DNA Intercalators in Cancer Therapy: Organic and Inorganic Drugs and Their Spectroscopic Tools of Analysis

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Abstract: Since the discovery of the DNA intercalation process by Lerman in 1961 thousands of organic, inorganic octahedral (particularly ruthenium(II) and rhodium(III)) and square-planar (particularly platinum(II)) compounds have been developed as potential anticancer agents and diagnostic agents. The design and synthesis of new drugs is focused on bis-intercalators which have two intercalating groups linked *via* a variety of ligands, and synergistic drugs, which combine the anticancer properties of intercalation with other functionalities, such as covalent binding or boron-cages (for radiation therapy). Advances in spectroscopic techniques mean that the process of DNA intercalation can be examined in far greater detail than ever before, yielding important information on structure-activity relationships. In this review we examine the history and development of DNA intercalators as anticancer agents and advances in the analysis of DNA-drug interactions.

Key Words: DNA intercalation, bis-intercalation, synergistic, anticancer, cytotoxicity, spectrometry, review.

INTRODUCTION

Cancer accounted for 7.1 million deaths world-wide in 2002, and ranks as three of the ten leading causes of death among people over the age of 60 [1]. Seventeen per cent of all cancer deaths relate to respiratory cancer, accounting for 928,000 deaths in 2002, followed by stomach cancer (605,000) and colorectal cancer (477,000) (Table **1**). Around 11 million people are diagnosed with cancer each year, and by 2020 the World Health Organization estimates that this number will grow to 16 million [1].

The causes of cancer remain unclear, but many things are thought to stimulate cancerous behaviour, including changes to cellular DNA from: radiation (e.g. sunlight and radio frequency devices), chemical carcinogens (e.g. polycyclic aromatic hydrocarbons) or mutagens and viruses (e.g. Human Papilloma viruses, the cause of cervical cancer). As such, many of the drugs used in chemotherapy specifically target DNA (Fig. **1**). Currently, there are around 130 drugs[1] approved by the Food and Drug Administration (FDA, USA) for use in oncology, including platinum based drugs (cisplatin, oxaliplatin and carboplatin), polyaromatics (daunorubicin and doxorubicin) and mustards (meclorethamine and uracil mustard). These drugs all target DNA, but do so *via* different mechanisms. The platinum-based drugs form coordinate covalent bonds with the purine bases of DNA [2], the mustards alkylate DNA bases and the polyaromatics intercalate into the DNA double helix [3].

Intercalation describes the reversible insertion of a guest molecule into a lamellar host structure. It is almost 50 years since Lerman proposed an hypothesis on intercalation to explain the strong affinity and binding of planar aromatic chromophores to DNA [4, 5], and there is now a large body of evidence which validates, refines and extends this concept [5-7].

In specific terms, intercalation is the insertion of a planar aromatic ring system in between consecutive base-pairs of DNA. Partial intercalation can also occur where the introduction of bulky substituents on flat aromatic molecules prevents their full insertion between the base-pairs [8, 9]. DNA-drug intercalation is stabilised by significant π-electron overlap, hydrophobic and polar interactions, as well as the electrostatic forces of cationic intercalators with polyanionic nucleic acid [10, 11]. The insertion of an intercalator between adjacent base-pairs results in a substantial change in DNA structure, causing lengthening, stiffening and unwinding of the DNA helix [12]. As a result of intercalation, DNA shows a loss of regular helical structure in its backbone; as indicated by fibre diffraction patterns which exhibit a loss of resolution in their inner layer lines to give a spacing greater than 10.2 Å. The sugar-phosphate torsional angles change in order to accommodate the aromatic compound, causing separation of the base-pairs with a lengthening of the double helix and a decrease in the helix diameter (i.e. unwinding) at the intercalation site [13].

In the classical model of intercalation the helix is lengthened by the theoretical length of 3.4 Å per intercalated drug [14]. In contrast, electric dichroism measurements suggest a range of possible helix extensions, from 2.0 to 3.7 Å, depending on the intercalating compound [15]. The usual rotation of approximately 36º of one base-pair with respect to the next is decreased to 26º as a result of intercalation [16-19].

Within the human body, DNA can be found in many conformations, such as C-, D- and T-DNA, although the three

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Table 1. Estimation of the Number of World-Wide Deaths Due to Cancer by Cause and Sex in 2002 (Adapted from World Health Organization Report 2003)

Cancer Type	Both Sexes		Men		Women	
	No. of deaths	% Total	No. of deaths	% total	No. of deaths	% total
Trachea/bronchus/lung	1,239,000	2.2	886,000	3.0	353,000	1.3
Stomach	849,000	1.5	523,000	1.7	326,000	1.2
Colorectal	620,000	1.1	321,000	1.1	299,000	1.1
Liver	619,000	1.1	428,000	1.4	191,000	0.7
Breast	477,000	0.3	3,000	0.0	474,000	1.8
Oesophagus	446,000	0.8	284,000	0.9	161,000	0.6
Lymphomas, multiple myeloma	334,000	0.6	168,000	0.6	165,000	0.6
Mouth and oropharynx	317,000	0.6	220,000	0.7	97,000	0.4
Prostate	268,000	0.5	268,000	0.9	…	…
Leukaemia	264,000	0.5	146,000	0.5	117,000	0.4
Cervix uteri	239,000	0.4	…	…	239,000	0.9
Pancreas	229,000	0.4	121,000	0.4	109,000	0.4
Bladder	178,000	0.3	125,000	0.4	53,000	0.2
Other neoplasms	148,000	0.3	74,000	0.2	74,000	0.3
Ovary	134,000	0.2	…	…	134,000	0.5
Corpus uteri	71,000	0.1	…	…	71,000	0.3
Total	7,106,000	12.5[a]	3,963,000	13.2	3,144,000	11.6

[a] Percentage of total word-wide deaths with cancer as the primary cause.

most prevalent forms are A-, B- and Z-DNA [20]. The different conformations of DNA are largely due to the sugar groups which dictate whether the helix is right- or left-handed, as the phosphate groups and nucleosides themselves have no chirality at all. Changes in the sugar pucker from a C3′-*endo* to C2′-*endo* base-pair tilt varies the distance between consecutive base-pairs and the degree of rotation of the helix per residue. The changes in sugar pucker can rise for various reasons, including: a change in salt or drug concentration, relative humidity and base-composition. Intercalation-induced structural changes to DNA have also been examined by near-infrared Raman spectroscopy, which clearly indicate a structural transition from the B-form to an A-like form with increasing intercalator concentration [21]. Combined together, these changes in DNA secondary structure by intercalators is believed to prevent replication of DNA and thereby inhibit further growth of cancers or cause cell death. Intercalation has also been reported as a preliminary step in mutagenesis [22].

The presence of an intercalator between two base-pairs excludes the nearest neighbouring sites from being occupied by another drug and this phenomena is commonly referred to as the Nearest Neighbour Exclusion Principle [23]. This results in periodic intervals of 10.2 Å between two drugs, which corresponds to 3 × 3.4 Å intercalation sites [13, 23, 24]. Support for this principle has been obtained from x-ray diffraction studies which have demonstrated an upper binding limit of one drug molecule for every four nucleotides [25, 26].

In this review, we examine the development and use of simple DNA intercalators as anticancer agents and diagnostic probes, through to current research into bis-intercalators and synergistic intercalators. We also examine the spectroscopic techniques that are used commonly to monitor DNA-drug interactions, focusing in particular on new techniques that provide more extensive and informative data on DNA-drug binding.

ORGANIC INTERCALATORS

Lerman was the first to demonstrate the process of DNA intercalation through his studies into the binding of acridines to DNA [5], and his later examination of the x-ray scattering of Proflavine (**7**) and DNA [4]. Over the last 45 years, the number of reported organic intercalators has grown into the thousands, but, in general, all of them have chemical structures based on six different intercalator frame works: anthracenes, acridines, anthraquinones, phenanthridines, phenanthrolines and ellipticines (Fig. **2**).

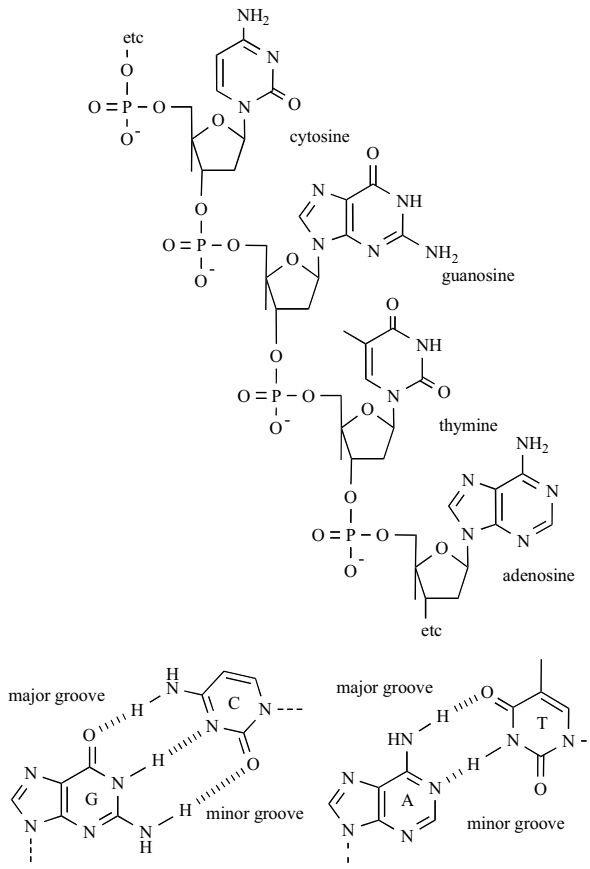

Fig. (1). The chemical structure of DNA (top) showing the sugar-phosphate backbone and the purine bases guanosine (G) and adenosine (A) and the pyrimidine bases thymine (T) and cytosine (C); and (bottom) the Watson-Crick base pairing between guanosine and cytosine bases, and adenosine and thymine bases.

Currently there are four organic intercalators approved by the FDA for the treatment of human cancers (Fig. **3**). Daunorubicin (**9**, sold under the brand names of Cerubidine® and Daunoxone® and the generic name Daunorubicin hydrochloride) is a natural product produced by the bacterium *Streptomyces coeruleorubidus* and is used in the treatment of non-lymphocytic leukaemia (myelogenous, monocytic, erythroid) in adults and acute lymphocytic leukaemia in children and adults [27-29]. The drug is administered as an intravenous injection in 25 to 60 mg/m^2 doses, depending on the age of the patient, over three consecutive days for the first treatment and over two consecutive days for subsequent courses. A liposomal preparation of the drug is also available, which is used as a first line treatment for advanced Human Immunodeficiency Virus (HIV) related Kaposi's sarcoma [30].

Doxorubicin (**10**, sold under the brand name Adriamycin PFS®, Adriamycin RDF®, Rubex® and the generic name Doxorubicin hydrochloride) is a natural product obtained from the bacterium *Streptomyces peucetius* var. *caesius* and is used in the treatment of a large number of human cancers. These include: acute lymphoblastic leukaemia, acute myeloblastic leukaemia, neuroblastoma, soft tissue and bone sarcoma, breast carcinoma, ovarian carcinoma, transitional

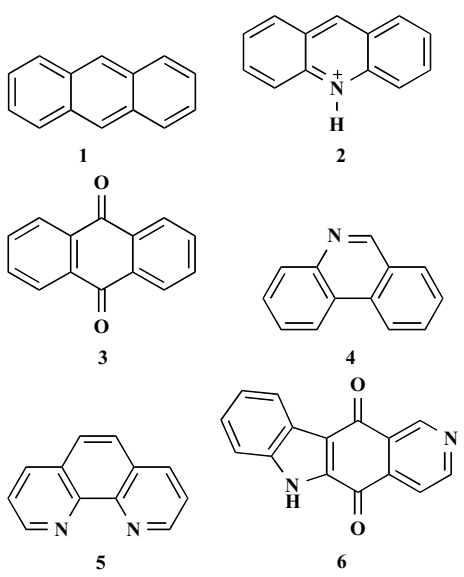

Fig. (2). Some examples of organic intercalators showing the basic chemical structures of (**1**) anthracenes, (**2**) acridines, (**3**) anthraquinones, (**4**) phenanthridines, (**5**) phenanthrolines and (**6**) ellipticines.

cell bladder carcinoma, thyroid carcinoma, gastric carcinoma, malignant lymphoma, broncogenic carcinoma and Hodgkin's disease [29]. The fact that daunorubicin has such a limited range of effectiveness in the treatment of cancers compared to doxorubicin is surprising, given the intercalators only differ in structure by one functional group (H → OH). Doxorubicin is administered as a single 60 to 75 mg/m^2 dose every 21 days. A liposomal version of doxorubicin (Doxil®) is also available and is used to treat ovarian cancer, HIV-related Kaposi's sarcoma and in the secondary treatment of patients intolerant of other therapies [31]. Doxil is given as a single 20 to 50 mg/m^2 dose every four weeks.

