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Review

Application of high-performance liquid chromatography for recognition of covalent nucleic acid modification with anticancer drugs

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ABSTRACT

Covalent modification of DNA by antineoplastic agents represents a potent biochemical lesion which can play a major role in drug mechanism of action. The ability to measure levels of DNA covalent modifications in target cells *in vivo* may, therefore, be seen as the ultimate form of therapeutic drug monitoring. Additionally, elucidation of the structure of critical DNA adducts and definition of their role in tumour cell cytotoxicity will provide more selective targets for rational drug design of new cancer chemotherapeutic agents. High-performance liquid chromatography has contributed significantly to all these areas. *In vivo* levels of nucleic acid covalent modifications are in the range of 1 in 10^5–10^8 nucleotides precluding the use of conventional high-performance liquid chromatographic detection methods. Several classes of natural product anticancer drugs have been shown to bond covalently to nucleic acids under optimal laboratory conditions. These have proved more accessible to high-performance liquid chromatographic analysis because of their lipophilicity and strong UV chromophores. However, the majority of experimental evidence to date suggests that with the exception of mitomycin C and morpholino-anthracyclines these compounds do not exert their primary mechanism of action through nucleic acid covalent modification. DNA adducts of alkylating and platinating agents are more difficult to detect by high-performance liquid chromatography and can be chemically unstable. These compounds interact with DNA on the basis of chemical kinetics. Thus, the principle sites of attachment tend to be with the most nucleophilic base (guanine) at its most reactive centre (N-7 position). Limited *in vivo* high-performance liquid chromatographic studies with all classes of anticancer drugs indicate a much more complex pattern of adductation than would have been anticipated from *in vitro* studies alone. Some of these differences are probably due to methodological artefacts but these studies stress the need for sensitive detection methods and reliable sample preparation (nucleic acid extraction and digestion techniques) when attempting to determine nucleic acid covalent modifications by anticancer drugs.

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LIST OF ABBREVIATIONS

AP	Alkaline phosphatase
BCNU	Bis-chloroethylnitrosourea
BFNU	Bis-fluoroethylnitrosourea
CBDCA	*cis*-Diammine-1,1-cyclobutane-dicarboxylatoplatinum(II)
CCNU	Chlorocyclohexylnitrosourea
cDDP	*cis*-Diamminedichloroplatinum(II), cisplatin
DDTC	Diethyldithiocarbamate
^{3}[H]-DEP	*cis*-[^{3}H]Dichloro(ethylenediamine)-platinum(II)
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DOX	Doxorubicin, adriamycin
DAUNO	Daunorubicin
FPLC	Fast protein liquid chromatography
GF-AAS	Graphite furnace atomic absorption spectroscopy
HPLC	High-performance liquid chromatography
HRP	Horse radish peroxidase
ICP-MS	Inductively coupled plasma mass spectroscopy
MMC	Mitomycin C
MNU	Methylnitrosourea
NME	N_2-Methyl ellipticine
P1	Nuclease P1
PFM	Porfiromycin
RNA	Ribonucleic acid
RP-HPLC	Reversed-phase HPLC
SVD	Snake venom diesterase
TBA	Tetrabutylammonium phosphate
TLC	Thin-layer chromatography
UV	Ultraviolet
3-D TLC	Three-dimensional thin-layer chromatography

1. INTRODUCTION

Out of the 50 or so cytotoxic drugs that are commercially available to treat cancer, the majority of these interact with DNA resulting in an alteration in its functionality to act as the template for the genetic code. One form of nucleic acid modification, covalent bonding, the subject

of this review, is generally considered to be a critical event in drug mechanism of action because of its persistence. In fact, it has been estimated that only one molecule of the antineoplastic agent cisplatin is required to bind covalently to one molecule of DNA in a chinese hamster ovary cancer cell in order to produce cell death [1]. It is now apparent that cytotoxicity is not brought about exclusively through covalent binding but requires a series of downstream biochemical events which to date are only poorly characterised [2,3]. As a consequence, levels of DNA covalent adducts need not necessarily always correlate with levels of drug therapeutic activity [4,5], and this is an important consideration that has to be borne in mind when performing such determinations. An equally significant concern, with compounds where irreversible binding to nucleic acids occurs under optimal laboratory conditions, is whether DNA modification *in vivo* is important in the drug's mechanism of action. Whilst a high degree of covalent binding to DNA and RNA has been demonstrated *in vitro* with doxorubicin after enzyme catalyzed anaerobic bioreduction [6,7], no increases in the levels of four detectable drug/DNA adducts were recorded *in vivo* in tumour tissue [5] after the same form of metabolism.

In this review on HPLC techniques for recognition of covalent nucleic acid modification with anticancer drugs, we intend to focus on the three major issues raised above:

(1) Since adducts can be pharmacologically relevant at very low concentrations, are HPLC detection methods available which are sufficiently sensitive and reliable to measure these levels?

(2) Are covalent DNA modifications implicated in the drug's mechanism of action: *i.e.* is it worthwhile attempting the difficult process of measuring the adducts in the first place?

(3) Once individual adduct levels have been determined how do these relate as indices of therapeutic activity?

In the first part of the review we will deal, in general, with HPLC methodologies and sample preparation techniques examining particularly their suitability for *in vivo* application. The second part concentrates in depth on those established classes of anticancer drugs where a large body of evaluable experimental data has been reported on the role of DNA adducts in drug mechanism of action.

2. DETECTION METHODS

A number of detection options are available and the pros and cons of these are summarised in Table 1. Only two techniques have been applied *in vivo* with any degree of success. First, immunologic methods (polyclonal rabbit antisera) for cisplatin/DNA adducts coupled to fast protein liquid chromatographic (FPLC) separation of nucleotides [8–10]. Second, ^{32}P-postlabelling coupled either to three-dimensional (3-D) TLC separations (using PEI-cellulose plates) for mitomycin C/DNA adducts [11,12] or gradient elution, ion pairing, reversed-phase HPLC for doxorubicin/DNA adducts [5]. The limitations of both these techniques are highlighted in Table 1. For immunologic detection, column fractions have to be collected which are then reacted with antibodies. A major drawback with this approach is that antibodies are not generally available, thus, different laboratories have had to raise their own preparations with the resultant problems of differential specificity [13 and refs. therein]. ^{32}P-Postlabelling is discussed in more detail below (Section 2.1.).

Electrochemical and fluorescence detection methods have had only limited application with anticancer drugs. The former technique has been used extensively to measure DNA adducts produced by environmental carcinogens [14]. UV–visible detection is probably the most extensively used method, but is restricted mainly to the analysis of *in vitro* incubations performed in aqueous media with drug and naked nucleic acid, oligonucleotides or single nucleotides/nucleosides (and activating system if required). A level of modification several orders of magnitude greater than achieved *in vivo* is necessary in order to detect adducts [15]. These incubations take little account of the biological milieu in which nucleic acid modifications occur *in vivo*, the presence of nuclear proteins [16], nucleic acid sequence [17] and

TABLE 1

DETECTION METHODS USED IN HPLC RECOGNITION OF ANTICANCER DRUG/NUCLEIC ACID COVALENT ADDUCTS

Detection method	Limit of detection		Maximum nucleic acid loading (μg)	Applicability *in vivo*	Specificity	Limitations	Ref.
	On column	Adducts/ nucleotide					
UV–visible	pmol	$1/10^4$	100	No	Not specific for modified nucleic acids	Adduct standards required	16
Atomic absorption	pmol	$1/10^6$		No	Highly specific	Limited mainly to platinum containing compounds	162
^{14}C-labelling of precursor	pmol	$1/10^6$	100	Yes	Identifies individual adducts	Limited sensitivity	163
^{3}H-labelling of precursor	fmol	$1/10^7$	100	No	Identifies individual adducts	Prone to errors due to exchange of label	163
Fluorescence	fmol	$1/10^7$	100	Yes	Identifies individual adducts	Limited number of anti-cancer drugs fluorescen	164
Electrochemical	fmol	$1/10^7$	10–20	Yes	Identifies individual adducts	Electroactive guanosine causes interferences/ ultra pure reagents required. Very limited application in cancer drug studies	14
Immunological	low fmol	$1/10^8$–$1/10^{10}$	50	Yes	Can be highly specific for adducts	Problems are encountered with both cross-reactivity and lack of reactivity with individual adducts	13
Fluorescence postlabelling	pmol	$1/10^8$	100	Yes	Highly specific for adducts	Relatively new technique requires two column steps	165
^{32}P-Postlabelling	low amol	$1/10^{10}$	10	Yes	Preference for aromatic/ hydrophobic adducts	Most sensitive technique available	20

the tertiary structure of chromosomes [15] all of which can alter patterns of adductation. Consequently, molecular biology techniques are being increasingly applied to drug/DNA adduct measurements where it is becoming possible to identify lesions in specific genes [18,19]. Nonetheless, *in vitro* incubations and HPLC analyses using UV detection are regarded as a useful first stage in the elucidation of the structure of potential adducts and in characterising potential mechanisms of drug activation.

2.1. ^{32}P-Postlabelling

^{32}P-Postlabelling is a technique where a modified DNA specimen is digested to 3′-monophosphates and then using T4 polynucleotide kinase ^{32}P-phosphate is transferred from ATP to the available 5′-site [reviewed in 20]. The net result is that cold nucleotides are converted into labelled nucleotides which can then be detected with high sensitivity. The method was developed originally for 3-D TLC, where the overwhelming majority of radioactivity associated with unmodified bases could be washed off the plate on to filter paper by two preliminary elutions run in different directions [21–23]. Subsequently, modified bases, which should remain at the centre of the layer, were resolved by two further elutions. In this conventional format ^{32}P-postlabelling is not suitable for HPLC because of the problem of eliminating the vast excess of radioactivity associated with the

unmodified bases. With the advent of the nuclease P1 enhancement of the technique [24] (P1 removes the 3′-phosphate group only from unmodified bases which then take no further part in the labelling reactions resulting in only modified bases becoming labelled) it has been possible to employ HPLC. Despite the prospect of better resolution, HPLC has not been used widely with postlabelling in the determination of anticancer drug/nucleic acid modifications [20].

There are two potential pitfalls with ^{32}P-postlabelling and these particularly effect HPLC applications. First, whilst it has been shown that many aromatic and bulky non-aromatic DNA adducts are resistant to nuclease P1 digestion (which is a prerequisite for HPLC) [24], certain smaller adducts such as those produced by alkylating agents like nitrogen mustards and nitrosoureas are substrates [20]. Therefore caution has to be recommended when using nuclease P1 treatment for adducts whose chemical identity (and therefore ability to resist P1 digestion) is unknown. It has been reported that major cisplatin/DNA adducts are only labelled with 0.1% efficiency [25]. Secondly, not all modified bases are equally good substrates for T4 kinases, introducing further quantitative uncertainties into this method. Significantly, it is with the aromatic anticancer drugs mitomycin C, doxorubicin and nitracrine [26] (and aziridinyl benzoquinones, personal communication, John Butler, Paterson Institute of Cancer Research, Manchester, UK) where positive identification of DNA adducts has been reported using ^{32}P-postlabelling.

