplasm, with little change in cell morphology compared to untreated cells. In EFO-21 and EFO-21^{r}CDDP^{2000} cells, there was clearly greater uptake of the more toxic **Ir-2N** compared to **Ir-2S**. This is impressive given the tenfold difference in concentrations. In EFO-27 cells, the **Ir-2N** uptake was only slightly higher than that of **Ir-2S**, while in EFO-27^{r}CDDP^{2000} cells, the total level of uptake was approximately equal. However, in the latter case, distribution of **Ir-2S** was notably punctate compared to a more even distribution of **Ir-2N**. Whilst further analysis would be required to depict what may cause these discrepancies, this indicates cell line-specific differences in cellular uptake and intracellular distribution, which may cause the differences observed in toxicity.

Conclusions

All compounds tested showed notable toxicity. On the one hand, this shows that use of similar thiourea and guanidine structures in materials applications should be accompanied by caution about release. On the other hand, these structures could be useful scaffolds for cancer chemotherapeutics; in particular for drug resistance – the series **1S-4S** showed minimal reduction in efficacy in drug resistant cell lines, and **Ir-2S** was in fact more effective in one resistant subline. Notably, cisplatin is a mainstay of ovarian cancer therapy. Although initial response is common, resistance formation and therapy failure are typically inevitable[39,40,43]. Hence, cisplatin-resistant ovarian cancer is an unmet clinical need and novel therapies are urgently needed.

The mechanism of action of these compounds is unknown, but we have shown that uptake of the metal complexes at least is efficient. In summary, the investigated thiourea and guanidine derivatives uncovered promising low dose responses in the early research stages of human *in vitro* cell assays, with the data collected providing a start to new potential therapeutic pathways in the battle against drug-resistant ovarian cancer.

Acknowledgements

SJT thanks SACO AEI Polymers UK for funding. JC thanks Frankfurter Stiftung für krebskranke Kinder, and Hilfe für krebskranke Kinder Frankfurt e.V for funding.

References

1 National Library of Medicine, ChemIDplus, https://chem.nlm.nih.gov/chemidplus/rn/startswith/57-13-6, (accessed 26 April 2019).

2 T. Güthner, B. Mertschenk and B. Schulz, in *Ullmann's Encyclopedia of Industrial Chemistry*, Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, Germany, 2006.

3 B. Mertschenk, A. Knott and W. Bauer, in *Ullmann's Encyclopedia of Industrial Chemistry*, Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, Germany, 2013, pp. 1–15.

4 C. J. Weber, The Determination of the Guanidine Bases in Urine, *J Biol Chem*, 1928, **78**, 465–

473.

5 J. F. Van Pilsum and E. A. Wolin, Guanidinium compounds in blood and urine of patients suffering from muscle disorders, *J. Lab. Clin. Med.*, 1958, **51**, 219–223.

6 T. Ishikawa and T. Isobe, Modified Guanidines as Chiral Auxiliaries, *Chem. - A Eur. J.*, 2002, **8**, 552–557.

7 W. A. Rightsel, J. R. Dice, R. J. McAlpine, E. A. Timm, I. W. McLean, G. J. Dixon and F. M. Schabel, Antiviral effect of guanidine., *Science*, 1961, **134**, 558–9.

8 W. Huang and X. Sun, Adhesive properties of soy proteins modified by urea and guanidine hydrochloride, *J. Am. Oil Chem. Soc.*, 2000, **77**, 101–104.

9 B. Balónová, D. R. Martir, E. R. Clark, H. J. Shepherd, E. Zysman-Colman and B. A. Blight, Influencing the Optoelectronic Properties of a Heteroleptic Iridium Complex by Second-Sphere H-Bonding Interactions, *Inorg. Chem.*, 2018, **57**, 8581–8587.

10 C. J. Dunn and D. H. Peters, Metformin, *Drugs*, 1995, **49**, 721–749.

11 M. Said, A. Badshah, N. Shah, H. Khan, G. Murtaza, B. Vabre, D. Zargarian and M. Khan, Antitumor, Antioxidant and Antimicrobial Studies of Substituted Pyridylguanidines, *Molecules*, 2013, **18**, 10378–10396.