Mitoxantrone (**11**, sold under the brand name Novanthrone® and its generic name) is primarily used to treat Multiple Sclerosis (MS) but is also used in conjunction with other anticancer drugs for the initial treatment of acute non-lymphocytic leukaemia in adults [32-34]. Amsacrine (**12**) has previously been used to treat acute adult leukaemia and malignant lymphomas, but has poor activity in the treatment of solid tumours, and is classified by the FDA as an *orphan drug* (a drug where the cost of production, registration and marketing outweighs the likely revenue from sales and is therefore subject to special privileges from the US federal government).

These organic drugs all bind by intercalation from the DNA minor groove, where binding is stabilised by electrostatic interactions between positive charges on the drug and the negatively charged phosphate backbone [35], although some, like mitoxantrone have also been shown to bind DNA by non-intercalating, electrostatic interactions [36-40]. Importantly, organic intercalators are also known to act by inhibiting topoisomerase (a nuclear enzyme that regulates DNA topology through single- (topoisomerase I) or double-strand (topoisomerase II) breaks) [41-44].

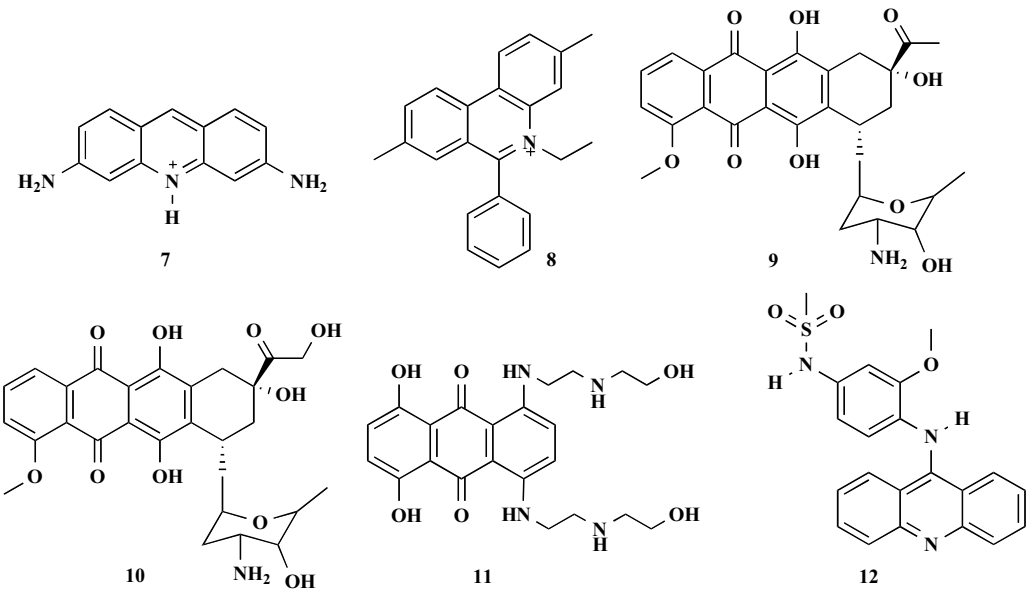

Fig. (3). Some examples of organic intercalators showing the first organic intercalator (**7**) Proflavine, the most commonly used intercalator (**8**) ethidium, and the clinical agents (**9**) daunorubicin, (**10**) doxorubicin, (**11**) mitoxantrone and (**12**) amsacrine. Anions have been omitted for

While these four drugs are particularly useful in the clinic, their use is limited by major side-effects as well as acquired resistance to the drug [44]. The principle dose limiting toxicity (DLT) is myocardial toxicity (heart damage), which may cause death through congestive heart failure [45, 46]. Patients can experience heart failure during treatment and at any stage up to two years after completing treatment. This toxicity is also cumulative; the risk of heart failure rises after every subsequent treatment and for a patient with a cumulative dose of around 400 to 550 mg/m^2 their chance of heart failure increases by as much as 20 per cent. Because these drugs are also mutagenic [44], prolonged treatment can lead to the formation of resistant tumours [47], and/or secondary cancers [48]. Treatment with organic intercalators has been shown to increase the chance (1 to 2 in every 100 patients) of developing secondary acute myelogenous leukaemia (AML) or myelodysplastic syndrome (MDS), and for patients who received concurrent treatment with cyclophosphamides or radiotherapy the chance of AML/MDS is even higher.

Recently, researchers have synthesised new organic intercalators [49-51], examined ways to increase the localisation of organic intercalators in cancer cells [52] and studied the interactions of organic intercalators with DNA to a much greater extent than before [51, 53-56]. Rescifina *et al.* have synthesised a series of isoxazolidinyl intercalators that display high cytotoxicity in MOLT-3, THP-1, U937 and Vero cell lines [49]. Results from ultraviolet-visible (UV-Vis) spectrophotometry showed that the isoxazolidinyl compounds intercalate DNA with binding constants of the order 6×10^3 M^{-1}. Inge *et al.* have designed and synthesised doxorubicin derivatives to overcome multi-drug resistance [50]. Two of these drugs (WP744 and WP769) were found to be 2 to 36 times more active than doxorubicin in the advanced neuroblastoma cell lines tested.

METALLOINTERCALATORS

Octahedral Metallointercalators

Originally, the ability of octahedral transitional metal complexes to intercalate into DNA was debated extensively. Dwyer *et al.* showed a difference in the biological activity of the Δ- and Λ-enantiomers of $[Ru(phen)_3]^{2+}$, phen = 1,10-phenanthroline (Fig. **4**, **13**) in mice [57]. While this metal complex had no obvious effect on the health of the mice, inspection of their faeces revealed that the ruthenium(II) complexes were excreted by the animals intact. From this observation, it was inferred that the interaction of these metal complexes within the body was physical, rather than chemical. Rehmann and Barton were the first to suggest that binding of this complex to DNA could occur through two possible modes - intercalation and surface interaction [58, 59].

Incomplete or partial intercalation, caused by the limited aromatic overlap between the intercalating ligands of $[Ru(phen)_3]^{2+}$ and the base-pairs of DNA led to the development of a series of structural analogues. Although Dwyer had already synthesised a metal complex incorporating 3,4,7,8-tetramethyl-1,10-phenanthro-line, the next generation of metal complexes focused on utilising intercalating ligands with larger aromatic surface areas [60]. The first complex to emerge was $[Ru(phen)_2dppz]^{2+}$ (dppz = dipyrido [3,2-a:2',3'-c]phenazine), where the dppz ligand possesses two additional aromatic rings when compared to phenanthroline [61]. Other variants include: dpq (dipyrido[3,2-d:2'3'-*f*]quinoxaline) [62], dpqc (dipyrido [6,7-d:2'3'-*f*]6,7,8,9-tetrahydro)phenazine) [62], and more recently, *p*-npip (2-(4-nitrophenyl)inidazo [4,5-*f*]-1,10-phenanthro-line) [63].

For the metal complexes $[Ru(phen)_2dppz]^{2+}$ and $[Ru(bipy)_2\ dppz]^{2+}$ (**14**) (bipy = 2,2′-bipyridine), despite their extended aromatic ring systems a number of conflicting theories emerged regarding their DNA binding preference(s)

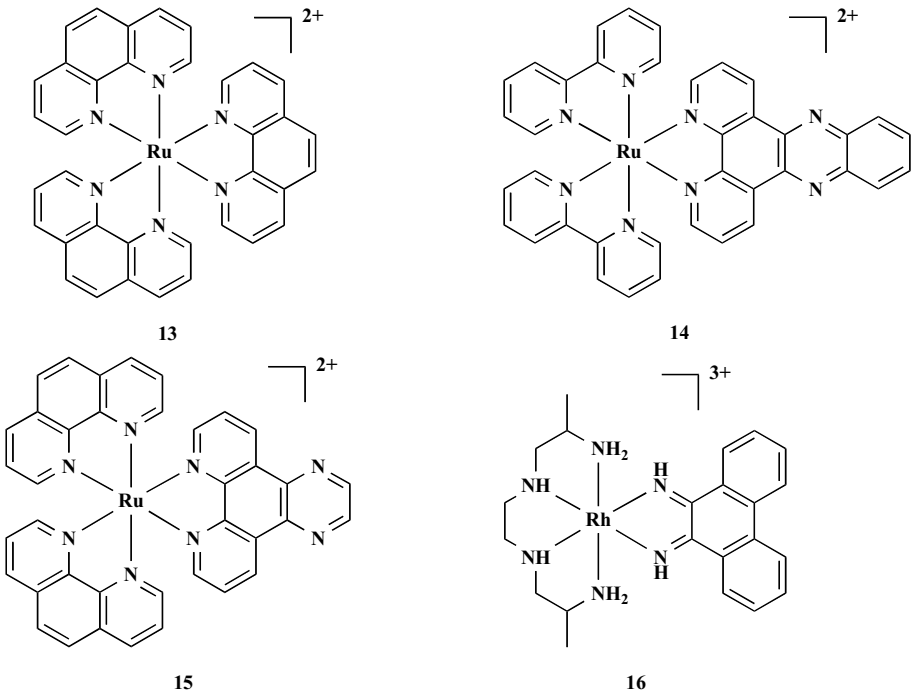

Fig. (4). Some examples of octahedral metallointercalators of ruthenium(II) and rhodium(III). Anions have been omitted for clarity.

[61]. Friedman *et al.* demonstrated that metal complexes containing dppz bind to DNA by intercalation [61], with a binding constant greater than 10^6 M^{-1} [64].

Barton and Dupureur [65, 66] demonstrated, by nuclear magnetic resonance spectroscopy (NMR), that the binding of both the Δ- and Λ-enantiomers of $[Ru(phen)_2dppz]^{2+}$ to the hexanucleotide $d(GTCGAC)_2$ occurred by intercalation from the DNA major groove [65]. In contrast, Nordén and co-workers proposed that the binding of $[Ru(phen)_2dppz]^{2+}$ occurred to DNA from the minor groove [67, 68]. To aid the characterisation of the DNA adduct, Dupureur and Barton experimented with selective deuteration of the ligands used to form the metal complexes [66]. By focusing on the dppz ligand, they determined that the complex bound to the oligonucleotide from the DNA major groove [66].

Octahedral intercalators containing the dpq ligand have also been shown to readily intercalate with DNA, but with more base sequence selectivity than dppz [69]. Δ-$[Ru(phen)_2dpq]^{2+}$ was shown to exhibit some sequence selectivity for purine-purine and pyri-midine-pyrimidine sequences, although the specific binding model could not be determined due to the exchange broadening of many of the resonances in ^{1}H NMR spectra [69]. The addition of the methyl groups to the non-intercalating ligand in $[Ru(dmphen)_2dpq]^{2+}$ (**15**) (dmphen = 2,9-dimethyl-1,10-phenanthroline) provided a better binding model for the complex with DNA. The results from DNA binding experiments suggested that both enantiomers of $[Ru(dmphen)_2dpq]^{2+}$ intercalate from the DNA minor groove, with the Δ-enantiomer binding more deeply than the Λ-enantiomer [62].