3. SAMPLE PREPARATION

HPLC determination of DNA adducts *in vivo* necessitates extracting the nucleic acid from the tissue or tumour of interest and in many cases digesting the nucleic acid to constitutive nucleotides/nucleosides. Due to the instability of many anticancer drug/DNA adducts (nitrogen mustard and cyclophosphamide N-7 guanine monoadducts have half lives of 1–3 h at room temperature [27], doxorubicin cross-linked adducts are unstable at alkaline pH [28]), specimens should be analysed as quickly as possible with the minimum of storage (at −80°C).

3.1. Extraction of DNA from tissue and tumour

Modified methods based on that first described by Marmur are most commonly used to isolate DNA [29]. Proteinase K is included to digest protein and a variety of ribonucleases (A and T1) are included to remove RNA. Samples are extracted with chloroform–isoamyl alcohol and the DNA is precipitated from the aqueous phase with ethanol. This technique is rather time consuming and requires that the isolated DNA is redissolved in a buffer before further sample preparation is possible. Ethanol precipitation of samples containing small amounts of DNA can be inefficient [30]. An alternative (and faster) approach is to use hydroxyapatite column chromatography [31], but difficulties can be encountered with contamination from RNA. More recently HPLC methods have been published using DEAE-anion exchange columns [30] for rapid isolation of DNA from small amounts of tissue. Such methods require that the HPLC column is fully conditioned and free from material that can remain adsorbed on these columns. With non-porous DEAE resins (NPR) manufacturers recommend washing the column at the end of each run [32].

3.2. Digestion of nucleic acids

Different digestion techniques can produce a three-fold variation in the levels of specific DNA adducts measured [33]. We have also observed that a particular protocol which gives rise to a high yield for one class of compound (doxorubicin) need not necessarily produce high yields for other classes of compound (cisplatin) (unpublished observations).

Monofunctional adducts of alkylating and platinating agents can remain chemically active unless quenched by addition of a nucleophile and can inactivate the enzymes present in digestion cocktails. Certain workers recommend the addition of "top up" enzymes during the digestion of heavily modified nucleic acids [34]. Digestion

protocols should be validated by incorporating a radioactive label into intact DNA (normally [^{3}H]thymidine). Specific examples of digestion methods for individual anticancer drugs are to be found below in the appropriate sections.

4. BIOREDUCTIVE ALKYLATING AGENTS

Bioreductive alkylating agents are compounds that contain latent functionalities which only upon reduction are activated into DNA reactive species. Many of these compounds have been shown to be activated preferentially under anaerobic conditions and to be more effective against cells grown under low oxygen tension [35]. This has lead to the concept that these drugs may be selective against hypoxic cell populations present in solid tumours which are difficult to treat with conventional cytotoxic drugs and radiotherapy. The selectivity of an anaerobic bioreductive alkylating agent is defined by the hypoxic cell cytotoxicity ratio (HCR), which is the ratio of the dose of drug required to kill 50% of the cell population grown under oxic conditions divided by the dose of drug required to kill 50% of the cell population grown under hypoxic conditions (assuming all else is equal).

4.1. Mitomycins

The original mitomycins are natural products which were isolated from *Streptomyces caespitosis*

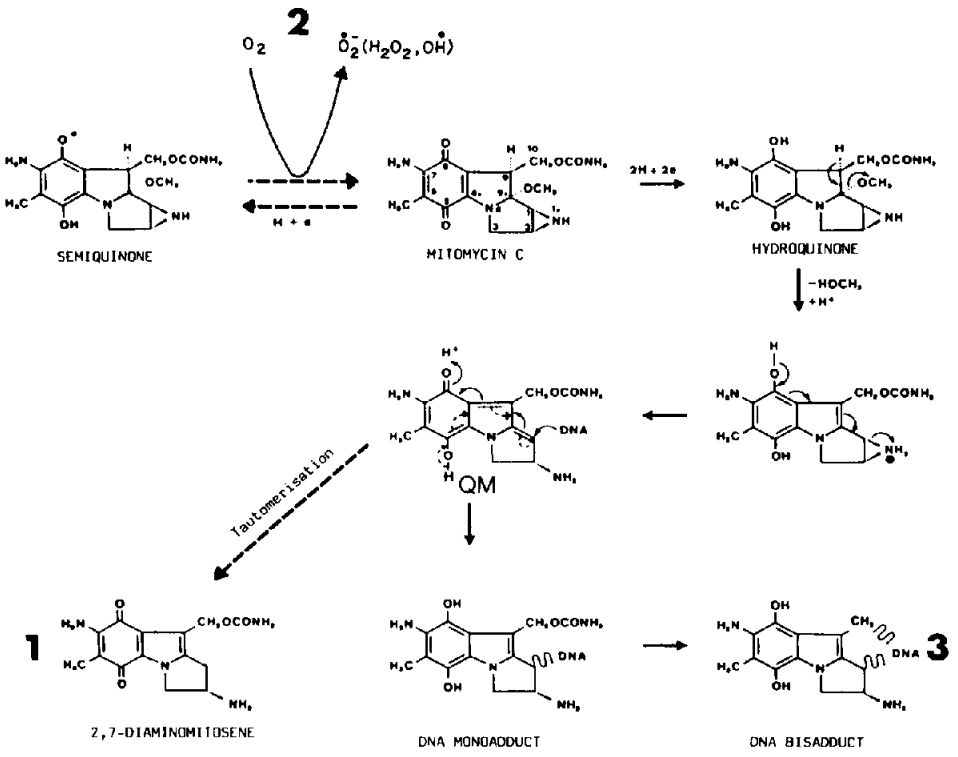

Fig. 1. Proposed pathways of metabolic activation of mitomycin C to DNA reactive species [41–44]. Under aerobic conditions one electron reduction produces the semi-quinone free radical which redox cycles with molecular oxygen to generate a cascade of reactive oxygen species (pathway 2). Under anaerobic conditions for one electron reduction and aerobic or anaerobic conditions for two electron reduction a quinone methide intermediate is formed (QM). Acting as an electrophile the QM can alkylate DNA at C-1 (solid line, pathway 3) or water to give the *cis*- and *trans*-1-hydroxy-2,7-diaminomitosene metabolites. Acting as a nucleophile the QM reacts with a proton (broken line) to yield 2,7-diaminomitosene (pathway 1). The C-1 drug monoadduct then reacts with a second site on DNA at C-10 after decarbamoylation.

in 1956 [36]. The first two compounds were designated mitomycin A and B and although they exhibited potent antitumour activity, they were not pursued as anticancer drugs because of very high toxicity in animals. This high toxicity has been attributed to non-selective metabolic activation and cross-linking of DNA in both aerobic and anaerobic cells occurring as a direct consequence of a higher redox potential [37]. Structure activity studies indicate that a favourable quinone reduction potential, good water solubility and low lipophilicity improve antitumour activity [38].

4.1.1. Mitomycin C

Mitomycin C (MMC, structure contained within Fig. 1) was isolated in 1958 and shown to have superior antitumour activity with less toxicity compared to other mitomycins [39]. It remains an important component in combination chemotherapy of breast and prostate cancer, is one of only a few drugs with even marginal activity against colorectal cancer, and is probably the drug of first choice for intravesical administration in superficial bladder cancer [40]. MMC is the only clinically useful natural product anticancer drug believed to work exclusively through DNA alkylation. The unique biologic properties of MMC are due to three significant chemical features: a quinone moiety capable of free radical formation and redox cycling with molecular oxygen; a fused aziridine ring which can promote DNA alkylation; and a carbamate function which can undergo SN_2 reactions and is also implicated in bifunctional alkylation of DNA.

MMC is the prototype bioreductive alkylating agent and its proposed mechanism of action involves drug metabolism via quinone reduction, operating preferentially (or exclusively) under anaerobic conditions, resulting in activation of bonding moieties at C-1 and C-10 [41–44]. The reaction pathway accepted by most investigators is shown in Fig. 1 although other schemes have been proposed [45–48]. A number of different monofunctional and bifunctional DNA adducts have been identified [34,49–53] but cross-links are generally considered to be the critical DNA lesion in MMC induced cytotoxicity [54]. These data firmly establish the case of measuring DNA modifications induced by MMC and analogues.

Perhaps, with no other single anticancer drug has HPLC made such a large contribution to our understanding of molecular mechanisms involved in drug metabolic activation and the factors which govern covalent binding to DNA. This is mainly due to two beneficial characteristics for the HPLC analyst. First, rather than depositing a small alkyl moiety on to nucleic acids, MMC alkylates DNA with its ring system intact (see Fig. 1). Thus, MMC-nucleotide/nucleoside covalent adducts are lipophilic and can be readily resolved from unmodified species by reversed-phase HPLC. Second, MMC has a complex, highly characteristic UV–visible chromophore which is sensitive to changes in the bonding configuration of the mitosane ring system. Therefore, structural changes can be identified with a diode array detector without recourse to purification of peaks and the use of a battery of spectroscopic techniques. HPLC has been employed in four major ways: (1) in the identification of the various reaction intermediates and products that form after activation of MMC; (2) in determining the base preference of the monofunctional alkylation process; (3) in determining the sequence selectivity of the bifunctional process; and (4) in the purification and structural characterization of adducts.

4.1.1.1. Specific HPLC techniques. The first reports of the use of HPLC to measure MMC/DNA adducts formed after reductive activation under neutral conditions (5% palladium catalyst on charcoal and H_2 gas) were provided by Hashimoto *et al.* [49,50,55]. DNA was digested with nuclease P1 and nucleotides were chromatographed on a Polygosil C_{18} reversed-phase column (25 cm × 5 mm I.D.) using a mobile phase of 10% acetonitrile in 0.3% ammonium chloride (flow-rate 0.8 ml/min). Detection was by UV at 310 nm (λ_{max} for the reduced mitosene ring which becomes adducted to DNA, see Fig. 1). Three adducts were resolved with retention times (t_R) of 5, 6 and 7 min, respectively. These were purified and identified (in decreasing order of importance) as O-6 guanine, N-2 adenine and N-2 guanine

attached to the C-1 position of MMC. A similar profile of adducts was observed in digested DNA extracted from the liver of rats but levels of modification *in vivo* were 1–2 per 10^4 nucleotides.

