Cancer Chemother Pharmacol (1996) 38: 431–438

ORIGINAL ARTICLE

M. I. Walton · P. Goddard · L. R. Kelland
D. E. Thurston · K. R. Harrap

Preclinical pharmacology and antitumour activity of the novel sequence-selective DNA minor-groove cross-linking agent DSB-120

Received: 5 November 1995/Accepted: 30 January 1996

Abstract We examined the in vitro cytotoxicity, antitumour activity and preclinical pharmacokinetics of the novel sequence-selective, bifunctional alkylating agent DSB-120, a synthetic pyrrolo[1,4][2,1-*c*]benzodiazepine dimer. DSB-120 was shown to be a potent cytotoxic agent in vitro against a panel of human colon carcinomas [50% growth-inhibitory concentration (IC_{50}) 42 ± 7.9 n*M*, mean ± SE, $n = 7$] and two rodent tumours (L1210 and ADJ/PC6). Antitumour activity was assessed in the bifunctional alkylating-agent-sensitive murine plasmacytoma ADJ/PC6 using a variety of administration protocols. The maximal antitumour effects were observed following a single i.v. dose but the therapeutic index was only 2.6. DSB-120 was less effective when given i.p. either singly or by a daily × 5 schedule. After a single i.v. dose at the maximum tolerated dose (MTD, 5 mg kg^{-1}) the plasma elimination was biphasic, with a short distribution phase ($t_{1/2\alpha}$ 4 min) being followed by a longer elimination phase ($t_{1/2\beta}$ 38 min). Peak plasma concentrations were 25 μg ml^{-1}, the clearance was 1.3 ml g^{-1} h^{-1} and the $AUC_{0-\infty}$ was 230 μg ml^{-1} min. Concentrations of DSB-120 in ADJ/PC6 tumours were very low, showing a peak of 0.4 μg g^{-1} at 5 min. The steady-state tumour/plasma ratio was about 5% and the AUC was only 2.5% of that occurring in the plasma. DSB-120 appeared to be unstable in vivo, with only 1% of an administered dose being recovered unchanged in 24-h urine samples. Plasma protein binding was extensive at 96.6%. In conclusion, the poor antitumour activity of DSB-120 may be a consequence of low tumour selectivity and drug uptake as a result of high protein binding and/or extensive drug metabolism in vivo.

Key words DSB-120 · Pharmokinetics · Cytotokicity · DNA cross-linking · Sequence selectivity

M.I. Walton (✉) · P. Goddard · L.R. Kelland · K.R. Harrap
CRC Centre for Cancer Therapeutics, Institute of Cancer Research, E block, 15 Cotswold Road, Belmont, Surrey, SM2 5NG, UK

D. E. Thurston
School of Pharmacy and Biomedical Science, University of Portsmouth, Park Building, King Henry 1st Street, Portsmouth PO1 2DZ, UK

Introduction

The pyrrolo[2,1-*c*][1,4]benzodiazepines (PBDs) are a group of naturally occurring, monofunctional DNA-alkylating antitumour antibiotics [17, 20]. These compounds bind exclusively to the exocyclic N^2 of guanine in the minor groove and span 3 bp in a sequence-specific manner (5′PuGPu). Although they exhibit potent in vitro cytotoxicity, their in vivo antitumour effects have been limited through cardiotoxicity and related problems [20].

DSB-120 is a synthetic derivative of two PBD molecules (DC-81) [1] that have been linked through their C8-position via three methylene groups to form a minor-groove DNA cross-linking agent [2]. Recent studies have shown that DSB-120 is highly cytotoxic to a panel of human ovarian tumour cell lines in vitro, primarily as a result of efficient DNA interstrand cross-linking [19]. Furthermore, this compound exhibited enhanced sequence selectivity as compared with simple PBDs, preferentially recognising the sequences 5′PuGATCPy or 5′PyGATCPu. Molecular modeling [8] and nuclear magnetic resonance (NMR) studies [14] have shown that DSB-120 binds symmetrically to the N^2 guanine position in the minor groove with minimal distortion to the helix [2]. DNA cross-linking studies in naked calf-thymus DNA and in vitro cell studies have established that DNA is extensively and irreversibly cross-linked [3], with minimal repair occuring after up to 48 h in human K562 leukaemia cells [19]. Sequence searches in gene data banks have shown that DSB-120 possesses sufficient sequence selectivity

(i.e. 6-bp span with 4 bases recognised) to have a number of different binding sites on different oncogene fragments [15]. Thus, DSB-120 possesses several features that make it a potentially useful sequence-selective antitumour agent [4].