Other transition metal intercalators, such as Δ- and Λ-$[Rh(phi)(R,R\text{-}Me_2trien)]^{3+}$ (**16**) (where *R*,*R*-Me_2trien = 2*R*, 9*R*-2,9-diamino-4,7-diazadecane and phi = 9,10-diaminophenanthrene) were also studied extensively by Barton and co-workers [70, 71]. This metallointercalator was designed specifically to target 5′-TGCA sequences in DNA by intercalation from the major groove [70, 71]. NMR spectroscopy, photocleavage data and a crystal structure with DNA confirmed that the molecule bound to DNA through hydrogen bonding and van der Waals interactions [72]. The crystal structure represented the first high-resolution view of an octahedral metallointercalator bound to DNA and provided confirmation for the proposed binding modes of octahedral metal complexes.

A number of groups have attached single amino acids and short peptides to transition metal complexes [73-83]. Recently, Karidi *et al.* reported the first example of an amino acid chain attached to a chloro(polypyridyl) ruthenium(II) complex [84]. The complex, $[Ru(terpy)(4\text{-}CO_2H\text{-}4'\text{-}Mebpy\text{-}Gly\text{-}L\text{-}His\text{-}L\text{-}LysCONH_2)Cl]^+$ (where terpy = 2,2:6',2'-terpyridine) was found to be soluble in aqueous solvents, with the chloro group being displaced by water after several hours. Many more mononuclear ruthenium intercalators with a wide variety of structures and DNA binding properties have been synthesised than can be discussed within the scope of this review [10, 85-89].

Unfortunately, octahedral metallointercalators have not shown particular promise in overcoming drug resistance, but they may have other uses in cancer therapy. $[Ru(phen)_2dppz]^{2+}$ and $[Ru(bipy)_2dppz]^{2+}$ have been shown to act as "molecular light switches" for DNA [61]. While the metallointercalators show appreciable solvatochromic luminescence in organic solvents, they exhibit low photoluminescence in aqueous solution but luminesce brightly upon intercalation with DNA. A correlation was subsequently established between the extent of protection from the aqueous

solvent and the luminescence characteristics [61]. Therefore, octahedral metallointercalators which are sequence selective might be developed as diagnostic agents, where in a simple *in vitro* test they fluoresce strongly in the presence of cells with DNA markers for cancers (e.g. mutant p53 genes [90] or extended telomere regions [91]).

Square-Planar Metallointercalators

The intercalation of square-planar transition metal complexes was first demonstrated by Jennette *et al.* with [Pt(terpy) $S(CH_2)_2$ OH)]$^+$ ([Pt(terpy)(HET)]$^+$) [92] (Fig. **5**, **17**). Like organic intercalators, this metallointercalator was demonstrated to intercalate double stranded DNA by UV-vis, viscosity, circular dichroism (CD), fluorescence displacement and unwinding experiments [92]. It reversibly bound to calf thymus DNA (ct-DNA), increasing the helical length, stiffness and DNA stability (as measured by DNA melting temperature which increased 5 to 6 ^oC) [92, 93]. [Pt(terpy)(HET)]$^+$ was also found to bind ct-DNA with a binding constant in the order of 2×10^5 M^{-1}, not significantly different to that for ethidium bromide (Etbr, **8**) (3.9×10^5 M^{-1}) [92]. Other ligands containing planar, π-electron rich rings have also been shown to intercalate DNA including: phenanthroline [94] and 2,2′-bipyridine (bipy),[95] when incorporated into square-planar platinum complexes; however, when coordinated onto non-square planar metals, such as copper, these ligands do not intercalate with DNA [95].

Square-planar metallointercalators, particularly of platinum, show a strong DNA sequence selectively for 5′-GpC sites [94]. GpC sites are preferred because of their higher dipole moment. By studying platinum complexes with different intercalating ligands, Howe-Grant and Lippard demonstrated that whilst most platinum metallointercalators exhibit similar binding constants (~ 10^4 M^{-1}), the binding strength decreased in the order phen>terpy>bipy [94]. The increased binding of phenanthroline-platinum complexes appears to be a function of its extended aromatic system and higher charge [94].

Interestingly, square-planar metallointercalators can bind DNA with a number of orientations; from either the DNA major or minor grooves, or by partial intercalation. From the crystal structure of a dinucleotide (CpG) with [Pt(terpy)(HET)]$^+$ Wang *et al.* showed that the platinum intercalator bound from the DNA major groove [96]. Intercalation induced a C3′-*endo* pucker of the deoxycytidine instead of the normal C2′-*endo* pucker. This change may be stabilised by the hydrogen bond observed between the ancillary ligand hydroxy group to the cytosine O2 atom. In contrast to these results, Collins *et al.* demonstrated *via* two dimensional NMR that platinum metallointercalators, containing phenanthroline and phenanthrenequinone diamine, bound the oligonucleotide $d(GTCGAC)_2$ from the DNA minor groove [97]. Nuclear overhauser effect (NOE) cross peaks to DNA major groove protons were observed from the leading edge protons of the intercalating ligands as well as NOE cross peaks from the ancillary ligand to the minor groove sugar protons. Finally, where metallointercalators contain chiral, bulky ancillary ligands, these groups may prevent full insertion of the intercalating ligand into the DNA helix. Jaramillo *et al.* have demonstrated that the inclusion of 1,3-diamino-

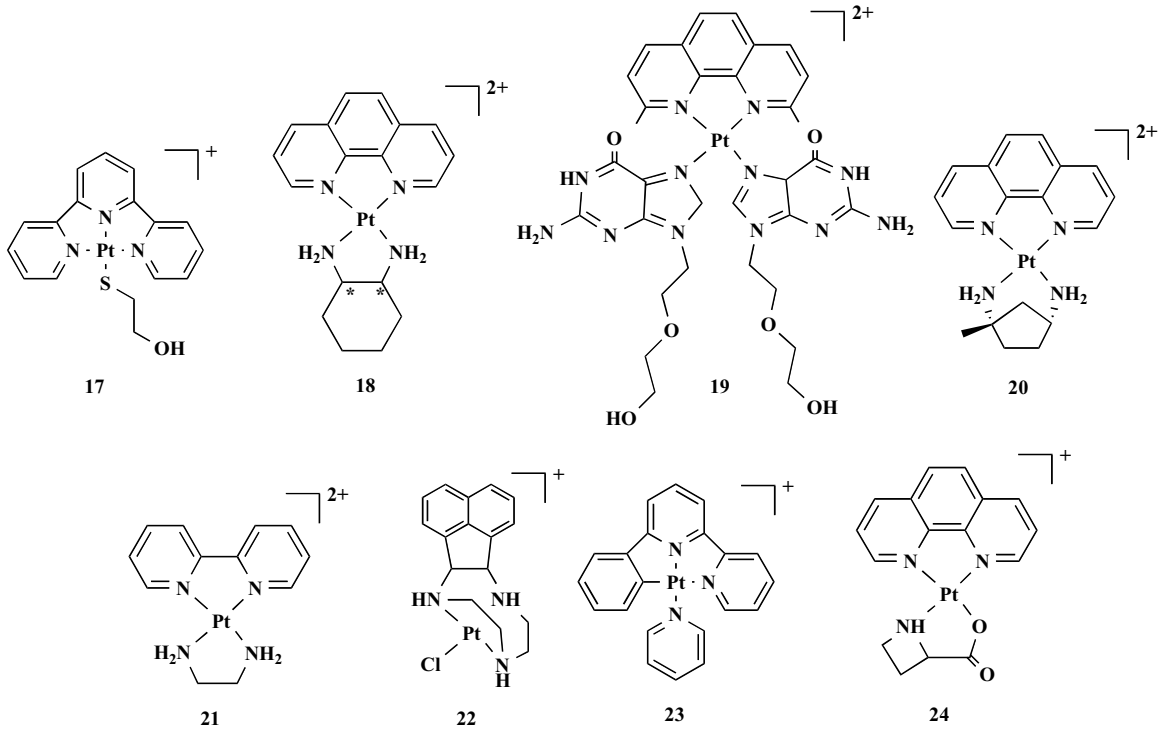

Fig. (5). Some examples of square-planar metallointercalators showing platinum(II) complexes with phenanthroline, bipyridine and terpyridine intercalating ligands. * indicates a chiral centre (*R* or *S*). Anions have been omitted for clarity.

1,2,2-trimethylcyclopentane (**20**, *R*,*S*- and *S*,*R*-tmcp) prevented full insertion of the methylated phenanthroline ligand into the DNA helix to differing extents [98]. The metallointercalators instead bound by partial intercalation and in a "side-on" fashion [98].

To improve the extent of intercalation square-planar metallointercalators with extended aromatic ring systems, similar to those used for the octahedral metallointercalators, have been used. Recently, these have included dpq [99], dppz [99-101], benzodipyrido [*b*:3,2-*h*:2′,3′-*f*]phenazine (bdppz) [99], naptha[2,3-*f*][1,*w*]phenan-throline (np) [100], naphtho[2,3-*f*][1,10]phenanthroline-9-carbo-nitrile (CN-np) [100], naphtho[2,3-*f*][1,10]phenanthroline-9,14-dicarbonitrile (CN_2-np) [100], benzo-[*f*][1,10]phenanthroline (bp) [100], and a terpyridine derivative: 2,6-(bis[benzimidazo-2-yl)pyri-dine (bzimpy) [102]. Interestingly, despite the extended ring systems of the metallointercalators incorporating np, CN^2-np, dppz and CN-np some demonstrate reduced binding constants (10^3 and 10^4 M^{-1}) with DNA [100]. Most platinum(II) intercalators have binding constants in the range of 10^4 to 10^7 M^{-1} [72, 94, 103, 104].

Through the use of substituted intercalating ligands (particularly those incorporating methylated phenanthroline ligands) and the use of chiral ancillary ligands, researchers are now able to tune the cytotoxicity of platinum intercalators; such metallointercalators are active at much lower concentrations than cisplatin, and able to overcome cisplatin resistance in several cancer cell lines (see Table **2**). Extending the earlier work of McFadyen *et al.* [105], Aldrich-Wright and co-workers have examined the structure-activity relationship of platinum intercalators with methylated phenanthroline intercalating ligands and chiral diamino ancillary ligands (see Fig. **5**) [97, 98, 106, 107]. Based on their results, a lead compound, $[Pt(5,6\text{-}Me_2\text{-phen})(S,S\text{-dach})]^{2+}$ (**18**, where 5,6-Me_2-phen = 5,6-dimethyl-1,10-phenanthroline and *S,S*-dach = 1*S*,2*S*-diaminocyclo-hexane), has been developed which is 12 times more cytotoxic that cisplatin [108], and shows cytotoxicity in L1210 (murine leukaemia), A-427 (human lung cancer), RT-112 and RT-4 (human bladder cancer), KYSR-70 (human esophagus cancer), SISO (human cervical cancer), MCF-7 (human breast cancer), 2008 (human ovarian carcinoma) cell lines. More importantly, the intercalator also shows activity in cell lines with acquired and intrinsic resistance to chemotherapy, such as: L1210/DDP (murine leukaemia with acquired resistance to cisplatin), C13*5 (human ovarian carcinoma with acquired resistance to cisplatin) and SKOV-3 (human ovarian carcinoma with intrinsic resistance to cisplatin) [106].

A structure-activity relationship has been developed which defines those characteristics which impart high cytotoxicity. First, the most active ancillary ligand isomers are in an *S*,*S* configuration, which is, on average, 10-times more active than the *R*,*R* isomers [106]. Chiral ancillary ligands generally display better cytotoxicity than non-chiral ligands, particularly when compared to ethylenediamine [106]; however, the reason for this chiral activity is currently unknown. Oxaliplatin forms a coordinate covalent DNA adduct where the *R,R*-dach ancillary ligand points into the DNA major groove, which helps to prevent DNA transcription and replication [109]. The platinum intercalators, on the other hand, intercalate in such a way that the *S,S*-dach ligand would reside in the DNA minor groove. It may be that *S,S*-dach has the same effect in the DNA minor groove as *R,R*-dach in the major groove, or it could be that the *S,S*-dach ligand acts by some other mechanism (e.g. assists in drug transport).

Secondly, cytotoxicity is dependent on both the number of methyl groups substituted onto the phenanthroline intercalating ligand, and their substitution position. The most active compounds, compared to non-methylated phenanthroline contain either one or two methyl groups while the attachment of four methyl groups greatly decreases the activity of a compound. The most active methylation positions are the five and six positions [107]. Complexes that incorporate methylation at the four and seven positions are less active [107].