Using an earlier developed gradient separation method employing a 10 cm × 8.0 mm I.D. Radial-Pak C_{18} (10 μM particle size) column module and gradient elution (buffer A, 10 mM potassium phosphate (pH 7.0); buffer B, 50% methanol–50% 10 mM potassium phosphate (pH 7.0); linear gradient from 0% B to 100% B over 13 min, flow-rate 3 ml/min), O-6 guanine attached to C-1 MMC was also identified as the major monofunctional adduct [51,56]. Based on resistance to nuclease digestion it was estimated that 50% of all MMC bound to DNA was present as cross-linked drug. The major adduct which had a t_R of 14.0 min as a nucleoside was purified by preparative HPLC and characterised tentatively by NMR. Three different digestion techniques were investigated: nuclease P1 (as described by Hashimoto and Shudo [55]; S1 nuclease; and a combination of DNAase I, snake venom phosphodiesterase (SVD) and alkaline phosphatase (AP). Each gave different results. Nuclease P1 and S1 nuclease treatment produced four adducts peaks (t_R 7.4 min, 7.9 min, 8.2 min and 8.6 min) whilst the DNAase protocol yielded the single adduct at 14.0 min. Further incubation of S1 or P1 digests with DNAase I converted the four adduct peaks into the single adduct peak and it was concluded that these two nucleases were not suitable for MMC-modified DNA due to incomplete digestion. This gradient methodology has become the standard for the analysis of patterns of MMC metabolism after *in vitro* enzyme catalysed bioreduction [47,57–59]: particularly for the identification of the end product of C-1 electrophilic substitution with H^+ (2,7-diaminomitosene, 2,7-DM, Fig. 1), and the end products of nucleophilic substitution of C-1 with water (1,2-*cis* and 1,2-*trans*-1-hydroxy-2,7-diaminomitosene). Recently, an isocratic method has been reported for the *in vivo* determination of these three products after MMC administration to rats or mice (see Fig. 2 and ref. 60).

In careful studies from the laboratory of Tomasz *et al.* [34] and employing isocratic HPLC (stationary phase, Beckman Ultrasphere ODS, 25 cm × 1 cm I.D.; mobile phase, 8% acetonitrile–92% 20 mM potassium phosphate (pH 5); flow-rate, 2 ml/min; detection, UV at 254 nm) a single monofunctional MMC/DNA adduct (t_R 45 min) was also identified after enzymatic activation (cytochrome P-450 reductase or xanthine oxidase) or chemical reduction (H_2/PtO_2). This single

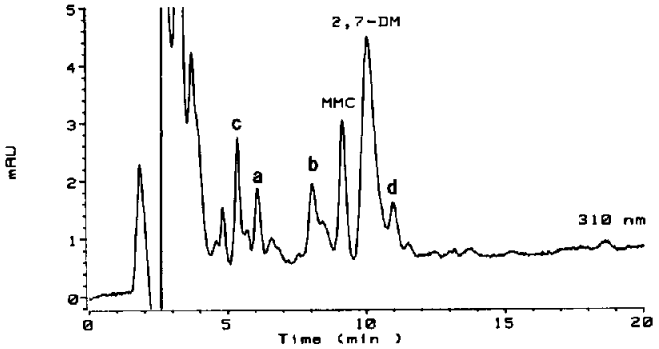

Fig. 2. HPLC analysis of an *in vivo* drug-treated Sp 107 rat mammary carcinoma specimen collected 1 h after intra-tumoural administration of 450 μg of mitomycin C (MMC, taken from Cummings *et al.* [60]). Sample preparation and HPLC are described in ref. 60. The chromatogram was monitored at 310 nm and five putative drug metabolite peaks were detected (peaks a–d and 2,7-DM) along with MMC. MMC (t_R 9.2 min) is mitomycin C (2.63 μg/g tissue). 2,7-DM (t_R 10.1 min) is believed to be the key metabolite product of MMC anaerobic quinone bioreduction (see Fig. 1) 2,7-diaminomitosene (1.04 μg/g). Peak a (t_R 6.1 min, 0.20 μg/g) is 1,2-*trans*-1-hydroxy-2,7-diaminomitosene and peak b (t_R 8.1 min, 0.20 μg/g) is 1,2-*cis*-1-hydroxy-2,7-diaminomitosene. Peaks c and d are unidentified metabolites of MMC.

adduct accounted for virtually 100% of MMC covalently attached to DNA. Using preparative HPLC it was purified and identified by NMR, mass spectroscopy and Fourier transform infrared spectroscopy as the stereo-specific product N2-(2″β,7″-diaminomitosene-1″α-yl)-2′-deoxyguanosine (N2-guanine-C1 MMC). Subsequent studies by independent laboratories have confirmed N-2 guanine as the principal site of covalent attachment of MMC [see ref. 61]. The four adducts of Hashimoto and Shudo [55] were shown to be dinucleotide artefacts (all containing dG and a combination of the four constitutive bases) produced due to incomplete digestion by nuclease P1. The digestion technique adopted by Tomasz *et al.* [34] employed DNAase I, SVD and AP in a 24 h incubation with enzyme replacements. No evidence of bis-adducts was detected using this technique.

Applying the same analytical techniques as above but a different method of drug activation (the chemical reducing agent sodium dithionite) Tomasz *et al.* [52] reported the first direct identification of an MMC covalent cross-linked DNA adduct (t_R 54 min). This adduct was the sole species produced under these conditions and it was confirmed as the N-2 guanine-MMC-N-2 guanine bisadduct. The second site of attachment on MMC was the C-10 position as originally hypothesised by Iyer and Szybalszi in 1964 [41]. Computer models indicated that this bisadduct fits snugly into the minor groove of DNA oligonucleotides with minimal distortion to the structure of B-DNA and provided a rationale for the base stereo-specificity of MMC alkylation. In this classic paper it was also demonstrated by HPLC that the bis N-2 guanine adduct could be detected in digests of DNA extracted from the liver of rats treated with 30 mg of MMC. The digests (containing 11 mg of DNA starting material) had to be first pre-fractionated on Sephadex G-25 (an 800-ml fraction was collected) and concentrated (by freeze drying) prior to loading on to the analytical HPLC column for detection by UV at 254 nm. This same HPLC methodology was utilised further to understand features of the monofunctional alkylation process [44,62,63].

Reddy and Randerath [11] using their ^{32}P-post-labelling TLC method studied the pattern of MMC DNA adducts in the tissues of non-tumour bearing rats treated with 9 mg/kg. An even tissue distribution of ten adducts was observed (9–22 per 10^7 nucleotides). One major species accounted for 71% of all adducts. Using extracted rat liver DNA and a series of homo-oligonucleotides it was shown that the *in vivo* adduct profile seen was qualitatively identical to that produced *in vitro* after chemical reduction of MMC with sodium borohydride and that greater than 90% of *in vivo* adducts were with guanine. Although the major *in vivo* adduct and *in vitro* adduct proved to be different, these data confirm *in vivo* the remarkable specificity of MMC for guanine.

Employing an extensive series of specially synthesised oligodeoxyribonucleotides the sequence selectivity of MMC cross-link formation was studied after chemical activation with sodium dithionite [64,65]. The oligonucleotides were chromatographed by HPLC using a Beckman C-3 Ultrasphere column (25 cm × 4.6 mm I.D.) and gradient elution: buffer A was 0.1 *M* triethylamine (pH 7.0); buffer B was acetonitrile; the normal gradient was 6% B to 18% B in 24 min (native oligonucleotides t_R 12–13 min, covalently cross-linked oligonucleotides t_R 14–16 min). These studies showed that duplexes containing a single central CG.CG gave high yields of cross-links between the two guanines while those having GC.GC gave none. Further, a four-base-pair sequence preference was observed with PuCGPyr.PuCGPyr and this was explained on the basis of reaction kinetics. Absolute sequence selectivity for CG has been confirmed by several independent laboratories using molecular biology techniques [66–68]. Based on molecular modelling studies and energy minimised structures Tomasz *et al.* [69] have proposed an orientation model to explain the sequence selectivity of MMC cross-links. They claim that the C-1 monofunctional adduct complexed at N-2 guanine (which always forms first) can only point in one direction where the C-10 carbamate group of MMC is facing the 3′ direction of the minor groove. Therefore, only in the case where a G is situated 3′ of the monofunc-

tional adduct in the complementary strand of DNA can a cross-link possibly form. This case is only satisfied with CG.CG or less frequently with GG.GG.

Recently, using HPLC, chemical reduction with sodium dithionite, but a less severe digestion protocol, a dGpdG intrastrand MMC cross-link has been identified [53]. The inability to detect this species in the past was explained by the fact that the DNAase I 24 h digestion protocol employed hydrolysed this adduct artefactually into the cross-linked species. Due to its increased water solubility the standard HPLC conditions of Tomasz *et al.* [34] were modified: the stationary phase remained the same but gradient elution was employed: buffer A was 30 m*M* potassium phosphate (pH 5.0); buffer B was acetonitrile and the gradient was from 6% B to 18% B over 30 min at 1 ml/min. The t_R of all major guanine adducts were as follows: inter-strand cross-link, 20 min; monoadduct, 18 min; the new intra-strand adduct, 13 min; and a sulphate adduct produced due to reaction with dithionite, 9 min. Digestion conditions were: nuclease P1 for 2 h at 55°C; SVD for 1 h at 37°C and AP for 2 h at 37°C. In these studies it was shown that inter-strand adductation normally predominates over intra-strand adductation by a ratio of 3.6:1.

4.1.2. Porfiromycin

Porfiromycin (N-1a-methyl-mitomycin C, PFM) is a close structural analogue of MMC, and although not widely used clinically, has similar efficacy and toxicity but is tolerated better by patients [70]. PFM has been shown to be metabolised to three main mitosene products analogous to MMC: the *cis* and *trans* isomers of 7-amino-1-hydroxy-2-methylaminomitosene after nucleophilic attack by water on C-1, and the product of electrophilic H^+ substitution 7-amino-2-methylaminomitosene [71]. These have been identified *in vitro* after enzyme catalysed quinone reduction using the gradient elution HPLC methodology of Andrews *et al.* and Pan *et al.* described for MMC [56,57]. Applying the same methodology Pan and Iracki [71] have identified the major DNA adducts produced after enzyme catalysed activation and nucleic acid digestion with DNAase I, SVD and AP. Four peaks were detected at t_R 19.4 min, 19.6 min, 20.5 min and 21.3 min. One adduct (t_R 19.4 min) was present in overwhelming majority; it was purified and identified as the N-2 guanine C-1 PFM monofunctional alkylation product. Adduct 3 (t_R 20.5 min) was identified as a cross-linked species and it predominated after chemical reduction with sodium dithionite.