12 F. Sączewski and Ł. Balewski, Biological activities of guanidine compounds, 2008 – 2012 update, *Expert Opin. Ther. Pat.*, 2013, **23**, 965–995.

13 R. G. S. Berlinck, A. E. Trindade-Silva and M. F. C. Santos, The chemistry and biology of organic guanidine derivatives, *Nat. Prod. Rep.*, 2012, **29**, 1382.

14 Y. Qian, H.-J. Zhang, P.-C. Lv and H.-L. Zhu, Synthesis, molecular modeling and biological evaluation of guanidine derivatives as novel antitubulin agents, *Bioorg. Med. Chem.*, 2010, **18**, 8218–8225.

15 A. A. Legin, M. A. Jakupec, N. A. Bokach, M. R. Tyan, V. Y. Kukushkin and B. K. Keppler, Guanidine platinum(II) complexes: synthesis, in vitro antitumor activity, and DNA interactions, *J. Inorg. Biochem.*, 2014, **133**, 33–39.

16 R. K. Kumar and D. R. Davis, *Synthesis and studies on the effect of 2-thiouridine and 4-thiouridine on sugar conformation and RNA duplex stability*, Oxford University Press, 1997, vol. 25.

17 C. J. Serpell and P. D. Beer, in *Comprehensive Supramolecular Chemistry II*, 2017.

18 L. J. White, S. N. Tyuleva, B. Wilson, H. J. Shepherd, K. K. L. Ng, S. J. Holder, E. R. Clark and J. R. Hiscock, Towards the Prediction of Global Solution State Properties for Hydrogen Bonded, Self-Associating Amphiphiles, *Chem. – A Eur. J.*, 2018, **24**, 7761–7773.

19 Z. Zhang and P. R. Schreiner, (Thio)urea organocatalysis—What can be learnt from anion recognition?, *Chem. Soc. Rev.*, 2009, **38**, 1187.

20 A. Nagasaka and H. Hidaka, Effect of antithyroid agents 6-propyl-2-thiouracil and l-methyl-2-mercaptoimidazole on human thyroid iodide peroxidase, *J. Clin. Endocrinol. Metab.*, 1976, **43**, 152–158.

21 W. Liu, J. Zhou, T. Zhang, H. Zhu, H. Qian, H. Zhang, W. Huang and R. Gust, Design and synthesis of thiourea derivatives containing a benzo[5,6]cyclohepta[1,2-b]pyridine moiety as potential antitumor and anti-inflammatory agents, *Bioorg. Med. Chem. Lett.*, 2012, **22**, 2701–

2704.

22 R. Vig, C. Mao, T. . Venkatachalam, L. Tuel-Ahlgren, E. A. Sudbeck and F. M. Uckun, Rational design and synthesis of phenethyl-5-bromopyridyl thiourea derivatives as potent non-nucleoside inhibitors of HIV reverse transcriptase, *Bioorg. Med. Chem.*, 1998, **6**, 1789–1797.

23 A. Shakeel, Thiourea Derivatives in Drug Design and Medicinal Chemistry: A Short Review, *J. Drug Des. Med. Chem.*, 2016, **2**, 10.

24 I. L. Goncalves, G. O. de Azambuja, D. F. Kawano and V. L. Eifler-Lima, Thioureas as Building Blocks for the Generation of Heterocycles and Compounds with Pharmacological Activity: An Overview, *Mini. Rev. Org. Chem.*, 2017, **15**, 28–35.

25 Biology and brimstone, *Nat. Chem. Biol.*, 2006, **2**, 169–169.

26 R. Tokala, S. Bale, I. P. Janrao, A. Vennela, N. P. Kumar, K. R. Senwar, C. Godugu and N. Shankaraiah, Synthesis of 1,2,4-triazole-linked urea/thiourea conjugates as cytotoxic and apoptosis inducing agents, *Bioorg. Med. Chem. Lett.*, 2018, **28**, 1919–1924.

27 P. Liao, S.-Q. Hu, H. Zhang, L.-B. Xu, J.-Z. Liu, B. He, S.-G. Liao and Y.-J. Li, Study on Anti-Proliferative Activity in Cancer Cells and Preliminary Structure-Activity Relationship of Pseudo-Peptide Chiral Thioureas, *Bull. Korean Chem. Soc.*, 2018, **39**, 300–304.