Table 2. The Cytotoxicity of a Variety of Square-Planar Platinum Complexes in a Number of Different Cancer Cell Lines, with the Cytotoxicity of Cisplatin in the Same Cell Line Given for Comparison. IC_{50} is Defined as the Concentration of Metal Complex Required to Inhibit Cell Growth by 50%

Intercalator	Cell line	IC_{50} (μM)	Standard	IC_{50} standard (μM)	Reference
18 (*S,S*-dach)	L1210	0.13	cisplatin	0.5	[106]
18 (*R,R*-dach)	L1210	1.5	cisplatin	0.5	[106]
19	L1210/0	6.31	n.r	n.r	[216]
19	TLX5	3.94	n.r	n.r	[216]
20	L1210	11.2	cisplatin	0.5	[98]
21	L1210	33	n.r	n.r	[105]
22	A2780cisR	3.6	n.r	36	[217]
23	HL60	1.0	cisplatin	4.2	[123]
24	Molt-4	9.8	n.r	n.r	[218]

n.r is not reported.

BIS-INTERCALATORS

While many groups continue to pursue mononuclear metallointercalators by modifying the intercalating and ancillary ligands and adding additional functionality through amino acid conjugation, such enhancements may not significantly improve cytotoxicity. As such, some groups have begun to examine the utility of bis-intercalators (two sets of polyaromatic rings joined *via* a linking chain) for both metallointercalators and organic compounds.

Bis-intercalators may have improved cytotoxicity because of their ability to bind a greater number of DNA base-pairs simultaneously, and their increased DNA binding affinity. By binding a larger number of base-pairs, bis-intercalators may have improved DNA specificity and selectivity over mononuclear intercalators, allowing for the design of drugs that can target specific gene sequences, and through this, specifically target cancerous cells. The increased DNA binding affinity of bis-intercalators may increase the number of DNA-drug adducts formed and/or make DNA-drug adducts harder to repair. The improved DNA binding affinity may also induce larger/different conformational changes in DNA, again bypassing or hindering DNA repair.

Ruthenium Bis-Intercalators

Nordén *et al.* has focused primarily on two bis-intercalators derived from $[Ru(phen)_2dppz]^{2+}$, $[\{Ru(phen)_2\}_2\mu\text{-}(didppz)_2]^{4+}$ (**26**) and $[\{Ru(phen)_2\}_2\mu\text{-}C4(cpdppz)_2]^{4+}$, where didppz = 11,11′-bis (dipyrido[3,2-*a*:2′3′-*c*]phenazinyl, and C4(cpdppz)$_2$ = *N,N*′-bis(12-cyano-12,13-dihydro-11H-8-cyclopenta[*b*]dipyrido[3,2-*h*:2′,3′-*j*]phe-nazine-12-carbonyl) (Fig. **6**) [110-115]. Using CD, linear dichroism (LD) and luminescence spectroscopy, all three stereoisomers of $[\{Ru(phen)_2\}_2\mu\text{-}(didppz)_2]^{4+}$ ΔΔ-, ΛΛ- and meso- (ΔΛ/ΛΔ-) have been shown to form an initial groove-bound adduct with ct-DNA [116]. The geometry of the intercalative binding subsequently reorganises slowly, taking almost a day to reach equilibrium [111]. This mode of binding with ct-DNA has also been observed with poly(dA-dT)$_2$, poly(dG-dC)$_2$ and poly(dI-dC)$_2$, although with poly (dA-dT)$_2$, no initial groove-bound species was detected [113]. The dissociation of the bound bis-intercalator from DNA has been shown to be remarkably slow, with the complex requiring several days for SDS-induced (sodium dodecyl sulfate) dissociation to occur [113]. The rearrangement from groove to intercalative binding was proposed to occur *via* the threading of one of the Ru(phen)$_2$ moieties through the DNA duplex. The final geometry of the DNA-bound bis-intercalator is characterised by the intercalation of the didppz ligands in an anti-conformation, with a small rotation around the central bond bridging the two dppz ligands [113]. The complex intercalates asymmetrically, with one of the ruthenium centres situated deeply in the DNA minor groove, while the other centre projects through the core of the DNA helix into the major groove.

The binding constants of ΔΔ- and ΛΛ-$[\{Ru(phen)_2\}_2\mu\text{-}(didppz)_2]^{4+}$, and ΔΔ- and ΛΛ-$[\{Ru(bipy)_2\}_2\mu\text{-}(didppz)_2]^{4+}$ were determined by CD, LD and UV-vis spectrophotometry, together with displacement experiments on intercalated $[Ru(dpq)_2dppz]^{2+}$ (measured by emission quenching) [110-113]. For the didppz complexes, the binding constants were found to be very high, in the order of 10^{11} to 10^{13} M^{-1} and the order of binding affinity was found to be ΛΛ-$[\{Ru(bipy)_2\}_2\ \mu\text{-}(didppz)_2]^{4+}$ ≈ ΔΔ-$[\{Ru(bipy)_2\}_2\mu\text{-}(didppz)_2]^{4+}$ > ΔΔ-$[\{Ru(phen)_2\}_2\mu\text{-}(didppz)_2]^{4+}$ > ΛΛ-$[\{Ru(phen)_2\}_2\ \mu\text{-}(didppz)_2]^{4+}$ [113].

Platinum Bis-Intercalators

Lowe and co-workers have developed a series of bis-platinum intercalators, with substituted terpyridine intercalating ligands, linked *via* a variety of rigid, variable length hydrophobic and hydrophilic chains (Fig. **7**) [117-120]. Their cytotoxicity was examined in a panel of six human ovarian cancer cell lines; two that are sensitive to cisplatin (CH1, A2780), two with resistance to cisplatin (CH1cis and A2780 cis), one with resistance to doxorubicin (CH1dox) and the cancer cell line SKOV-3 (which is the most cisplatin resistant line available) [117]. The degree of cytotoxicity is dependent on three variables: chain length, charge density and the counter ions. First, those bis-intercalators with shorter chains were more cytotoxic than those with longer chains. Secondly, the more highly charged bis-intercalators were more cytotoxic than those with lower charges, or lower charge density. Finally, the tetrafluoroborate salts were generally more cytotoxic than their corresponding water soluble nitrate salts [117]. All the bis-intercalators were found to have little, to no, cross-resistance in the matched cell lines (as indicated by resistance factor (Rf) values between 0.6 to 4). One bis-intercalator, $[\{Pt(terpy)\}_2\mu\text{-}4,4'\text{-vinylenedipyridine}]^{2+}$ (**29**) was up to 13 times more cytotoxic than cisplatin [117] (see Table **3**). In another study by Lowe using three glioblastomas (NCH37, NCH82 and NCH 89) and two head and neck squamous cell carcinoma cell lines (HNO97 and HNO199) the bis-inter-calators were found to have IC_{50} values between 2.5 and 6.2 μM (no control drug value was reported) [119].

Interestingly, Lowe's bis-intercalators appear to derive their anticancer activity from non-classical DNA intercalation mechanisms. For one, the linking chains of the bis-intercalators appear to be labile, yielding a reactive electrophile in solution that is able to form coordinate covalent binds with DNA [121]. In a study with $[\{Pt(terpy)\}_2\mu\text{-}4,4'\text{-vinylenedipyridine}]^{2+}$ and the oligonucleotide d(CGTACG), the bis-intercalator broke apart, with a terpyridine-platinum group forming a coordinate covalent adduct through the N7 position of guanosine [121]. *In vivo* these bis-intercalators have also been shown to inhibit thioredoxin reductase (TrxR), which is a key component in human tumours [119]. The authors proposed that TrxR binding is the major target of these bis-intercalators, but they also continue to inhibit DNA replication, thus producing an improved cytotoxic effect when compared to cisplatin [119]. Finally, the transport of these platinum bis-intercalators by human serum albumin may also play a role in explaining their improved activity [122].

More recently, Chan *et al*. have reported the synthesis, DNA binding and cytotoxicity of three bis-intercalators linked *via* a flexible alkane chain (**33**) [123]. These metal complexes were shown to intercalate DNA, by viscosity experiments, with binding constants between 10^6 to 10^7 M^{-1}, considerably higher than that for the corresponding mononuclear intercalator (10^4 M^{-1}), have cellular uptake equal to or

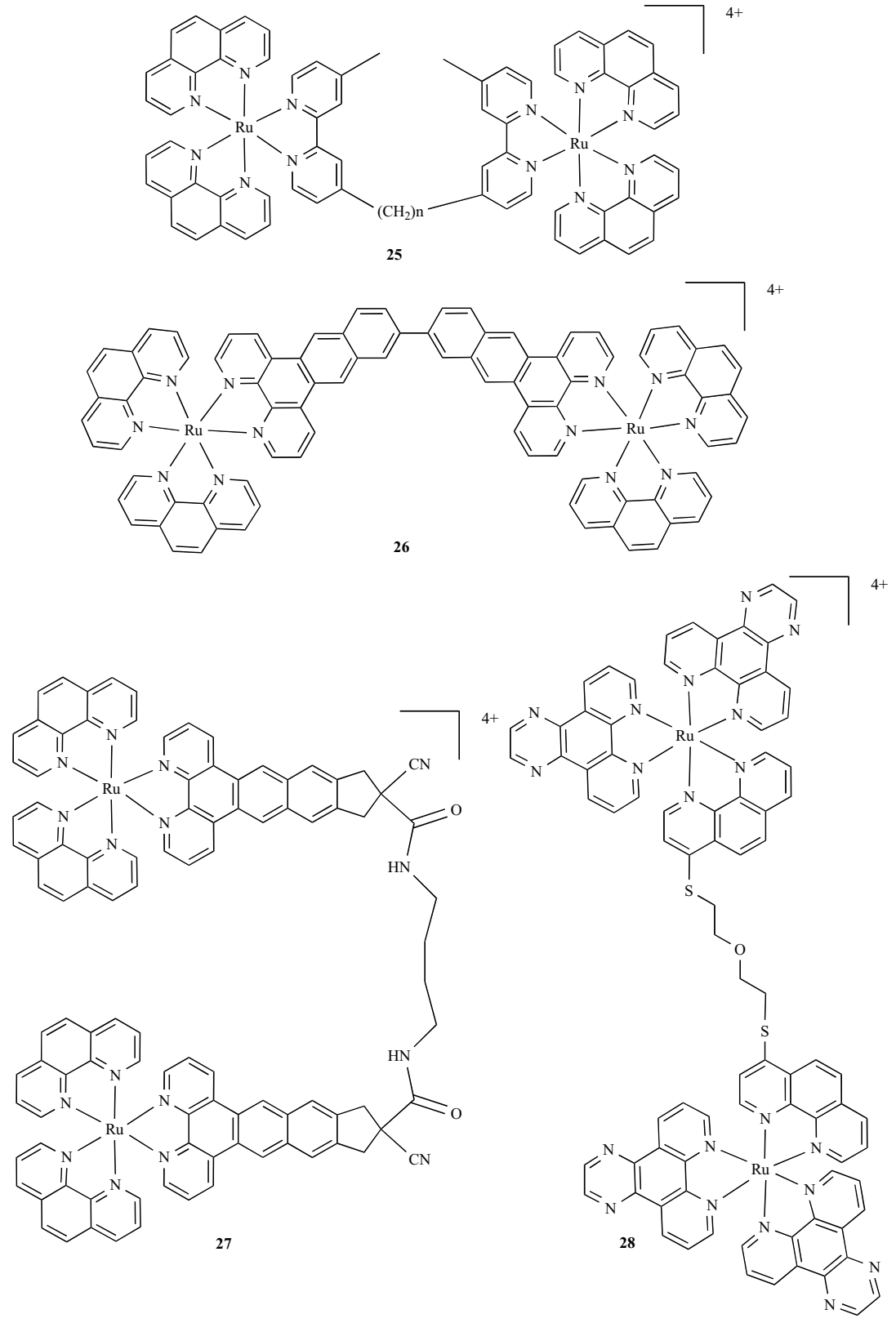

Fig. (6). Some examples of octahedral ruthenium(II) bis-intercalators with inflexible and flexible linking chains. Anions have been omitted for clarity.