Two major *in vivo* studies with cancer cells growing in culture have been performed with radiolabelled PFM and HPLC separation of individual adducts, and these have provided insights into the relationship between events recorded *in vitro* under laboratory conditions compared to events that occur *in vivo* at therapeutic drug concentrations [16,72]. In both studies isotopic labelling was carried out by N-methylation of MMC. Radiolabelled MMC has not been readily available for comparative studies. In the first study a C-14 label was employed, enzyme digestion and HPLC were as previously described [56,57,71] but, uniquely, the products of alkylation of chromatin (DNA wrapped around a protein core) were defined as additional standards [16]. Five adducts were identified in P388 murine leukemia cells (I, 11 min; II, 13.4 min; III, 14.5 min; IV, 15.5 min and V, 17 min) at levels in the range 1 in 10^5 nucleotides well below the limit of detection of the UV monitor. Adduct II was the N-2 guanine C-1 PFM monofunctional product; adduct III was also a guanine product; IV was the cross-linked species and these were analogous to their respective *in vitro* products. Adduct I was unique to *in vivo* and adduct V, the major *in vivo* adduct, was related to alkylation of chromatin. Thus, differences do exist between *in vitro* and *in vivo* patterns of adductation, suggesting caution when interpreting the clinical relevance of *in vitro* derived results. Also of interest, it was shown that in cells grown under hypoxic conditions DNA alkylation increased by a factor of ten in line with an eight-fold increase in cytotoxicity.

Using a tritium label, PFM cross-linked at C-1 and C-10 to the N-2 position of two guanines was detected *in vivo* [72]. HPLC conditions were similar to those previously reported by Tomasz *et*

al. [34]. In this work considerable exchange of tritium into thymidine occurred which then became incorporated into DNA confirming this as a poor label (see Table 1). Approximately ten-fold greater alkylation was observed under hypoxic conditions concomitant with increased cytotoxicity against the EMT6 mouse mammary tumour cells. A total of only three adducts were detected *in vivo*: the cross-linked N-2 guanine species, monofunctional N-2 guanine, and decarbamoyl monofunctional N-2 guanine. The ten guanine adducts observed by Reddy and Randerath *in vivo* [11] by ^{32}P-postlabelling were explained as dinucleotide artefacts produced by spleen diesterase and micrococcal nuclease since these enzymes cannot digest the 3'-internucleotide phosphate group of MMC adducts [72]. When cells were incubated with dicoumarol, an inhibitor of DT-diaphorase the obligate two electron reducing enzyme, levels of all three adducts fell and a completely new adduct t_R 45 min appeared, again highlighting additional complexity *in vivo*.

4.1.3. Miscellaneous

HPLC has been applied less frequently to other bioreductives under clinical development. A variety of reversed-phase stationary phases and elution protocols have been employed to study the binding kinetics of the nitroimidazole aziridine RSU 1069 to 2'-deoxyribonucleoside 5'-monophosphates [73,74] and isotopically labelled drug in conjunction with Sephadex G-50 column chromatography has been applied to study DNA alkylation by the aziridinyl-quinone AZQ [75].

5. ANTHRACYCLINES

The anthracyclines consist of a large group of clinically active, naturally occurring and semi-synthetic antibiotic compounds based on a benzanthraquinone nucleus and carbohydrate side chain and are structurally and functionally divided into three classes [76]. Class I compounds, including doxorubicin (DOX) and daunorubicin (DAUNO) the prototypes for the whole family, are all monosaccharides. The class II compounds, for example aclacinomycin and marcellomycin, are polysaccharides and are distinguished functionally from class I in being more effective inhibitors of RNA synthesis. Included in this class is the non-sugar containing semi-synthetic antibiotic menogaril. The third class has more recently emerged and is characterised by high activity *in vitro* and by a mechanism which probably involves DNA cross-linking. These compounds are based on class I but with substitution of morpholino and cyano-morpholino ring systems onto the amino group of the daunosamine sugar of DOX and DAUNO.

5.1. Doxorubicin and daunorubicin

The major difficulty in measuring DOX nucleic acid covalent modifications is the unambiguous removal of non-covalently bound intercalated drug from DNA. During the earlier part of the 1980s a large number of reports appeared indicating that DOX binds covalently to DNA and RNA (and proteins) [6,7,28,77–83]. With one exception [82] intact nucleic acid was investigated and covalent binding was measured by indirect methods using either isotopically labelled drug (normally ^{14}C) or by measuring the absorbance of the DOX chromophore (visible λ_{max} 480 nm for the native drug; 506 nm for drug bound non-covalently to DNA by intercalation). Long, complex and severe sample preparation techniques potentially harmful to the covalent adducts, which have been shown to be both temperature sensitive (100°C) and alkaline labile (0.3 *M* sodium hydroxide) [28], had to be applied to the nucleic acids in order to remove non-covalently bound intercalated drug. These techniques included incubation overnight at pH 9.0 and washing nucleic acid with 10 *M* HCl [82], and washing nucleic acids with boiling methanol [78,80]. DOX is well known to be extremely difficult to remove from biomolecules by washing techniques due to strong binding through non-covalent interactions [84,85]. It is therefore very likely that these indirect measurements either (a) missed potential adducts due to degradation or (b) greatly overestimated the amount of covalent binding due to incomplete removal of intercalated drug. Sinha and co-

workers [6,7] actually reported covalent modification of DNA as high as 1 adduct per 12 nucleotides after chemical reduction using sodium borohydride or NADPH-enriched liver microsomes and a dependence on anaerobic conditions, both of which have never been confirmed independently [5,28,79,82,83,86].

5.1.1. Determination of covalent modification to intact nucleic acid by intercalators

Recently an HPLC method has been developed which can determine covalent binding to intact nucleic acid by intercalating anticancer drugs (which include anthracyclines, actinomycin, bleomycins, ellipticines (see below) and acridines) quickly, accurately and with virtually no sample preparation required [86]. This is because after priming with 1 mg of DNA, the HPLC column (2 μm non-porous anion exchange resin with DEAE as the bonded phase) behaves as an intercalator affinity column stripping the nucleic acid of non-covalently bound drug during chromatography with 99.8% efficiency but allowing the nucleic acid (up to 50 000 bases in composition) to elute normally (retention mechanism illustrated schematically in Fig. 3). Retained drug is

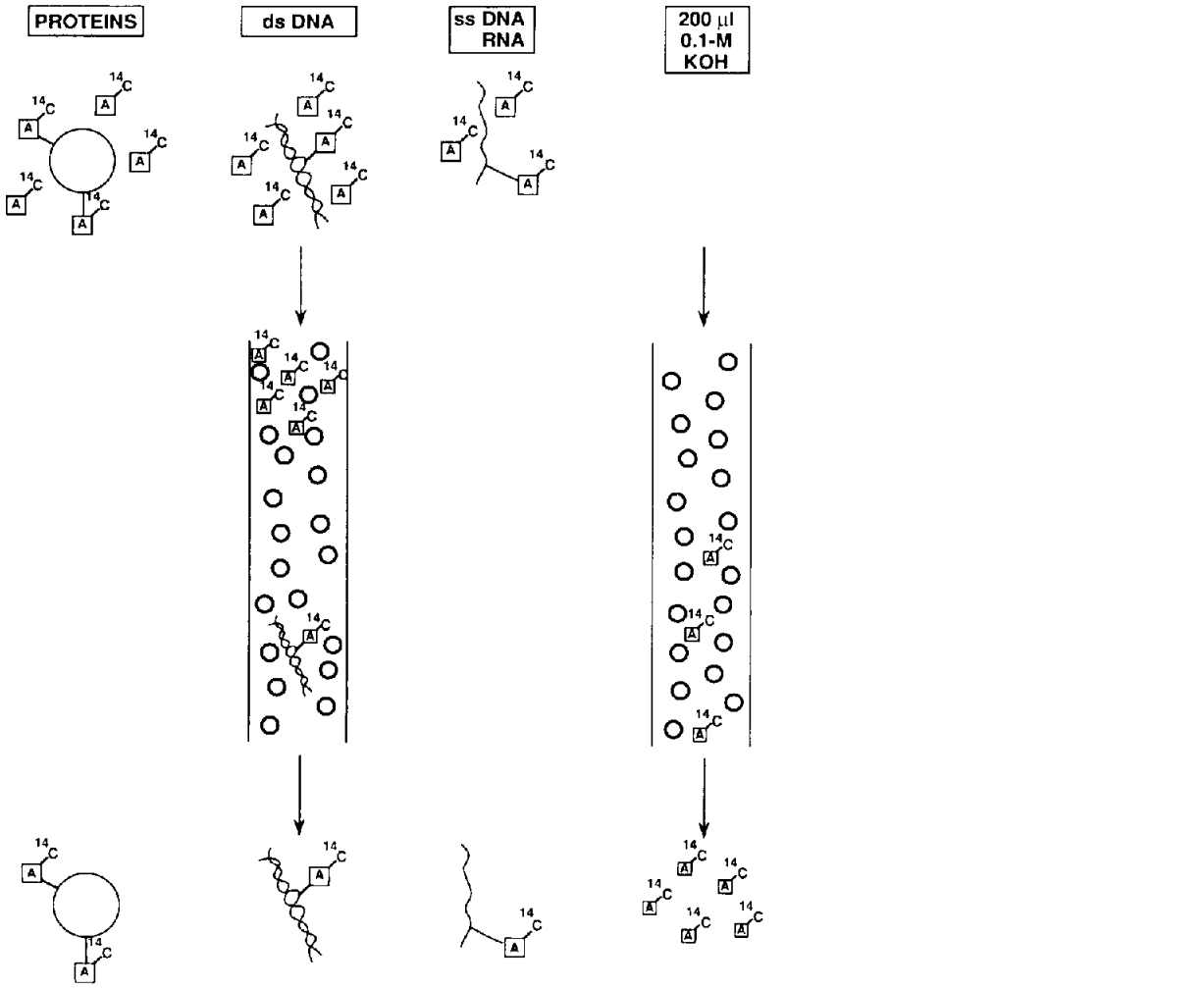

Fig. 3. Schematic representation of the retention mechanism of the Gen Pak FAX column (2 μm NPR resin bonded with DEAE; 10 cm × 4.6 mm I.D.) acting as an intercalator affinity column. The column can strip 99.8% of non-covalently bound drug from the intact nucleic acid allowing for an accurate determination of covalent binding of radiolabelled anticancer drugs (A) to be performed with minimal sample preparation.

removed from the column with an injection of potassium hydroxide. The stationary phase that achieves this effect is a non-porous 2-μm DEAE anion exchange resin (10 cm × 4.6 mm I.D.). Gradient elution is employed in a Tris–HCl buffer (pH 8.0) with increasing sodium chloride concentration. The exceptional ability of this column to resolve nucleic acids is shown in Fig. 4.

Applying the above methodology, no evidence of covalent binding to DNA was observed *in vitro* using NADPH-cytochrome P-450 reductase as the activating system under both aerobic and anaerobic conditions. Low levels of DNA covalent modifications (18.6 pmol/100 μg) were measured in MCF-7 human breast cancer cells after exposure to frankly cytotoxic drug concentrations (50 μ*M*) and this is a common feature with DOX that covalent binding is only detected in biological systems when very high levels of drug are employed [87].