In view of the potent cytotoxicity, novel sequence selectivity and remarkable stability of the DNA interstrand cross-links formed by DSB-120, its antitumour activity together with its pharmacokinetics and metabolism were studied in vivo. Cytotoxicity was assessed in a panel of human colon-tumour cell lines and selected mouse tumour lines. Antitumour and pharmacokinetics studies were carried out in BALB/c mice bearing ADJ/PC6, a tumour that is sensitive to many bifunctional DNA cross-linking agents [6].

Materials and methods

Drug supply and administration

DSB-120 was synthesised as previously described (Fig. 1) [2]. Drug material was provided as a lyophilised yellow powder, which was dissolved in absolute ethanol prior to use. Cisplatin was provided by The Johnson Matthey Technology Centre (Sonning, UK) and was made up in sterile saline immediately prior to use. Doxorubicin was obtained from Sigma (Poole, UK) and was made up in sterile water prior to use. DSB-120 was stable in ethanol at $-20^{\circ}C$ for at least 4–6 weeks without loss of activity.

For in vitro studies, DSB-120 was made up as a 5-m*M* stock solution in ethanol and serial dilutions were made in growth medium immediately before addition to cells. For in vivo studies, DSB-120 was dissolved in dimethylsulfoxide (DMSO) or ethanol ($50\ mg\ ml^{-1}$) and then diluted 1:10 in physiological saline immediately prior to use. Drug solutions were given either i.p. or i.v. via the tail vein in a volume of $0.01\ ml\ g^{-1}$ body weight.

Cytotoxicity studies

Drug-induced cytotoxicity was determined using a 96-h exposure sulforhodamine B (SRB) growth-delay assay as previously described [11]. Cells were plated at $0.5–1.0 \times 10^4$ in 96-well microtitre plates and left overnight to attach. Drugs were added in medium. Cells were exposed continuously to different drug concentrations and growth inhibition was assessed by SRB protein staining. IC_{50} values (50% growth-inhibitory concentrations) for L1210 and ADJ/PC6 cells were obtained by cell counting (Coulter counter) following 48-h and 72-h periods of exposure, respectively.

Cell culture

The human colon-cancer cell lines were grown as monolayer cultures in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum, gentamicin at $50\ \mu g\ ml^{-1}$, amphotericin B at $2.5\ \mu g\ ml^{-1}$, 2 m*M* glutamine, insulin at $10\ \mu g\ ml^{-1}$ and hydrocortisone at $0.5\ \mu g\ ml^{-1}$ in an atmosphere of 10% CO_2: 90% air at 37°C.

The mouse-leukaemia cell line L1210 and the mouse-plasmacytoma cell line ADJ/PC6 were grown as suspension cultures in DMEM containing 20% horse serum, 5 m*M* glutamine, insulin at $10\ \mu g\ ml^{-1}$, hydrocortisone at $0.4\ \mu g\ ml^{-1}$, 5 m*M* non-essential amino acids, streptomycin at $100\ \mu g\ ml^{-1}$, neomycin at $50\ \mu g\ ml^{-1}$ and penicillin at $60\ \mu g\ ml^{-1}$.

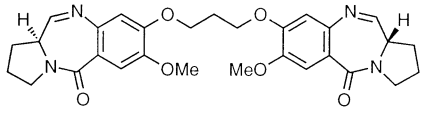

Fig. 1. Structure of DSB-120

Mice and tumours

Adult female BALB/c mice were obtained from the MRC (Mill Hill, London, UK) and adult male DBA-2 mice, from OLAC (Bicester, UK) and all animals were housed in plastic cages containing paper chips. Mice were allowed food (Expanded Rodent diet) and water ad libitum and were used in experiments at 6–12 weeks of age.