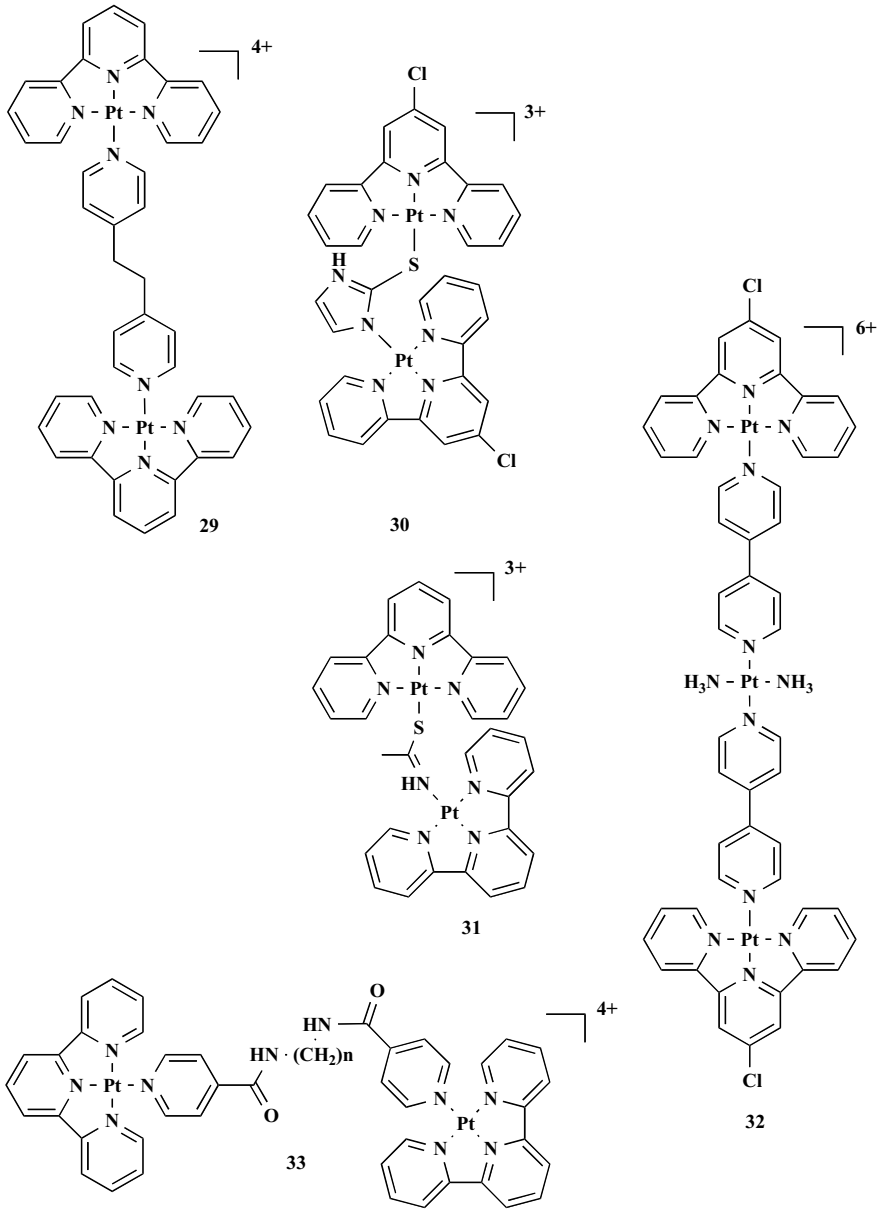

Fig. (7). Some examples of square-planar platinum(II) bis-intercalators with flexible and inflexible linking chains. Anions have been omitted for clarity.

better than cisplatin, and cytotoxicity levels equal to, or slightly less than, cisplatin in five human tumour cell lines (KB-3-1, KB-V1, CNE3, Hep G2 and HL60) [123]. Unlike Lowe's intercalators, these bis-intercalators were found to have increasing cytotoxicity with increasing linking chain length.

Organic Bis-Intercalators

Finally, some groups have synthesised bis-intercalating organic drugs [124-131] (see Fig. **8**). These include compounds with rigid, but variable chains joining acridine and phenanthridium intercalators [126, 128] and daunorubicin intercalators linked *via* a flexible chain [125, 127, 130, 131]. In the latter case two lead bis-intercalators, WP631 (**34**) and WP762, were designed and synthesised based on the binding characteristics of native daunorubicin [129, 132]. It was found that two daunorubicin molecules bound a six base oligonucleotide with the amino groups of each compound pointing into the DNA minor groove [132], such that the intercalators were separated by 7 Å. Two daunorubicin intercalators cross-linked through their sugar amino group with a *p*-xylenyl linker produced WP631, while those linked together with a *m*-xylenyl linker produced WP762 [127].

WP631 and WP762 intercalate DNA with binding constants of 3.1×10^{11} and 7.3×10^{12} M^{-1}, respectively [127, 131], and have been shown to bind DNA *via* intercalation using UV melting, differential calorimetry and viscosity ex-

Table 3. The Cytotoxicity of a Variety of Platinum and Organic Bis-Intercalators in a Number of Different Cancer Cell Lines, with the Cytotoxicity of Cisplatin, Daunorubicin or Doxorubicin in the Same Cell Line Given for Comparison. IC_{50} is Defined as the Concentration of Metal Complex Required to Inhibit Cell Growth by 50%

Intercalator	Cell line	IC_{50} (μM)	Standard	IC_{50} standard (μM)	Reference
31	NCH37	5.7	n.r	n.r	[119]
31	HNO97	4.8	n.r	n.r	[119]
32	CH1	0.55	cisplatin	0.4	[117]
32	CH1dox	0.42	cisplatin	0.5	[117]
33 n = 6	KB-3-1	24	cisplatin	5.5	[123]
33 n = 6	CNE3	16	cisplatin	11	[123]
34	Jurkat T	17.7 (nM)	daunorubicin	82.6 (nM)	[127]
34	MCF-7	4.8	doxorubicin	0.9	[125]
34	MCF-7/VP-16	2.5	doxorubicin	14.2	[125]

n.r is not reported.

periments [125, 127], and a series bisnapthalimide compounds have been shown to unwind DNA by a much greater extent (36-41°) than the mononuclear equivalent (19°) [124].

The bis-intercalators WP631 and WP762 have also been shown to overcome drug resistance in some cancer cell lines (see Table **3**). In the matched MCF-7 and MCF-7/VP-16 cell lines (where the latter has multi-drug resistance) WP631 displayed an Rf of just 0.5 compared to doxorubicin which has an Rf of 16 [125]. In Jurkat T Lymphocyte cells both WP631 and WP762 display a 3-5 fold higher cytotoxicity than daunorubicin and at nanomolar concentrations [127].

SYNERGISTIC INTERCALATORS

Over the last 20 years many researchers have examined the synergistic properties of combining a DNA intercalating compound with other functionalities (Fig. **9**). This includes

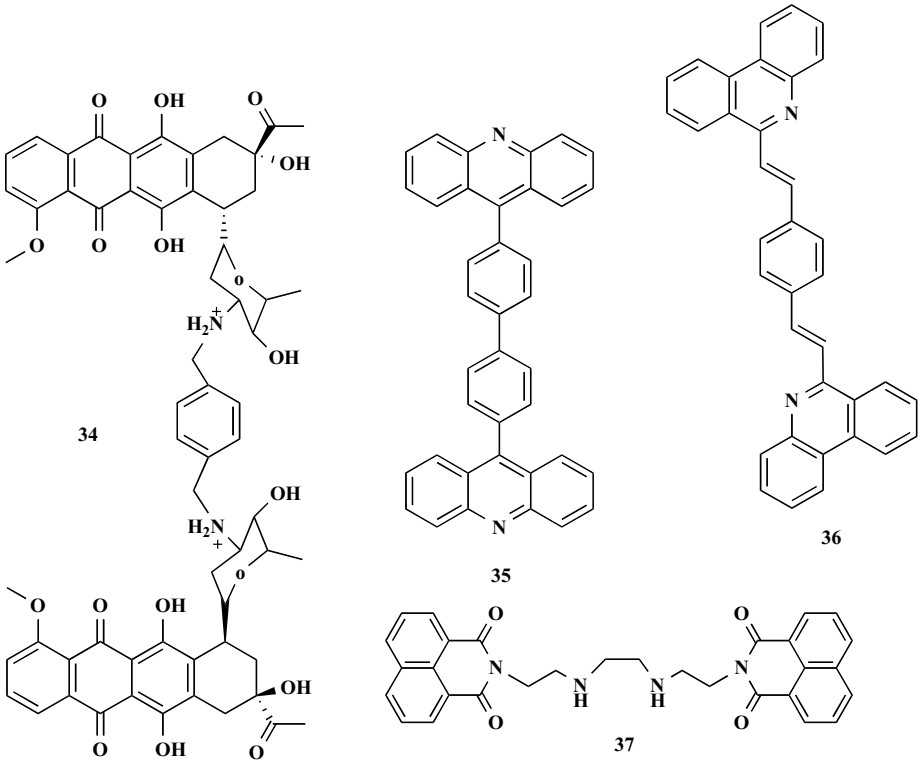

Fig. (8). Some examples of organic bis-intercalators with flexible and inflexible linking chains.

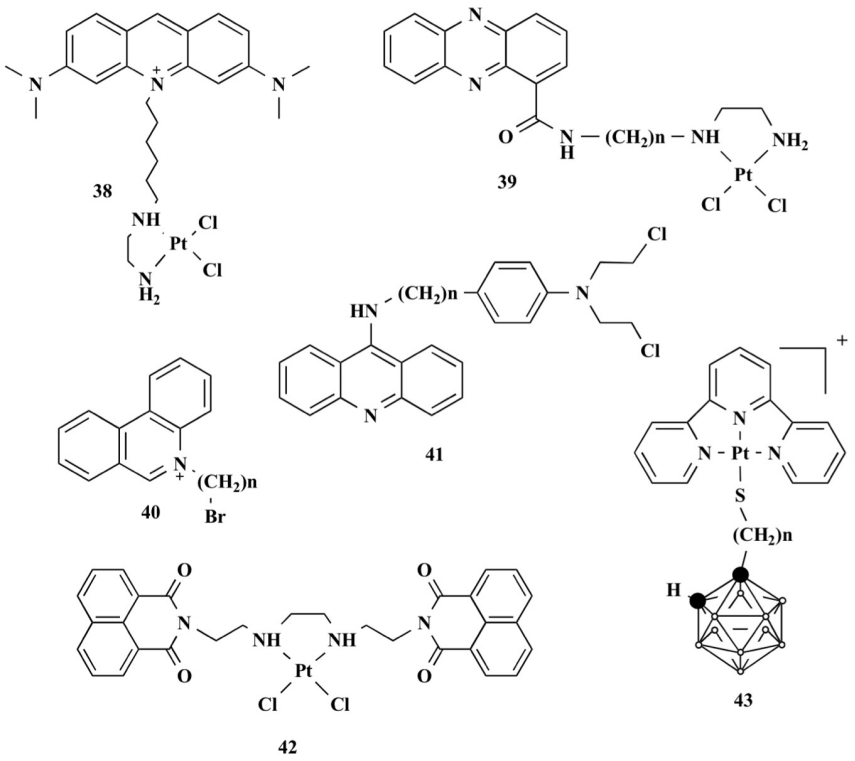

Fig. (9). Some examples of synergistic intercalators which combine a functional group capable of forming covalent or coordinate covalent bonds with DNA and an intercalating functional group. One compound (**43**) "where ○ = boron and ● = carbon" contains an intercalator linked to a carborane cage for use in boron neutron capture therapy. Anions have been omitted for clarity.

organic intercalators [133-150] and metallointercalators linked to cisplatin-like mole-cules [151-157], organic intercalators linked to transplatin-like mole-cules [158-160], organic intercalators linked to DNA-alkylating agents, such as mustards [161-165], and metallointercalators linked to carborane cages for use in radiation therapy [166-169]. These synergistic intercalators have been developed in an attempt to increase the DNA binding affinity, aid in the drug transport and/or to modify their DNA adducts/sequence selectivity.