Metabolic pathways of drug activation proposed to explain DOX covalent binding are illustrated in Fig. 5 [42,81]. The one electron reduction pathway produces a semi-quinone free radical intermediate which redox cycles under aerobic conditions and may bind to DNA under anaerobic conditions after chemical rearrangement. The two electron pathway produces a quinone methide intermediate which can either alkylate DNA acting as electrophile or abstract a solvent proton to produce the 7-deoxyaglycone metabolite acting as a nucleophile. Rapid disproportionation of the semi-quinone means that the quinone methide is probably a common intermediate (under anaerobic conditions) regardless of one or two electron reduction [88]. 7-Deoxyaglycone metabolites of DOX can be measured by HPLC in the serum of cancer patients and in the tissues of cancer patients after drug administration [89,90]. However, several studies employing HPLC have shown that the DOX quinone methide is not sufficiently reactive to alkylate DNA [91–93]. Therefore it is unlikely that DOX functions as an anaerobic bioreductive alkylating agent. Indeed, the majority of studies where positive detection of DOX covalent binding was achieved have shown a dependence on aerobic conditions [79,83,86].

5.1.2. Specific HPLC techniques

Confirmation that DOX is *not* an anaerobic bioreductive alkylating agent has been provided

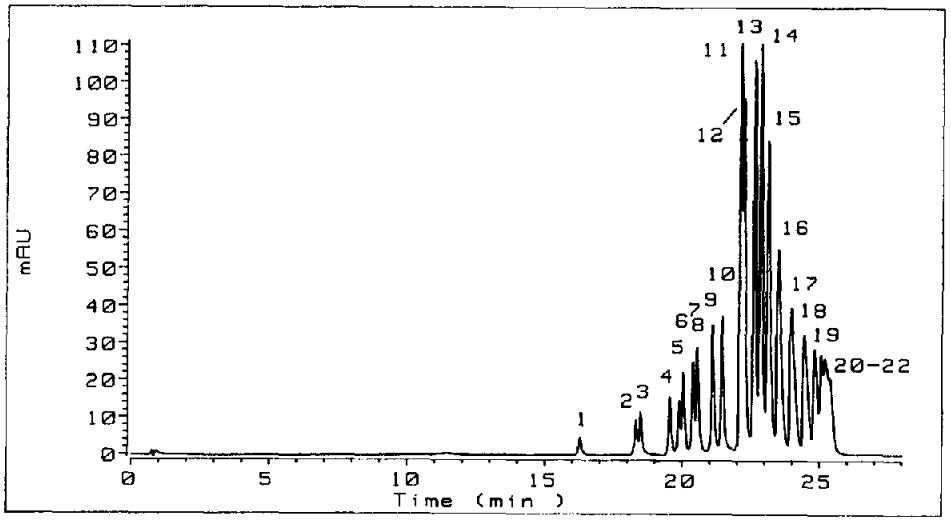

Fig. 4. Separation of 22 components present in a 1Kb DNA ladder (obtained from Gibco-BRL, Paisley, UK) using the Gen Pak FAX column (2 μm NPR resin bonded with DEAE; 10 cm × 4.6 mm I.D.). The mobile phase was 25 m*M* Tris–HCl and 1 m*M* EDTA (pH 8.0) and the flow-rate was 0.8 ml/min. The oligonucleotides were eluted with an increasing sodium chloride gradient (0.4 *M* to 0.8 *M* NaCl over 30 min). Peaks: 1 = 75 base pairs (bp); 5 = 220 bp; 10 = 506 bp; 12 = 1636 bp; 15 = 4072 bp; 17 = 6108 bp; 20 = 9162 bp; 22 = 11 198 bp.

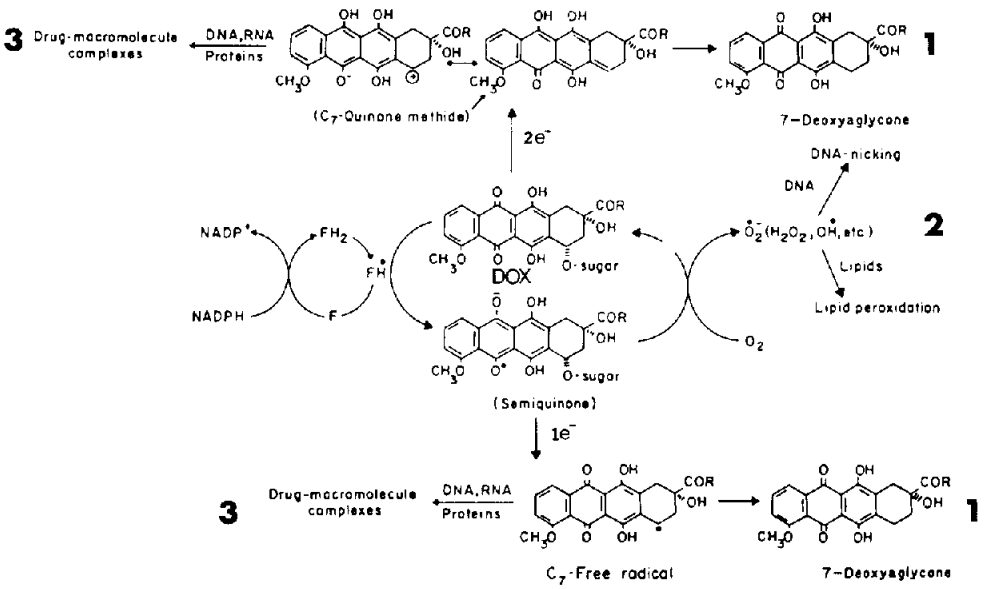

Fig. 5. Proposed pathways of metabolic activation of doxorubicin (DOX) to DNA reactive species [42,81]. Under aerobic conditions one electron reduction produces the semi-quinone free radical which redox cycles with molecular oxygen to generate a cascade of reactive oxygen species (pathway 2). Under anaerobic conditions for one electron reduction and aerobic or anaerobic conditions for two electron reduction a quinone methide (QM) intermediate is formed or less likely a C-7 carbon centred free radical. Acting as an electrophile the QM can alkylate DNA at C-7 (pathway 3); acting as a nucleophile the QM reacts with a proton to form the 7-deoxyaglycone metabolite (pathway 1).

recently *in vivo* in tumour tissue after administration of therapeutic, pharmacologically relevant, doses of the drug [5]. Further, it was shown that levels of DNA covalent modification do not correlate with antitumour activity suggesting that this process is not involved in the drug's mechanism of action (see Fig. 6). DNA adducts were determined by a ^{32}P-postlabelling technique espe-

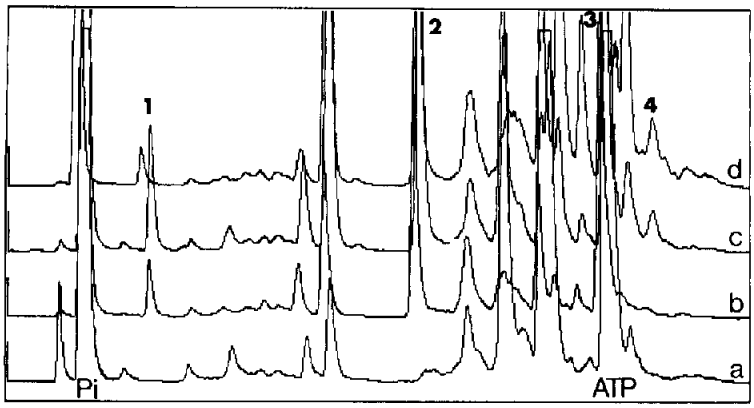

Fig. 6. Detection of doxorubicin–DNA covalent adducts *in vivo* by ^{32}P-postlabelling utilizing HPLC (taken from Cummings *et al.* [5]). For full description of methods see ref. 5. In all cases 10 μg of DNA extracted from the Sp 107 rat mammary carcinoma were postlabelled. Chromatograms: (a) control tumour; (b) 185 μg of doxorubicin administered intra-tumourally (48 h treatment); (c) and (d) 158 μg of doxorubicin incorporated in albumin microspheres also administered intra-tumourally which stimulated drug quinone reduction (see Fig. 5) by 155-fold (48 h treatment). Four putative adduct peaks (1–4) are identified which were absent in control tumour, however no increase in levels of these peaks occurred after intra-tumoural administration of the microspheres when quinone reduction was significantly increased. Time frame (*x*-axis) is 28 min; *y*-axis is radioactivity.

cially adapted for HPLC. DNA was extracted from tumours by the method of Marmur [29], digested using micrococcal nuclease and spleen nuclease, and postlabelled using nuclease P1 enhancement [5]. The HPLC stationary phase was Adsorbosphere nucleotide–nucleoside (7 μm; 25 cm × 4.6 mm I.D.) and gradient elution was employed at a flow-rate of 2 ml/min (buffer A, 60 m*M* ammonium dihydrogenphosphate and 5 m*M* tetrabutylammonium phosphate (TBA) (pH 5.0); buffer B, 5 m*M* TBA in methanol; gradient from 0% B to 36% B over 28 min). Four adduct peaks were detected (1, 4.7 min; 2, 15.3; 3, 21.4 min and 4, 24.3 min, see Fig. 6) which were not present in DNA extracted from non-treated tumours. Adduct levels were estimated to be in the range of 1 in 10^8 nucleotides. Uncertainties about labelling efficiencies with polynucleotide kinase and selectivity of nuclease P1 digestion made accurate quantitation impossible. The major species (15.3 min) eluted shortly after a dG standard (11.3 min) and may be related to a dG monofunctional adduct. Using HPLC, the closely related anthracycline menogaril has been shown *in vitro* to couple covalently onto dG at the N-2 position after chemical reductive activation under anaerobic conditions [94]. Adduct 4 (24.3 min) eluted later than ATP and the four unmodified monophosphate nucleotide standards and may be related to incomplete digestion of DNA.

In contrast to the above *in vivo* study Bartoszek and Wolf [95] have shown that metabolic activation of 50 μ*M* [^{14}C]-DOX by exogenously added cytochrome P-450 reductase (250 U/ml for a 3 h incubation) in the presence of aerobic MCF-7 cells produces a single DOX/DNA adduct HPLC peak (t_R 38 min). Here, DNA was digested by DNAase I, spleen exonuclease, SVD and AP and nucleosides were separated by gradient elution reversed-phase HPLC (stationary phase, Spherisorb ODS-2; buffer A, 50 m*M* sodium phosphate (pH 3.5); buffer B, methanol; gradient, 10% B isocratic for 10 min, then up to 75% B over 20 min, then isocratic at 75% B for 10 min). Curiously, this proposed adduct peak had a longer t_R than native DOX (32 min) which runs contrary to the behaviour of DOX/protein covalent adducts when chromatographed by reversed-phase HPLC [96].