28 H. Hu, C. Lin, M. Ao, Y. Ji, B. Tang, X. Zhou, M. Fang, J. Zeng and Z. Wu, Synthesis and biological evaluation of 1-(2-(adamantane-1-yl)-1H-indol-5-yl)-3-substituted urea/thiourea derivatives as anticancer agents, *RSC Adv.*, 2017, **7**, 51640–51651.

29 R. Pingaew, N. Sinthupoom, P. Mandi, V. Prachayasittikul, R. Cherdtrakulkiat, S. Prachayasittikul, S. Ruchirawat and V. Prachayasittikul, Synthesis, biological evaluation and in silico study of bis-thiourea derivatives as anticancer, antimalarial and antimicrobial agents, *Med. Chem. Res.*, 2017, **26**, 3136–3148.

30 S. Ghosh, Cisplatin: The first metal based anticancer drug, *Bioorg. Chem.*, 2019, **88**, 102925.

31 S. Dasari and P. Bernard Tchounwou, Cisplatin in cancer therapy: Molecular mechanisms of action, *Eur. J. Pharmacol.*, 2014, **740**, 364–378.

32 I. Rodríguez-Franco, I. Dorronsoro, A. Martínez and A. Castro, Studies on the reactivity of some *N* -aryl- and *N* -heteroaryl- *N''</i> -alkylthioureas towards electrophilic reagents. Synthesis of new* N *-pyridylthioureas and thiazolines maría*, *J. Heterocycl. Chem.*, 2001, **38**, 435–441.

33 N. Sato, S. Matsumoto, A. Tsunoda and N. Ishida, In vitro activity of some 2-N3-guanidinobenzimidazole derivatives against poliovirus, *J Antibiot*, 1966, **19**, 172–174.

34 T. Mukherjee, C. Ganzmann, N. Bhuvanesh and J. A. Gladysz, Syntheses of Enantiopure Bifunctional 2-Guanidinobenzimidazole Cyclopentadienyl Ruthenium Complexes: Highly Enantioselective Organometallic Hydrogen Bond Donor Catalysts for Carbon–Carbon Bond Forming Reactions, *Organometallics*, 2014, **33**, 6723–6737.

35 B. A. Blight, C. A. Hunter, D. A. Leigh, H. McNab and P. I. T. Thomson, An AAAA–DDDD quadruple hydrogen-bond array, *Nat. Chem.*, 2011, **3**, 244–248.

36 B. Balónová, H. J. Shepherd, C. J. Serpell and B. A. Blight, Ir^{III} as a Strategy for Preorganization in H-Bonded Motifs, *ChemRxiv*, 2019, 10.26434/chemrxiv.8312450.v1.

37 T. Mosmann, Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays, *J. Immunol. Methods*, 1983, **65**, 55–63.

38 M. Michaelis, J. Matousek, J. U. Vogel, T. Slavik, K. Langer, J. Cinatl, J. Kreuter, D. Schwabe and J. Cinatl, Bovine seminal ribonuclease attached to nanoparticles made of polylactic acid kills leukemia and lymphoma cell lines in vitro., *Anticancer. Drugs*, 2000, **11**, 369–76.

39 B. van Zyl, D. Tang and N. A. Bowden, Biomarkers of platinum resistance in ovarian cancer: what can we use to improve treatment, *Endocr. Relat. Cancer*, 2018, **25**, R303–R318.

40 G. Damia and M. Broggini, Platinum Resistance in Ovarian Cancer: Role of DNA Repair, *Cancers (Basel).*, 2019, **11**, 119.

41 D. Gerlier and N. Thomasset, Use of MTT colorimetric assay to measure cell activation, *J. Immunol. Methods*, 1986, **94**, 57–63.

42 S. Zhang, S. Ding, J. Yu, X. Chen, Q. Lei and W. Fang, Antibacterial Activity, *in Vitro* Cytotoxicity, and Cell Cycle Arrest of Gemini Quaternary Ammonium Surfactants, *Langmuir*, 2015, **31**, 12161–12169.

43 U. A. Matulonis, A. K. Sood, L. Fallowfield, B. E. Howitt, J. Sehouli and B. Y. Karlan, Ovarian cancer, *Nat. Rev. Dis. Prim.*, 2016, **2**, 16061.