Platinum centres have been attached to a variety of organic intercalators, including: doxorubicin [150], acridine [133, 147, 149], bis(naphalimide) [148], anilinoacridine [142], aminoacridine [134], phenazine [135, 136, 142], and proflavine [137]. The majority of studies, such as those by Denny and co-workers, have focussed on acridine and anilinoacridine [133, 142, 147, 149]. The first of these complexes developed linked acridine orange to platinum through ethylenediamine and a variable length alkane chain (**38**) [144-146]. Computational modelling and DNA binding experiments demonstrated that intercalation of the acridine orange and coordinate covalent binding of the platinum occur simultaneously, with separation of one to three base-pairs [145, 146]. This cooperative binding has a large affect on the DNA conformation, leading to enhanced unwinding of the helix [146]. In addition, where platinum binding usually results in a shortening of the helix due to kinking of the DNA, the attachment of the intercalator negates this, slightly increasing the length of the DNA [145]. In another study, Perez *et al.* demonstrated that a bis(naphalimide)-platinum complex unwound supercoiled DNA by 48°, a remarkable effect given that the unwinding caused by cisplatin and the free ligand are only 12° and 24°, respectively [148]. Similarly, an anilinoacridine-platinum complex induced DNA unwinding twice that of the native platinum complex [142]. Whilst platinum complexes containing two cis-chloro ligands preferentially form distinct adducts, like intrastrand GpG and ApG DNA adducts, the intercalator in synergistic complexes also changes the sequence-specificity of the platinum moiety. Transcription assays showed that these types of synergistic intercalators demonstrate less distinct binding with a greater number of low intensity blockage sites [133]. Whilst the major sequences targeted by synergistic complexes are similar to cisplatin, GpG [144] and GpC [136], some complexes do show a preference for GpA, GpT [144, 162] and even ApT sequences [147]. This indicates that the DNA binding is dominated by the groove and sequence recognition of the intercalator [147], with the platinum group targeting nucleophilic sites in the vicinity of the intercalator. The length of the linker chain also determines the sequence-specificity. The shorter the linker chain the more the DNA adducts differ from cisplatin, where the short length means the platinum binding site is dictated to a larger degree by the intercalator [141]. Synergistic intercalators have also been shown, by UV-vis and ^{195}Pt NMR, to bind simultaneously by intercalation and coordinate covalent bonds [142].

In transcription assays, synergistic intercalators are able to prevent DNA transcription in a time, as well as concentra-

tion, dependent manner. Transcription blockage bands have been observed shortly after the addition of the synergistic intercalators to DNA, the results showing initial intercalation, with the bands increasing in intensity as the platinum moiety reacts with the DNA [133, 134, 136-138, 147-149]. The coupling of an intercalator to platinum also increases the rate of DNA-platinum adduct formation [135-138, 141, 147]. Synergistic intercalators containing ethylenediamine platinum moieties react up to four-fold faster with DNA than the native dichloroethylenediammine platinum(II) [$Pt(en)Cl_2$] complex alone [138].

Synergistic intercalators are also much more effective DNA binding agents, resulting in increased DNA damage [136]. In DNA transcription assays, synergistic intercalators produced more intense and a greater number of blockages compared to the native intercalators and platinum complexes [136, 140]. In the human cancer cell line P388, phenanthridium-platinum complexes induce a six-fold enhancement in DNA damage compared to cisplatin at the same concentration [140]. Interestingly, the ability to induce DNA damage appears to correlate directly with linker chain-length. Unlike coordinate covalent binding multinuclear platinum drugs which cause more DNA damage with increasing chain length [109, 170], synergistic intercalators exhibit greater DNA damage with shorter linker lengths [140].

The cytotoxicity of synergistic intercalators varies greatly, but generally, all these compounds show significantly enhanced cytotoxicity compared to the native free intercalator [135, 143]. The anilinoacridine-platinum complexes of Palmer *et al.* are three- to five-fold more active *in vitro* than the free native intercalator in P388/W cells, but display only differential cytotoxicity in the cisplatin-resistant line P388/P [142]. The water soluble acridinecarboxamide-platinum complexes are equally active *in vitro* in both the P388/W and P388/P cell lines [143], and the P388/AMSA cell line which has resistance to amsacrine. In the latter case, the synergistic intercalators were ten-fold more active than cisplatin and [$Pt(en)Cl_2$] [143]. Significant cytotoxicity of synergistic intercalators has since been demonstrated *in vivo* [140], and in different cell lines, such as: HeLa [138], A2780 and A2780 cisR [148], CH1 [148], L1210 and L1210/DDP [150], and Pam 202-*ras* [148].

Cytotoxicity is related in part to the chain length and stereochemistry of the synergistic intercalators. In phenazine-1-carboxamide complexes cytotoxicity is dependent on the length of the polymethylene chain, with longer chains exhibiting greater activity than shorter chains [135]. In contrast, Holmes *et al.* have shown that for aminoacridine complexes the cytotoxicity of the drugs are enhanced with a shortening of the linker chain [139]. For acridine-n-carboxamide (where n is the 2 or 4 position), the stereochemistry affects cytotoxicity, where the complexes of the 4-carboxamide are more active than those of 2-carboxamide [143].

A series of octahedral metallointercalators coupled to cisplatin-like or platinum intercalators have been developed by several groups. A rhodium-platinum complex containing a chrysenequinonediimine ligand was synthesised by Petitjean and Barton, which was designed to transport platinum drugs to mismatch sites on DNA [151]. This heterodinuclear metallointercalator is capable of recognising mismatch sites in oligonucleotides where the distance between the mismatch and platinum binding site (a GpG sequence) is less than 20 Å (approx. six base-pairs). When the distance is greater than ten base-pairs, mismatch binding dominates and the binding preference is changed. This heteronuclear complex is also able to cleave DNA through photoactivation, thus greatly increasing the amount of DNA damage.

Other complexes containing ruthenium or osmium have been synthesised using flexible and inflexible linkers [152-156]. These complexes demonstrate increased DNA binding affinity and react faster than the native platinum complexes alone. The complexes are able to induce DNA damage through photoactivation, and at a level higher than that of native cisplatin [153, 154, 156].

Organic intercalators, including phenanthridinium [161, 162], and 9-aminoacridine [163, 164] have also been coupled to alkylating agents (e.g. **40** and **41**, Fig. **9**). These compounds derive their anticancer activity by binding purine bases in DNA, particularly at the N7 site, leading to depurination. Attachment of the intercalator, *via* a variable length polymethylene chain (n = 4, 5, 6, 8 or 10) to the alkylators significantly modifies the DNA sequence selectivity. Like native alkylators, these compounds target guanosine and adenosine, but the actual binding site on DNA is dependent on the linker length. For phenanthridinium compounds, short chains (i.e. n = 4) target GpA sites, but longer chains target GpT sites [161]. In contrast, for those compounds that utilise 9-aminoacridine, the short chained compounds target GpT sites while the longer chains target ApC sites [165]. Interestingly, while attachment of the intercalator increases the DNA affinity, only ten per cent of the compounds appear to form covalent adducts with DNA [161].

Rendina and coworkers have synthesised a series of platinum-based intercalators linked to a variety of carboranes for improved radiation oncology [166-169] (e.g. **43**). These include mononuclear complexes with a terpyridine-platinum group linked to 1,2-, 1,7- and 1,12-carborane through a variable length thioalkyl chain. Whilst high cytotoxicity in the absence of thermal radiation is not expected, and in fact not preferred (cell growth inhibition prevents accumulation of sufficient levels of ^{10}B nuclei within the cells), these synergistic intercalators are cytotoxic in ovarian cancer cell lines [169]. The level of cytotoxicity appears to be related to linker length and stereochemistry of the carborane cage, which relates directly to the degree of aqueous solubility. Cytotoxicity in the human ovarian carcinoma cell line 2008 decreases in the order 1,2- to 1,7- to 1,12-carborane, which is also the order of solubility. All compounds are poorly soluble in water and other polar solvents, although solubility can be improved through the addition of a pendant glycerol group to the carborane cage [168]. Interestingly, the shorter the thioalkyl linker length, the more cytotoxic the complex; for the intercalator that is separated from the carborane chain by one methylene group its cytotoxicity is three times higher than for the same intercalator with a three-methylene chain [169]. Whilst UV-vis experiments have shown that these metal complexes intercalate ct-DNA [166], it was expected that a longer linking chain would help prevent steric clashes

between the carborane and DNA; however, it appears that the cytotoxicity difference is a function of the reduced solubility of the longer chain [169]. Recently, Rendina has extended this work by developing a series of multinuclear intercalators linked to carborane cages. Again, the cytotoxicity of these synergistic intercalators is dependent highly on the stereochemistry of the carborane cage: 1,2- is much more cytotoxic than the 1,7- and 1,12-carboranes; however, the increased length of the thioalkyl linkers increased, rather than decreased, cytotoxicity to the extent that $[\{Pt(terpy)\}_2\mu\text{-}\{closo\text{-}1,2\text{-carborane}\}]^{2+}$ is as cytotoxic as cisplatin in the L1210 murine leukaemia cell line, and eight times more cytotoxic than cisplatin in the cisplatin-resistant cell line L1210/DDP [167].

SPECTROSCOPIC TOOLS OF ANALYSIS

Current research indicates that while transport of intercalators through the human body, across the cell membrane and within the cell itself [157] affects the cytotoxicity of intercalating drugs, it is their interaction with DNA, and through this, their ability to prevent DNA transcription and replication that is of most importance. Hence, examining the interaction of intercalating drugs with DNA provides an important step in determining structure-activity relationships. In this section we review the spectroscopic tools of analysis available to examine DNA intercalation. This discussion focuses on standard spectroscopic techniques and how they are applied to DNA-drug binding, but also examines recent advances that allow for more detailed analysis.

X-ray Diffraction

X-ray diffraction for crystal structure determination remains the most powerful tool for examining DNA-drug interactions because it can provide simultaneous information on the DNA binding site specificity, location and orientation, and any changes to the DNA helix conformation. Unfortunately, crystal structures are particularly difficult to obtain, because of the challenge in obtaining crystals of DNA-intercalator complexes. Lippard and co-workers at the Massachusetts Institute of Technology (USA) have achieved considerable success in "growing" crystals using a variety of techniques.

Crystals of platinum intercalators with DNA fragments have been produced by slow evaporation of a buffered solution of DNA [23, 171], or through vapour diffusion of 2-methyl-2,4-pentanediol (2-MPD) into a buffered solution of DNA [96]. Crystallisation can also be promoted by the addition of cacodylic acid, glycine, magnesium chloride and $[Co(NH_3)_6]Cl_3$ [172, 173].

Crystals of metal complexes bound (although not intercalatively) to DNA have also been grown using hanging [174] or sitting drop vapour diffusion [175], again with 2-MPD. In these experiments, a sample of the DNA-intercalator is made up in a very small volume (1 to 40 μL) of buffer and reagent (i.e. 2-MPD). The solution is then either pipetted onto a platform (sitting drop), or onto a glass cover that is inverted (hanging drop) in a sealed vial containing a reservoir of the reagent. Over time, the concentration of the reagent in the reservoir and in the DNA-intercalator solution come to equilibrium as water is drawn from the sample. This concentrates the sample causing precipitation and hopefully crystal growth.

Nuclear Magnetic Resonance

With the exception of x-ray diffraction, NMR spectroscopy is the most powerful tool for obtaining a detailed, three-dimensional picture of DNA intercalation. In some instances NMR may be more relevant than x-ray diffraction, in that it can provide details of DNA-drug interactions under physiological conditions as well as other information beyond the three-dimensional structure, such as binding constants. X-ray diffraction also provides only a snap-shot of DNA-drug interactions, whereas NMR provides a dynamic representation of binding. NMR, however, is a relatively insensitive technique. Many other spectroscopic techniques, like UV-Vis, CD and fluorescence require micromolar concentrations of intercalator and DNA, while NMR typically requires millimolar concentrations.