Other examples of the application of HPLC with anthracyclines include the use of a Vydac C-4 RP column and 5–20% acetonitrile gradient run at 0.3 ml/min over 90 min to detect covalent coupling of DAUNO to DNA hexamers after chemical treatment with formaldehyde. Here bonding occurred between the N-3′ of daunosamine on the drug and N-2 of guanine or 2-aminoadenine via a methylene bridge [97]. The *in vivo* relevance of this observation is unclear. A Lichrosorb RP-2 column together with a complex gradient utilising an increasing proportion of acetonitrile and a dual tritium labelling technique (either on the aglycone moiety or on the modified sugar moiety) has been employed to measure cyano-morpholino anthracycline covalent adducts [82]. Up to four adduct peaks were resolved and it was concluded that covalent bonding occurred exclusively between the morpholino ring systems and DNA nucleotides.

6. BIOOXIDATIVE ALKYLATING AGENTS

Oxidative activation of anticancer drugs to DNA alkylating species as a potential mechanism of action offers little tumour selectivity and therefore has not been avidly pursued as a course for rational design of new agents. Several classes of established agents have been shown to undergo oxidative metabolism and bind covalently to DNA and proteins. These include the acridines, a group of synthetic molecules designed rationally to act as DNA intercalators [for review see ref. 98], the epipodophyllotoxins [99–101] and the ellipticines (also potent DNA intercalators). The majority of experimental evidence favours these compounds working by inhibition of DNA topoisomerase II through stabilization of a ternary drug–nucleic acid–protein complex referred to as the cleavable complex [102]. Although the exact contribution of DNA alkylation to this proposed mechanism is unclear, it is likely that oxidative metabolism contributes significantly to toxicity profiles and the overall clinical pharmacology of these drugs.

6.1. Ellipticines

The ellipticines are semi-synthetic molecules based on the naturally occurring antitumour alkaloid ellipticine (5,11-dimethyl-6*H*-pyrido[4,3*b*]-carbazole) which was first described in 1967 [103]. The lead compound in this series is the N2-methyl-9-hydroxy derivative of ellipticine (9-OH-NME) and is currently used in the treatment of breast cancer. 9-OH-NME undergoes oxidation catalysed by horse radish peroxidase (HRP) and hydrogen peroxide (H_2O_2) at the 9-hydroxy group to yield a quinone imine intermediate with an electrophilic centre at C-10 [104]. Further oxidation produces the orthoquinone form 9,10-dioxo-NME. Reactivity of the proposed quinone imine derivative with cellular nucleophiles has been confirmed *in vivo* by HPLC [105]. Using a μ-Bondapak C_{18} column and a complex methanol gradient, three sulphydryl C-10 conjugates of 9-OH-NME have been identified in the kidneys of rats. Unexpectedly, 9-OH-NME has been shown by HPLC to alkylate purine nucleosides and nucleotides *in vitro* leading to regioselective adducts substituted only at the 2′-O position of ribose, in contrast to the alkylation specificity of most anticancer drugs [106,107]. Adducts were detected by HPLC using a μ-Bondapak C_{18} column and isocratic elution with a mobile phase consisting of ammonium acetate (pH 4.5)–methanol (50:50). Lack of alkylation at purine and pyrimidine was explained by the absence of a strong nucleophilic centre being present in these bases.

Covalent binding to intact DNA was subsequently shown to occur *in vitro* using tritium-labelled 9-OH-NME [108]. Fluorescence spectroscopy suggested that arylation occurred at the site of drug intercalation with limited denaturation of the double helix and that the covalent linkage was between C-10 of the drug and a primary amino group of DNA. HPLC conditions were also described for the detection of the orthoquinone species. In an elegant study [109] covalent binding of tritium-labelled 9-OH-NME to intact DNA and RNA extracted from L1210 cells was studied using a novel HPLC procedure to resolve the nucleic acids. Total cellular nucleic acids were loaded on to a Waters Protein Pak 125 column which was eluted isocratically with 15 m*M* phosphate buffer containing 0.1 *M* NaCl (pH 6.8) at 0.5 ml/min. Fractions were collected and counted to determine radioactivity (and therefore covalent binding) associated with the nucleic acids. The Protein Pak column was claimed to eliminate 98.0% of radioactivity associated with nucleic acids due to non-covalent binding via intercalation. This value, however, is not insubstantial and compares poorly to the 99.8% removal of radioactivity by the procedure of Cummings *et al.* [86]. Intact RNA and DNA co-eluted on the Protein Pak column (t_R 6 min) allowing total covalent binding to nucleic acid to be determined. The combined nucleic acids were then digested with RNAase T_2 overnight at 37°C and rechromatographed to give a single peak at 6 min corresponding to DNA. This allowed covalent modification of DNA to be determined selectively. Covalent binding to RNA was then derived by the difference between the two chromatographic runs. In this study kinetics of covalent modification to DNA and RNA were followed by this technique as well as the chemical stability of these adducts.

7. ORGANOMETALLIC ANTITUMOUR AGENTS

7.1. Cis-diamminedichloroplatinum(II) (cDDP; cisplatin) and analogues

cDDP is now one of the most widely utilised antitumour agents in clinical practice. Since its discovery in the 1960s [110] and subsequent licensing in 1979, a variety of organoplatinum analogues have been developed largely in an attempt to ameliorate the problematic nephrotoxicity of the lead compound. However, to date only the analogue carboplatin (*cis*-diammine-1,1-cyclobutane-dicarboxylatoplatinum II, CBDCA) has been adopted as an alternative to cDDP for routine clinical use, including those malignancies which demonstrate acquired resistance to cDDP.

Organoplatinum drugs are believed to exert their cytotoxic activity by the formation of DNA adducts followed by inhibition of transcription, replication and/or the corruption of the triplet

base code sequence leading to loss of gene function. Evidence for this is based upon the known genotoxic effects of cDDP. Genes can be directly mutated in experimental *in vitro* systems [111,112] and gross changes in chromosome morphology may result from exposure to cDDP [113]. Work by Roberts and Pascoe confirmed the ability of cDDP to cross-link DNA [114]. Consequently, there is great interest in the nature of the interaction between cDDP and its presumed cellular target, DNA.

The modification of bases by cDDP or carboplatin involves the addition of a relatively small group ($[NH_3]_2$–Pt=), which may bind through one or two covalent bonds. With cDDP, the parent drug undergoes aquation, with the loss of chloride, followed by loss of protons to leave one or two reactive hydroxyl groups (Fig. 7) prior to macromolecular binding [115]. The reaction with DNA is nucleophilic and hence binding to relatively strong nucleophiles such as the N-7 nitrogen in guanine is common. Both inter- and intrastrand cross-linking occurs with the latter being considered more critical for defining tumour cell cytotoxicity. If a bifunctional adduct is formed between $[NH_3]_2$ Pt= and two bases adjacent on the DNA strand, a closed ring structure may result upon digestion of the DNA strand (Fig. 8). Aquated cDDP will also readily bind to protein, and thus bifunctional adducts can also form with DNA and protein sulphydryl groups linked via the platinum-containing moiety. Thus, although the adducting group is fairly small, the effect on DNA structure and function is quite profound.

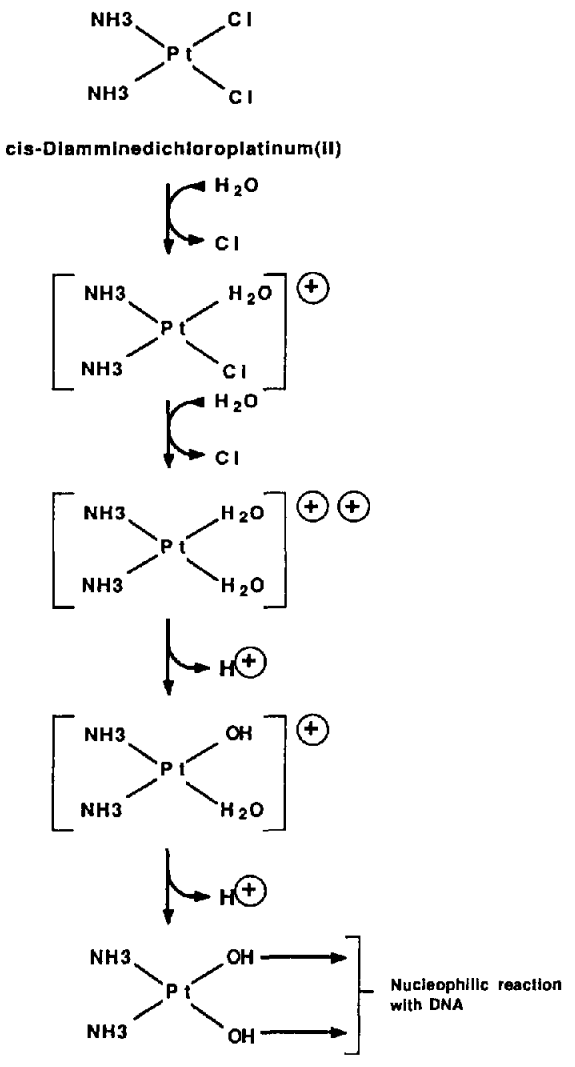

Fig. 7. Progressive aquation and loss of protons by *cis*-diamminedichloroplatinum(II), leading to formation of the highly reactive dihydroxy species which binds to cellular macromolecules.

The majority of the published analyses of organometallic complex–DNA adducts have involved treatment of purified DNA *in vitro* with platinum-containing compounds, usually cDDP or carboplatin. DNA isolation and digestion techniques are broadly similar to those used routinely to investigate other drug–DNA interactions; these methods have been outlined earlier in this review. Separation of platinated bases from unmodified residues is achieved in a variety of ways, but reversed-phase HPLC is the most popular technique. Most separation systems utilize elution mixtures composed of phosphate or acetate buffer and acetonitrile or ethanol in varying proportions. In 1982, Eastman [116] reported the separation of the reaction products of nucleosides and cDDP using an Altex Ultrasphere 25 cm ODS (C_{18}) column (5 μm particle size), with elution by 0.1 *M* ammonium acetate buffer and a 0–30% methanol gradient, and a flow-rate of 1 ml/min (over 30 min). The formation of a variety of adducts was demonstrated,

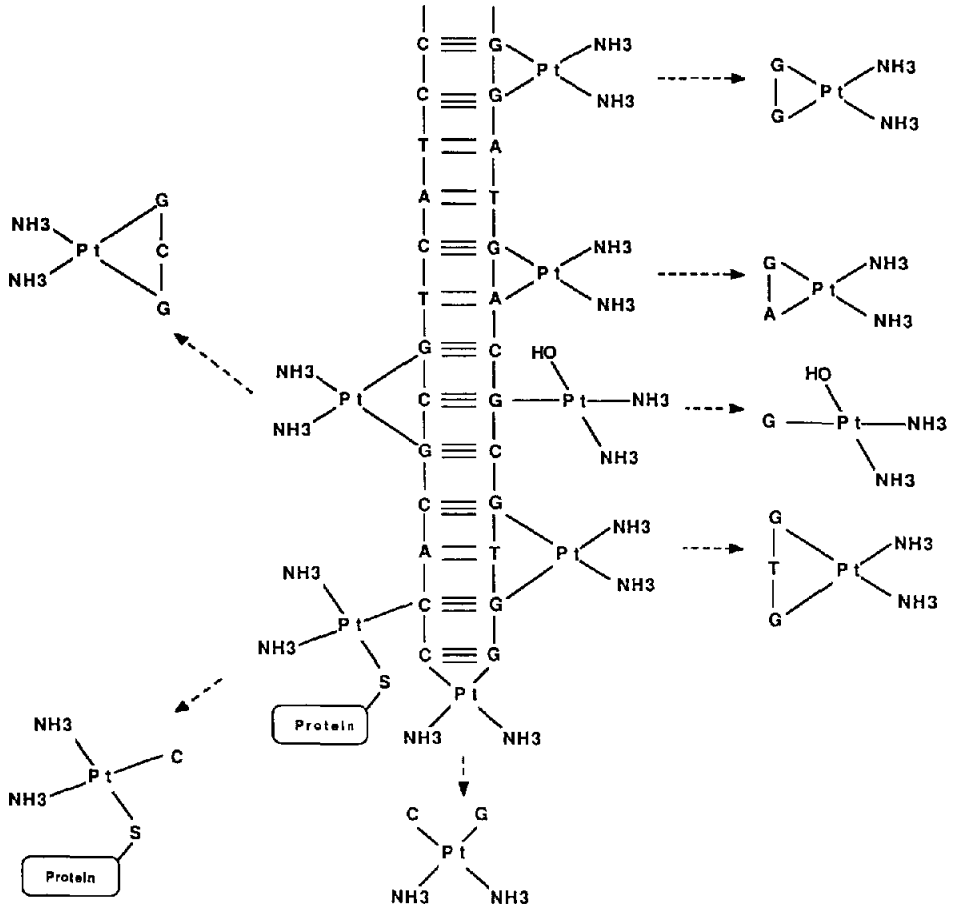

Fig. 8. Some products of digestion of DNA exposed to *cis*-diamminedichloroplatinum(II). Over 50% of the drug is released in the form of bifunctional guanine intrastrand cross-links. The cyclic guanine–Pt–guanine intrastrand adduct is not electrochemically active, and is therefore not detected by HPLC–ED techniques.

predominantly between deoxyguanosine–Pt and deoxyadenosine–Pt. Several bifunctional adducts (both Pt–base–Pt and base–Pt base) were identified in this study, but none of the cyclic adducts that might potentially arise as a result of the reaction of cDDP with DNA oligomers were apparent in these monomeric, free nucleoside incubations [116]. As discussed below, several problems exist with the sensitive detection of Pt–DNA adducts, especially when employing platinum isotopes. [^{3}H]-DEP, an analogue of cDDP, was used by Eastman to investigate platinum drug–DNA binding kinetics using tritium as a radiolabel in preference to ^{195}Pt in cDDP [117]. This report reveals that the ammonium acetate concentration of the eluting buffer may be critical when attempting to separate *cis*-[^{3}H]dichloroethylenediamineplatinum(II) (DEP) adducts of DNA. Ranges of ammonium acetate concentration from 0.5 m*M* to 0.5 *M* were required, depending upon the column employed, although other conditions including column type were similar to those previously reported in 1982 [116]. Other workers have modified Eastman's methodology and characterised the adducts formed by another analogue, *trans*-DDP, which has greatly attenuated antitumour activity when compared to cDDP [118]. Bergnes *et al.* [119] have similarly modified C_{18} reversed-phase separation methods and improved peak sharpness by use of a Waters

Resolve 5 μm C_{18} column (150 mm length). They have also improved resolution of dGMP from its platinated monoadduct using an LKB ODS (5 μm particle size) column (25 cm long). Variability of performance between columns of similar type but from different manufacturers is, of course, common and reflects unevenness in the coating of the silica gel support, leading to mixed retention mechanisms [15].

7.1.1. Detection of platinum DNA adducts

Direct detection of adducted platinum by physical techniques is difficult. The adducting group $[NH_3]_2$–Pt is an extremely poor UV chromophore, and fluorescence is limited to emissions by the platinum atom in the X-ray wavelength region [120]. The parent drug is electrochemically active, and both it and several hydration products have been detected by a number of on-line HPLC electrochemical detection systems including silver–mercury electrodes [121], hanging-drop mercury electrodes [115] and coulometric electrodes (Fig. 9), but the electrode potentials required for efficient detection are relatively high (+0.6 to +0.8 V). When adducted to nucleotides or bases, some UV absorbance and electrochemical activity is conferred upon the adducts, but this is merely a function of the bases' inherent physicochemical properties. *In vitro* incubations of DNA with cDDP have, upon digestion, yielded adducts detectable by both UV absorption [116] and electrochemical detection (unpublished observation). However, whilst this enables the resolution and identification of some adducts formed in simple *in vitro* incubations, the sensitivity of both UV and electrochemical detectors has proven too low to be useful for the analysis of samples from cell lines, animals or patients (see Table 1). Derivatization of the drug with diethyldithiocarbamate (DDTC) improves detection limits to 0.1 μg/ml [122]. However, this derivatization is inappropriate for use in the detection of cisplatin adducted to DNA, since it reacts with same functional groups on cDDP as the nucleic acid. *Ex vivo* detection of Pt–DNA adducts in rat urine has been achieved using UV detection, but this was subsequent to an i.p. administration of a specific guanine–Pt–guanine adduct, not the parent drug, and was therefore not indicative of *in vivo* cDDP-DNA modifications [123]. Radiolabelled drugs are frequently used in drug–DNA binding studies to improve sensitivity and specificity of adduct detection. However, the isotopes ^{191}Pt, ^{193}Pt and ^{195}Pt are not readily available and isotopically labelled platinum drugs have in the past been produced on a small scale, most notably by the Manchester Platinum Group. A technique for the synthesis of radiolabelled cDDP has been published [124]. The relatively short half-life of the isotopes (3–4 days) also limits the usefulness of radiolabelling for adduct detection purposes.

In the light of these problems, most analyses of Pt–DNA adducts have involved fraction collect-

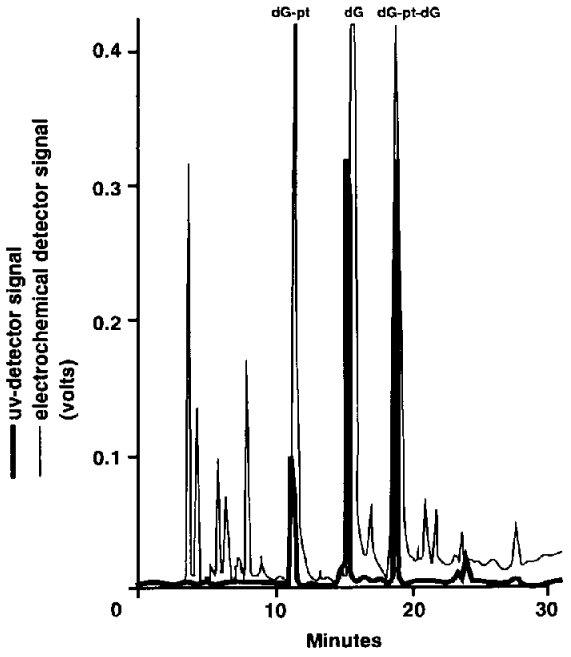

Fig. 9. HPLC separation of the reaction products of *cis*-diamminedichloroplatinum(II) with deoxyguanosine (dG): 2 mg/ml dG was reacted with 1 mg/ml cDDP for 48 h at 37°C in phosphate-buffered saline (pH 6.0). Detection of adducts was by UV absorbance (at 254 nm) and by coulometric electrochemical detection (+0.8 V). Separation of reaction products was on a Spherisorb ODS-2 column (25 cm × 4.5 mm I.D., 5 μm particle size) at room temperature, using a gradient of potassium phosphate buffer (20 m*M*, pH 4.5) in methanol (5–30% methanol over 35 min).

ing and subsequent off-line analysis. In this context, analytical techniques include graphite furnace atomic absorption spectroscopy (GF-AAS) and inductively-coupled plasma mass spectrometry (ICP-MS). Atomic absorption spectroscopy is not sufficiently sensitive to detect the low levels of platinated base adducts that occur *in vivo*. ICP-MS offers a 1000-fold increase in sensitivity, but hardware is expensive to purchase and maintain. Both ICP-MS and GF-AAS can suffer interferences from high salt concentrations, so choice of eluting buffers is restricted when these off-line detection systems are employed.

In the last ten years, highly sensitive immunochemical techniques have been developed to detect Pt–DNA adducts. A number of groups have now generated antibodies to Pt-modified DNA [10,13,125–129]; these vary in their sensitivity and specificity and can give rise to differences in adduct quantitation [130]. Nonetheless, limits of detection for these systems are in the fmol range, allowing for the detection of DNA platination *in vivo*.

7.2. Non-platinum organometallics

As the range of organoplatinum compounds has increased, so too has the number of metal complexes that utilize alternative transition metals at their centres. Antimony [131,132], copper, germanium, gold, hafnium, iridium, iron, molybdenum, niobium [133], palladium, rhenium, rhodium [134], ruthenium [135,136], silver, tin [131], titanium, vanadium and zirconium have all been utilised as centres of metal complexes that have demonstrated cytotoxic, cytostatic or antimetastatic [137] activity in a variety of model systems [138–140]. The titanium complex Budotitane has already entered clinical trials [141,142]. For many of these novel antitumour agents, the critical intracellular target is, in common with cDDP, thought to be DNA. However, some copper and gold complexes are believed to exert their antitumour activity by uncoupling oxidative phosphorylation [140,143,144]. As yet, HPLC has only been used to investigate the nature of the adducts formed between DNA and ruthenium complexes, specifically [*trans*-$RuCl_2(DMSO)_4$]. Much like the analyses of cDDP/DNA adducts, the use of μBondapak C_{18} columns and a gradient of methanol (0–20%) in 10 m*M* potassium chloride has resolved the products of DNA digestion after treatment with [*trans*-$RuCl_2(DMSO)_4$]. Interestingly, despite profound differences in the geometry of the drug complexes (cDDP is a square planar molecule, [*trans*-$RuCl_2(DMSO)_4$] has octagonal geometry) patterns of DNA modification are similar, with N-7-guanine being the most common site [145]. This finding reflects the predominately chemical nature of the interaction with DNA. Recently, the reaction product of [*trans*-$RuCl_2(DMSO)_4$] with d(GpG) in water has been shown to be the intrastrand cross-link and, in common with cDDP, these links may have a crucial role in the antitumour activity of the drug [146].

8. NITROGEN MUSTARDS

The nitrogen mustards have the same leaving groups as cDDP, chloride residues, and interact with DNA on the basis of chemical reaction kinetics. Utilising both functionalities of the mustard they can cross-link DNA in an inter- and intra-strand fashion. A variety of carrier molecules have been attached to the mustard group in order to improve its uptake into the tumour and modulate its degree of chemical reactivity.