Given the large number of protons in both intercalator compounds and DNA, and the high natural abundance of ^1H nuclei (99.985%) [176], ^1H NMR is typically the most extensively used experiment. When examining DNA intercalation, typical experiments utilise short segments of DNA (oligonucleotides) between 6 and 12 base-pairs in length. An oligonucleotide six base-pairs long has been shown to form a mini, normal B-type DNA helix with well defined minor and major grooves [65, 69, 177, 178]; a segment of DNA shorter than 6 base-pairs does not, whereas oligonucleotides larger than 12 base-pairs produce NMR spectra that are usually, but not always (using average field strength NMR spectrometers: 300-500 MHz), too complicated for detailed analysis. Use of modern high field NMR spectrometers, 800-900 MHz, may provide sufficient resolution to allow analysis of larger oligonucleotides.

In a typical one-dimensional experiment intercalation into the DNA helix induces large, selective chemical shift changes of the resonances from both the DNA and the intercalator (intercalator resonances and DNA sugar proton resonances shift upfield) [97]. The largest chemical shift changes are exhibited by those resonances on bases at the site of intercalation, and thus, give an indication of the DNA binding site. Further information on the binding site can be obtained through the use of two-dimensional nuclear overhauser enhancement spectroscopy (NOESY) and double quantum filtered correlated spectroscopy (DQFCOSY) experiments. NOESY provides *through-space* interactions between protons (see Fig. **10**). Where the distance between two protons is less than 5 Å [179], an NOE cross peak will be observed. As the strength of the NOE is related to r^{-6}, the volume of the cross peak can be used to determine proton-proton distances [179]. NOESY experiments can therefore be used to determine the exact binding site and orientation of an intercalator in the helix by observing NOE cross peaks between the intercalator and DNA resonances. For example, NOEs from the intercalator resonances to thymine C5-methyl, guanosine/adenosine H8-proton or the cytosine C5-proton resonances indicate interactions within the DNA major groove, whereas NOEs to any of the sugar H1′, H4′ or H5′/H5″ proton resonances indicate interactions and binding in the minor groove.

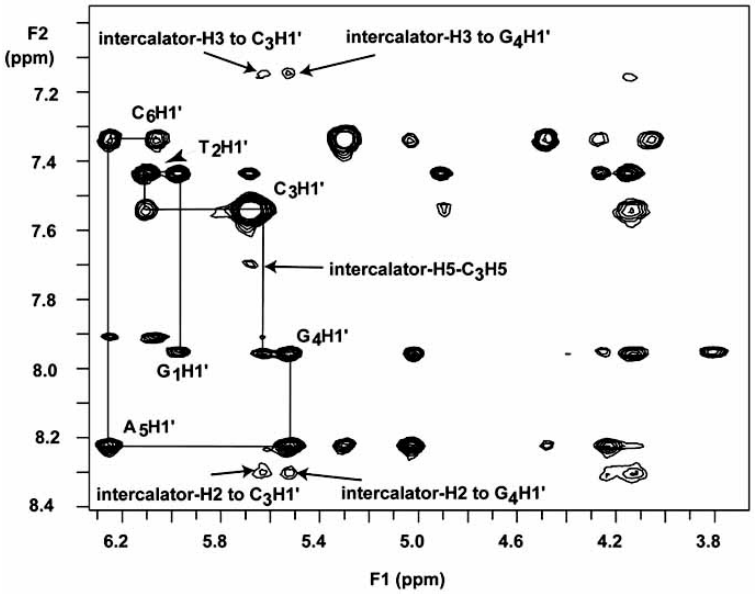

Fig. (10). A section of a ^{1}H NMR NOESY spectrum of the square-planar platinum intercalator $[Pt(phen)(en)]^{2+}$ and the oligonucleotide $d(GTCGAC)_2$, showing the DNA sugar proton (F1 axis) and DNA base/intercalator aromatic proton (F2 axis) NOE cross peaks. The spectrum displays three important pieces of information. The sequential oligonucleotide NOE cross peaks (connected *via* black lines) demonstrate that the DNA remains in a double helix. The indicated NOE cross peaks between the oligonucleotide and the intercalator demonstrate that the intercalator binds between the central GC base-pairs and that the intercalator binds from the DNA minor groove.

DQFCOSY experiments examine *through-bond* interactions between protons separated by three bonds (although long range coupling is sometimes seen up to four bonds), and provide information on the structural changes to the DNA helix. In normal B-type DNA, the coupling (*J*) of the H1′ proton to the H2″ proton is 10 Hz. If intercalation induces a conformational change in the DNA this will be observed through a change in the coupling constant. For example, in Z-type DNA, the coupling of the 3′-*endo* sugar H1′ to H2″ changes to 2 Hz.

Where the binding of an intercalator to DNA is both fast exchange on the NMR timescale and stoichiometric (1:1 binding intercalator and oligonucleotide) then a minimum binding constant can be estimated. When the binding is less than stoichiometric then the binding constant can be calculated [180, 181]. The binding constant (K_b) can be expressed as:

$$K_b = \frac{[\text{I-DNA}]}{[\text{I}][\text{DNA}]} \quad \text{Eq. 1}$$

where [I-DNA] is the concentration of intercalator bound oligonucleotide, and [DNA] and [I] are the concentrations of free oligonucleotide and intercalator, respectively. These concentrations can be estimated from the population-weighted chemical shift:

$$\delta_{obs} = P_f\delta_f + P_b\delta_b \quad \text{Eq. 2}$$

where δ_{obs} is the observed chemical shift of any given intercalator proton resonance, P_f and P_b are the mole fractions of free and bound intercalator and δ_f and δ_f are the chemical shifts of the same intercalator proton resonance of the free and bound drug.

Two emerging NMR techniques which will play an increasingly greater role in DNA intercalation chemistry in the future are heteronuclear single-quantum coherence (HSQC) and pulsed-gradient spin echo (PGSE). HSQC is of particular relevance given the advances in synergistic intercalators containing cisplatin- or transplatin-like groups, and when used with ^{1}H-^{15}N labelled compounds, can yield information on the pre-association, rate-of-aquation and rate of DNA adduct formation [182-191]. In these experiments, which can be performed at micromolar concentrations, the ^{15}N-chemical shift is influenced strongly by the ligand in the *trans*-position to the proton being monitored (in cisplatin- or transplatin-like compounds this would be an NH_2 group), and therefore chloro, aqua, hydroxy and nucleoside coordination resonances can be clearly resolved [182]. The ^{15}N resonances will also be shifted by nearby oxygen groups, which can then be used to examine hydrogen bonding between the intercalator and DNA [182].

PGSE (also commonly referred to as diffusion-ordered spectroscopy or DOSY) is the most common method for measuring the diffusion of small molecules through solution and studying drug binding to proteins and DNA [192-195]. This technique provides complementary information to that obtained from standard NMR experiments and can provide information on conformation (even for large molecules) which is particularly useful when individual resonances in a spectrum cannot be assigned [196, 197]. The basis of using diffusion measurements to probe such systems relies on the ability to separate the different species based on their diffusion coefficients, in the case of binding studies, on the exchange between free and bound states which modifies the observed diffusion coefficient [194]. Whilst PGSE techniques are not new, their application in protein-drug and

Table 4. The Cytotoxicity of a Variety of Synergistic Intercalators in a Number of Different Cancer Cell Lines, with the Cytotoxicity of Cisplatin or Chloroambucil in the Same Cell Line Given for Comparison. IC_{50} is Defined as the Concentration of Metal Complex Required to Inhibit Cell Growth by 50%

Intercalator	Cell line	IC_{50} (μM)	Standard	IC_{50} standard (μM)	Reference
39 n = 5	P388	0.044	cisplatin	0.3	[135]
39 n = 4	P388	0.082	cisplatin	0.3	[135]
40 n = 6	L1210	0.25	n.r	n.r	[161]
40 n = 10	L1210	0.150	n.r	n.r	[161]
41 n = 2	P388	0.061	chlorambucil	7.5	[164]
41 n = 4	P388	0.082	chlorambucil	7.5	[164]
42	A2780	0.25	cisplatin	0.3	[148]
42	A2780cisR	0.8	cisplatin	3.3	[148]
43 n = 1	2008	1.7	cisplatin	0.6	[169]
43 n = 1	C13	2.1	cisplatin	10	[169]

n.r is not reported.

DNA-drug chemistry has grown enormously in the last ten years, largely in part due to advances in NMR hardware. The uses of PGSE in monitoring DNA intercalation include determining the binding affinity of several different drugs at one given binding site (comparative binding affinity) and determining the preferred binding site of a particular drug where multiple drug binding sites exist.

iscosity

Viscosity experiments are an inexpensive means of demonstrating the ability of a polycyclic compound to intercalate into DNA [198], and has recently been used to study the intercalation of transition metal complexes [63, 199-202]. Cohen and Eisenberg were the first to show the relationship between viscosity and DNA length, from which the binding modes of a variety of ligands with DNA has been determined [203]. Viscosity experiments can now be used to distinguish three of the major types of DNA binding modes. The intercalation of a molecule into DNA results in a lengthening, unwinding and stiffening of the helix which increases the viscosity of the solution [204]. For compounds that form covalent or coordinate covalent bonds with DNA, like cisplatin, a decrease in the DNA-solution viscosity is generally observed because of kinking/bending of the DNA helix toward either the minor or major groove (depending on the location of the ligand) thus reducing the end-to-end length of the DNA molecule. Finally, groove binders, like netropsin and Hoechst 33258, do not change the end-to-end length of DNA and therefore have a negligible effect on viscosity.

As most chromosomal DNA is found in huge coils with large excluded volumes and molecular masses greater than one million, it is unsuitable for use in viscosity experiments. Sonicated DNA, where the typical molecular masses are around 5×10^5 g/mol and the overall end-to-end length is approximately 2000 Å (200 base-pairs) is ideal for viscosity experiments as the DNA is almost rod-like and a change in the axial length of the helix can be readily observed.

In a typical experiment, sonicated ct-DNA, in buffer, is passed through a viscometer, which is kept at a constant temperature by use of a thermostatic water bath. The viscosity of the unbound DNA (η_0) is the time difference in seconds between the DNA in buffer (t) minus the viscosity time for the buffer without DNA (t_0):

$$\eta_0 = t - t_0 \qquad \text{Eq. 3}$$

Addition of intercalating compound into the ct-DNA solution results in an increase in viscosity. Usually the viscosity is plotted with concentration on the x-axis (generally as the concentration of ligand divided by the concentration of DNA, [L]/[DNA]) and the viscosity ratio on the y-axis $(\eta/\eta_0)^{1/3}$, where η is the viscosity time in seconds of the fully bound ct-DNA solution (see Fig. **11**).

Circular Dichroism

CD is a useful technique for determining whether drug intercalation induces any global DNA conformational changes and for determining the binding affinity of a drug (Fig. **12**).

Due to their chirality, different DNA conformations are able to interact with polarised light in different ways, rotating it from its normal plane. This interaction gives rise to a CD spectrum, a measure of the difference in absorption of left and right handed circularly polarised light; however, the CD spectrum of DNA is also a function of the base composition and sequence [205].

Recently, Stootman *et al.* have reported a new method for determining the binding constant of achiral intercalators to DNA which utilises a least squares fitting method [206]. CD spectra are obtained by titrating an intercalator into a solution containing a fixed concentration of DNA. Subtraction of

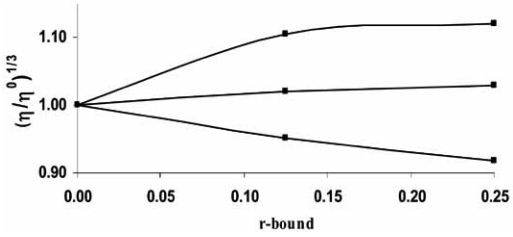

Fig. (11). A plot of r_b (number of bound drugs per DNA nucleotide) versus $(\eta/\eta_0)^{1/3}$ showing from the top down, the increase in viscosity with the addition of the intercalator ethidium bromide, the negligible effect on viscosity with the addition of the groove binder Hoechst 33258 and the decrease in viscosity with the addition of the coordinate covalent binder cisplatin.

the CD spectrum of the DNA in the absence of intercalator, from the spectra obtained with increasing intercalator concentration produces induced circular dichroism (ICD) spectra, from which a binding curve is generated. The binding constant is then determined from the change in molar ellipticity:

$$y = 0.5R\left\{A + B + x + \sqrt{(A + B + x)^2 - 4Bx}\right\} \qquad \text{Eq. 4}$$

where $A = 1/K_b$, B is the concentration of DNA, x is the variable concentration of intercalator, y is the change in molar ellipticity, and R is a scaling factor.