8.1. Cyclophosphamide and metabolites

Cyclophosphamide is a major antitumour agent which is known to require metabolic activation in order to express its antitumour activity. Products of cyclophosphamide metabolism include acrolein, phosphoramide mustard and nor-nitrogen mustard. These latter two metabolites differ only in the possession of a phosphoramide group and are thought to be the species mostly responsible for DNA adduct formation. Most of the work on cyclophosphamide–DNA interactions have investigated the products of *in vitro* incubations, although in contrast to cDDP, the availability of radiolabelled drug provides for limits of adduct

detection sufficiently sensitive for *in vivo* investigations (see below).

Low resolution separation of cyclophosphamide–DNA adduct products (from non-adducted bases and unreacted drug) after acid hydrolysis is achieved by Sephadex G-10 column chromatography, producing a single [^{3}H]-cyclophosphamide adduct radiolabelled peak which, when subjected to further separation by HPLC resolves largely into one major adduct, the monofunctional N-7 guanine nornitrogen mustard hydroxide (Fig. 10) [147]. HPLC separation is achieved with both reversed-phase (Partisil 10-ODS-2) and ion-exchange (Partisil 10-SCX) columns; detection is by both on-line UV and fraction collection and counting of radioactivity off-line. Acrolein is thought to bind most readily to proteins, but recent data suggests that it remains a mutagen in some cell lines [148,149]. In this context, ^{32}P-postlabelling of DNA adducts has proven to be a sensitive method for the assay of adducts separated by reversed-phase HPLC. Using postlabelling, final quantification is achieved after TLC separation of HPLC peaks and autoradiography [150].

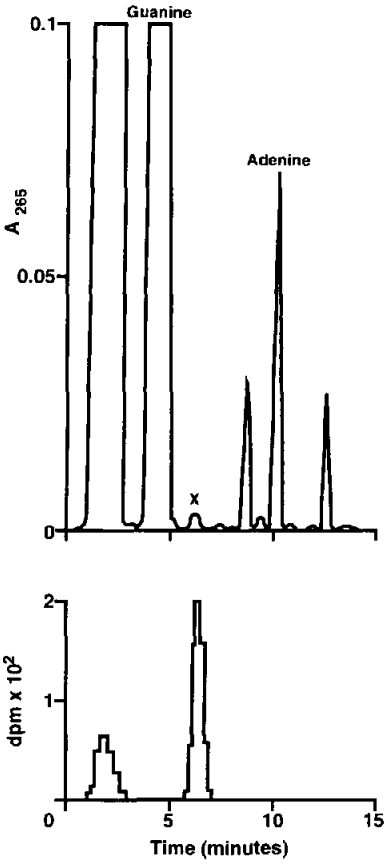

Fig. 10. HPLC separation of cyclophosphamide DNA adducts from rat kidney treated with drug *in vivo*. Detection was by use of radiolabelled [^{3}H]cyclophosphamide and by UV absorbance at 260 nm. X is the presumed major adduct, which co-chromatographed with an *in vitro* derived cyclophosphamide–guanine monoadduct. From ref. 147, permission applied for.

8.2. Chlorambucil

A rare example of the investigation, by HPLC, of drug–DNA adducts formed in humans after treatment with cytotoxic drugs is reported in the work of Bank *et al.* [151] with chlorambucil (Fig. 11). The separation technology is standard: gradient elution (8–50% acetonitrile over 60 min) in a potassium phosphate buffer (pH 5.0) and a semi-preparative C_{18} column (25 cm × 1 cm I.D.). The source of the DNA, from circulating chronic lymphocytic leukaemia leucocytes, presented the investigators with two distinct advantages: a ready supply of cellular material direct from patients, and access to the malignant cell type which is the target for cancer chemotherapy. Radiolabelled [^{14}C]chlorambucil allowed detection of adducts by scintillation counting off-line. Although individual adducts were not separately identified, the authors of this report claim that their data is evidence for the formation of multiple purine base-drug complexes *in vivo*.

9. NITROSOUREAS

9.1. Methylnitrosourea (MNU)

MNU is an alkylating agent of greater interest in a toxicological, rather than an antitumour context. However, it serves as a model for adduct formation by this class of compounds. As with other members of this class, most if not all of the adducts formed are modifications of the guanosine residues in DNA. Post-digestion, these can be separated by HPLC using ion-exchange columns (*e.g.* Dionex cation exchange DC-6A column [152]) and running a pH gradient separation

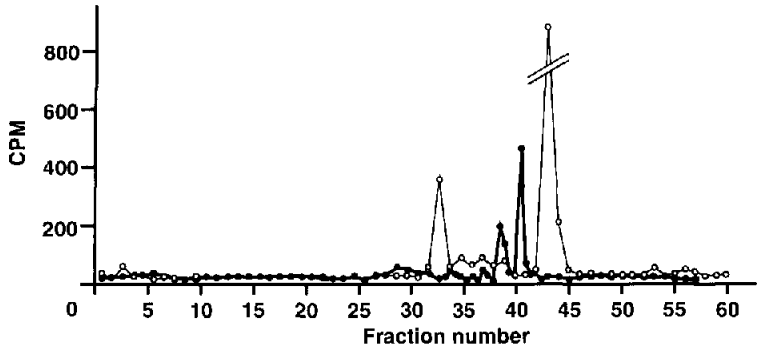

Fig. 11. HPLC separation of chlorambucil/DNA adducts. Detection was by use of radiolabelled [^{14}C]chlorambucil. Closed circles represent counts per minute from fractions collected after HPLC separation of DNA digested in a boiling water bath after treatment with [^{14}C]chlorambucil. The two peaks illustrate different drug–purine base adducts. Open circles represent counts per minute from fractions collected after incubation of drug alone without DNA. From ref. 151, permission applied for.

(4.2–8.5) in 0.2 *M* ammonium formate, at 30°C. In this report, formation of O-6 methylguanine was deemed to be a suitable index of exposure to MNU, and also correlated with exposure to other known potent toxins such as ethylmethane sulphonate. In contrast, the formation of another adduct evident after MNU treatment, 7-methylguanine, showed no such constant relationship.

9.2. Haloethylnitrosoureas

The differences in pK_a of modified DNA bases (*cf.* unmodified bases) can be utilised in their HPLC separation by the judicious use of buffers at appropriately selective pHs. The separation of DNA modified *in vitro* by haloethylnitrosoureas such as bis-chloroethylnitrosourea (BCNU) and chlorocyclohexylnitrosourea (CCNU) is achieved using reversed-phase (C_{18}) columns with acetonitrile gradients in potassium or ammonium phosphate buffers, and a variety of detection techniques including UV absorbance, fluorescence [153] and use of radiolabels with fraction collection [15]. This latter technique is of some importance as it can reveal adduct peaks which may co-elute with unmodified bases. Using these techniques, at least five adducts have been resolved after modification with BCNU, including hydroxyethyl and chloroethyl derivatives of guanine [154]. Interestingly, BCNU adducts of adenine have not been detected. By the use of ion-pairing agents (20 m*M* potassium phosphate buffer (pH 6.5) with 5 m*M* tetrabutylammonium hydrogensulphate, in a gradient elution system, 0–16.5% acetonitrile over 15 min) adducts arising as a result of the formation of interstrand cross-links were evident (detection was by fraction collecting and measurement of radiolabel off-line) [155]. With CCNU, single monofunctional adducts of adenine, cytidine and deoxyuridine have been identified (plus one bifunctional adduct deoxycytidyl–deoxyguanisonyl–ethane) whereas seven guanine adducts were detected in the same incubation. Di-guan-7-yl ethane has also been identified as a product of CCNU-DNA modification and represents an interstrand cross-link of potential cytotoxic importance. Once again, co-elution with unmodified species is a potential problem in this system, so positive identification of peaks was achieved by fraction collection, drying down and reconstitution prior to elution on a strong cation exchange HPLC column (Applied Science Absorbosphere 5 μm SCX) [156]. Other nitrosoureas studied by HPLC include another haloethylnitrosourea: bis-fluoroethylnitrosourea (BFNU) [157].

10. METHANESULPHONATES

These compounds have been developed as potentially more selective DNA alkylators than the nitrosoureas, although their mechanism of action is similar. 2-Chloroethyl (methylsulphonyl) methanesulphonate (clomesone) is effectively a "functional analogue" of BCNU. Its reactions *in vitro* with synthetic polynucleotides have been investigated, once again using reversed-phase HPLC. Digested DNA samples were loaded onto a 5 μm Altex Ultrasphere ODS column (25 cm × 4.6 mm I.D.) and eluted with 20 m*M* ammonium formate on a gradient of 0–100% acetonitrile. This methodology enabled Gibson *et al.* [158] to demonstrate that the products of clomesone-mediated alkylation of polynucleotides are restricted to chloroethylguanine only, with no potentially mutagenic hydroxyethyl products. However, more recently Struck *et al.* [159] have used several different elution systems to dispute these findings and suggest that small amounts of 7-(2-hydroxyethyl)guanine are produced by the reaction of clomesone with DNA. Gradient elution systems used ammonium phosphate, varying in concentration from 5 to 50 m*M*, and in pH from 2.8 to 4.5, run over a period of 20 min with acetonitrile (5–90%) and were able to resolve the hydroxyethyl adduct as both a peak in radioactivity and fluorescence. Detection was by UV absorbance or fluorescence (excitation at 250 nm, emission at 350 nm) and by scintillation counting of the fractions collected off-line.

11. DISTAMYCIN DERIVATIVES

A range of derivatives of distamycin, an oligopeptide antiviral antibiotic, have been synthesised, and screened for antitumour activity both *in vitro* and *in vivo*. Of the 14 compounds tested, most possessed chloride or hydroxide leaving groups attached to nitrogen at the N-terminus of the oligopeptide which, in a manner analogous to cDDP, allows the drug to bind covalently to cellular macromolecules including DNA. Separation of adducted nucleosides (DNA digested by refluxing in 1-butanol for 30 min) was achieved using an ion-pairing agent (6 m*M* tetrapropylammonium bromide in potassium phosphate buffer (pH 4.5), in a gradient elution against the same solution in 40% methanol) and a Novapak C_{18} column [160]. Regrettably, the authors were unable to correlate adduct formation to observed antitumour activity.

12. NEOCARZINOSTATIN

HPLC of small oligonucleotides treated with neocarzinostatin has demonstrated a base selectivity and the enhancement of binding in the presence of reduced thiols such as glutathione. Separation of adducts was achieved on a Rainin Microsorb C_{18} column (5 μm, 25 cm) using an elution gradient of 0–80% methanolic 5 m*M* ammonium acetate against ammonium acetate alone (pH 5.0). Detection was by fluorescence (excitation 340 nm, emission 418 nm) [161].

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