The concentration of DNA can be determined beforehand from UV-vis experiments:

$$C = \frac{A}{b\varepsilon} \qquad \text{Eq. 5}$$

where C is the oligonucleotide concentration, A is the measured absorbance at 260 nm, ε is the molar extinction coefficient and b is the cell path length (usually 1 cm). The molar extinction coefficient (at 260 nm) of the two most commonly used DNA types poly (dG.dC).poly(dG.dC) and ct-DNA are 1.48×10^4 and 1.32×10^4 M^{-1} cm^{-1}, respectively [207, 208]. Where the extinction coefficient is not known, it can be estimated by multiplying the number of nucleotides by 6600 M^{-1} cm^{-1}.

Fluorescence

Fluorescence spectrophotometry is primarily used to determine binding constants of intercalators with DNA, particularly where very small concentrations of DNA and intercalator are used. Many DNA intercalators are inherently fluorescent. The exception seems to be some platinum complexes, which are not fluorescent despite incorporating fluorescent ligands. DNA exhibits some intrinsic fluorescence, but the emission is too weak, and too deep in the ultra-violet spectrum for practical emission applications [209]. Instead, changes in the emission spectrum of the intercalators are monitored. The emission of many DNA intercalators is quenched in physiological solvents, ultra pure water or buffer, but increases when removed from an aqueous environment (i.e. upon intercalation into DNA, see Fig. **13**). For

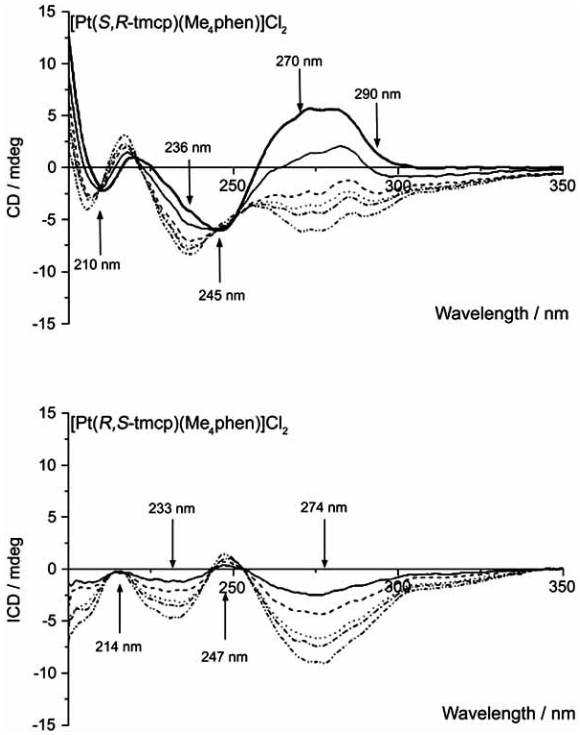

Fig. (12). The circular dichroism spectra (top) of ct-DNA (the solid black line indicates initial B-type DNA conformation) with increasing $[Pt(S,R\text{-tmcp})(Me_4phen)]^{2+}$ intercalator concentration and the induced circular dichroism spectra (bottom) showing the net effect of intercalation on DNA.

example, the fluorescence intensity for Etbr increases 30-fold upon intercalation and its emission lifetime increases from 1.7 to 20 ns [209]. A similar increase in fluorescence intensity is exhibited by the octahedral ruthenium-intercalator $[Ru(bipy)_2dppz](PF_6)_2$. Its fluorescence is nearly undetectable in aqueous solvents, but is greatly enhanced upon intercalation into double-stranded DNA [61, 210, 211]. The increase in fluorescence is attributable to the shielding of the dppz nitrogen atoms from water [209]. Thus the fluorescence intensity is directly related to the number of intercalator molecules bound to DNA.

The binding constant of fluorescent intercalators can be determined through the titration of the intercalator into a fixed concentration of DNA or through the titration of DNA into a fixed concentration of intercalator. The binding constant of the intercalator can then be determined [212-214]:

$$C_F = C_T(I/I_O\text{-}P)/(1\text{-}P) \qquad \text{Eq. 6}$$

where C_T is the concentration of added intercalator and C_F is the concentration of the free intercalator, I and I_O are the fluorescence intensities in the presence and absence of DNA, respectively, and P is the ratio of the observed fluorescence quantum yield of the bound intercalator to that of the free complex. The value of P is obtained from a plot of I/I_O versus 1/[DNA], such that it is the limiting fluorescence yield given by the y intercept. The amount of bound intercalator (C_B) at

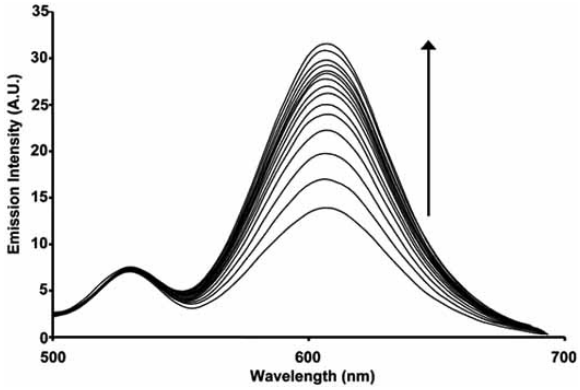

Fig. (13). Fluorescence spectra of the bis-intercalator [{Ru(dpq)$_2$}$_2$ μ-(phen-4-SOS-4-phen)]$^{4+}$ [219] showing the increase of fluorescence intensity with increasing ct-DNA concentration.

any concentration is equal to C_T-C_F. A plot of r/C_F versus r, where r is equal to C_B/[DNA] is plotted:

$$r/C_F = K_b(1\text{-}nr)\{1\text{-}nr)/[1\text{-}(n\text{-}1)r]\}^{n-1} \quad \text{Eq. 7}$$

where K_b is the binding constant of the intercalator and n is the binding site size in base-pairs.

Binding constants of non-fluorescent DNA intercalators can still be determined by fluorescence spectrophotometry through the use of fluorescent guest displacement experiments. In this case, a solution containing a fixed concentration of a fluorescent intercalator (usually Etbr) and DNA is prepared and the non-fluorescent intercalator is titrated into the solution. As the concentration of the non-fluorescent intercalator is increased, it displaces the fluorescent intercalator from the DNA helix, decreasing the fluorescent intensity until saturation of the DNA by the non-fluorescent intercalator is achieved [11, 215]. The change in fluorescence intensity is then used to calculate the binding constant *via* a simple Scatchard plot:

$$r_{Etd}/C_{Etd} = (n\text{-}r_{Etd})[K_{Etd}/(1\text{-}K_mC_m)] \quad \text{Eq. 8}$$

where r_{Etd} is the ratio of bound Etbr to total nucleotide concentration, C_{Etd} is the concentration of free Etbr, n is the maximum value of r_{Etd} (maximum number of binding sites), K_{Etd} and K_m are the intrinsic binding constants for Etbr and the non-fluorescent intercalator, respectively, and C_M is the concentration of free non-fluorescent intercalator. This requires that there is no metal complex/Etbr binding which can be determined by titration of the Etbr and metal complex without DNA.

Thermal Denaturation of DNA

Double-stranded DNA unwinds into single strands upon heating. Intercalation of a compound into DNA stabilises the helix, thus increasing the temperature at which it melts. As the intercalator concentration is increased, the degree of stabilisation also increases, such that the stabilising power of a series of intercalators can be compared by calculating the melting temperature (where the ratio of double-stranded to single-stranded DNA is 1:1) of DNA in the bound and unbound states. In a typical experiment a solution of ct-DNA in buffer is prepared and its absorbance at 260 nm measured using UV-vis spectroscopy. The intercalator is titrated into the DNA solution until there is no longer a change in the DNA melting temperature, which is given by the point of inflection on the sigmoidal graph.

CONCLUSIONS

DNA intercalators remain a powerful class of anticancer agents that have the potential to overcome the resistance to many drugs currently on the market for treating human cancers. In particular, chiral platinum intercalators, bis-intercalators and synergistic intercalators have shown high levels of activity in drug resistant cells *in vitro* and *in vivo*. Modern advances in spectroscopic techniques now mean that the interactions between DNA and intercalators can be examined at levels, and in ways, never before possible. New insights into their DNA binding continue to provide important information on structure-activity relationships, which drive the design and synthesis of new drugs that are not only able to overcome resistance, but also have lower, more manageable side-effects.

ABBREVIATIONS

2-MPD	=	2-methyl-2,4-pentanediol
A	=	Adenosine
AML	=	Acute myelogenous leukaemia
bdppz	=	Benzodipyrido[*b*:3,2-*h*:2′,3′-*f*]phenazine
bipy	=	2,2′-bipyridine
bp	=	Benzo-[*f*][1,10]phenanthroline
bpm	=	2,2′-bipyrimidine
bzimpy	=	2,6-(bis[benzimidazo-2-yl)pyridine
C	=	Cytosine
C4(cpdppz)	=	*N,N*′-bis(12-cyano-12,13-dihydro-11H-8-cyclopenta[*b*]dipyrido[3,2-*h*:2′,3′-*j*]phenazine-12-carbonyl)
CD	=	Circular dichroism
CN-np	=	Naphtho[2,3-*f*][1,10]phenanthroline-9-carbonitrile
CN_2-np	=	Naphtho[2,3-*f*][1,10]phenanthroline-9,14-dicarbonitrile
ct-DNA	=	Calf thymus-DNA
dach	=	1,2-diaminocyclohexane
didppz	=	11,11′-bis(dipyrido[3,2-*a*:2′3′-*c*]phenazinyl
DLT	=	Dose limiting toxicity
dmphen	=	2,9-dimethyl-1,10-phenanthroline
DOSY	=	Diffusion-ordered NMR spectroscopy
dpq	=	Dipyrido[*3,2-d*:2′*3′-f*]quinoxaline
dpqc	=	Dipyrido-6,7,8,9-tetrahydrophenazine
dppz	=	Dipyrido[*3,2-a*:2′*3′-c*]phenazine

DQFCOSY	=	Double quantum filtered correlated spectroscopy
en	=	Ethylenediamine
Etbr	=	Ethidium bromide
FDA	=	Food and Drug Administration (USA)
G	=	Guanosine
Gly	=	Glycine
HET	=	2-hydroxy-ethanethiol
His	=	Histidine
HIV	=	Human Immunodeficiency Virus
HSQC	=	Heteronuclear single-quantum coherence spectroscopy
ICD	=	Induced circular dichroism
LD	=	Linear dichroism
Lys	=	Lysine
MDS	=	Myelodysplastic syndrome
Mebipy	=	4-methyl-2,2′-bipyridyl
Me_2bipy	=	4,4′-dimethyl-2,2′-bipyridyl
MS	=	Multiple Sclerosis
NMR	=	Nuclear magnetic resonance spectroscopy
NOE	=	Nuclear overhauser effect
NOESY	=	Nuclear overhauser enhancement spectroscopy
np	=	Naptha[2,3-*f*][1,*w*]phenanthroline
p-npip	=	2-(4-nitrophenyl)inidazo[4,5-*f*]-1,10-phenanthroline
PGSE	=	Pulsed-gradient spin echo
phen	=	1,10-phenanthroline
phi	=	9,10-diaminophenathrene
Rf	=	Resistance factor
R,R-Me_2 trien	=	2*R*,9*R*-2,9-diamino-4,7-diazadecane
R,S-tmcp	=	1R,3S-1,3-diamino-1,2,2-trimethylcyclopentane
SDS	=	Sodium dodecyl sulphate
SOS	=	2-mercaptoethyl ether
T	=	Thymine
terpy	=	2,2:6',2'-terpyridine
TrxR	=	Thioredoxin reductase
UV-vis	=	Ultra violet – visible

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