The ADJ/PC6 mouse plasmacytoma [16] was grown by s.c. implantation of 1-mm^3 fragments in the flank of BALB/c mice and was treated at 20 days post-implantation as previously described [6].

Antitumour studies

The antitumour activity of DSB-120 was determined in BALB/c mice bearing the ADJ/PC6 plasmacytoma as previously described [6]. Mice bearing comparably sized tumours were randomised into treatment and control groups at 20 days post-tumour implantation and were drug-treated. Experiments were terminated at 30 days post-implantation. Drugs were given either i.v. or i.p. as a single dose or as five single daily doses. Animals were killed by cervical dislocation, tumours were removed and the weight of treated versus control tumours was compared. Antitumour activity was defined in terms of the therapeutic index (TI), defined as the ratio of the LD_{50} (the dose causing 50% lethality) to the ED_{90} (the dose required to reduce the tumour mass by 90%). Comparative data for cisplatin, carboplatin and Adriamycin were also obtained.

Sample preparation

Plasma and tissue samples were prepared using standard techniques [23]. Briefly, whole blood was obtained by cardiac puncture from CO_2-killed mice using heparinised syringes. Blood samples were cooled on ice and plasma was prepared immediately. Tumour samples were removed immediately and snap-frozen on dry ice.

Urine from individual mice was collected at room temperature at intervals of 0–8 and 8–24 h following drug administration. Cage washings were also taken and analysed. Urine was incubated overnight at 37°C in excess bovine liver β-glucuronidase (Sigma, Poole, UK) to reveal the glucuronidated parent compound. All tissues and fluids were stored at $-20^{\circ}C$ for up to 1 week prior to analysis.

All samples were thawed and handled rapidly on ice. Drug was extracted from plasma, urine and tissue homogenates (33%, w/v, in deionised water) by the addition of 2 vol. of ice-cold acetonitrile (MeCN). Samples were centrifuged at 14,000 rpm for 10 min and the clear supernatant was recovered for injection into the high-performance liquid chromatography (HPLC) system.

HPLC methods

Concentrations of DSB-120 in biological samples were determined by gradient HPLC. Separations were initially carried out on reverse-phase octadecylsilane C18 (polygasol) columns, but the peak shape

and resolution were poor. Separations were routinely carried out on 15-cm × 4.6-mm (inside diameter) columns packed with Spherisorb phenyl packing material (Spherisorb, 5 μm bead size) and protected with a 5-cm × 2.1-mm (inside diameter) precolumn packed with Whatman Pelicular ODS (C18, 37 to 53 μm bead size). Drugs were eluted using a mobile phase consisting of MeCN in 20 m*M* sodium phosphate buffer (pH 7.4). Gradient conditions were as follows: a linear gradient from 5% to 50% MeCN from 0 to 10 min, 50% MeCN from 10 to 14 min, followed by 50–55% MeCN from 14 to 16 min with re-equilibration from 16 to 20 min. DSB-120 eluted with a retention time of 7.5 min under these conditions. DSB-120 was routinely monitored using fluorescence detection on a Perkin Elmer LS4 Fluorimeter with excitation at 270 nm and emission at 405 nm. High concentrations of drug were also monitored using fixed-wavelength UV detectors at 254 and 280 nm. Peak identification was accomplished by co-chromatography with authentic material and by UV spectral properties.

Plasma protein binding

Fresh pooled BALB/c plasma was spiked with DSB-120 (10 ug ml^{-1}) and incubated at 37°C for 15 min to facilitate binding. Plasma proteins were removed via ultrafiltration across YMT membranes (molecular weight cut-off at 30,000) using the Amicon Micropartition System and were centrifuged at 1,500 rpm for 20 min. Spiked plasma ultrafiltrate was used to correct for the very small amount of non-specific membrane binding. Plasma and ultra filtrate were analysed by HPLC as described above.

Stability studies

Pooled DBA-2 whole blood and plasma were spiked with DSB-120 (10 μg ml^{-1}) and then incubated for up to 48 h in darkness. Plasma samples were incubated at 4, 22 (room temperature) and 37°C and whole-blood samples were incubated at 37°C. Aliquots were removed at appropriate intervals and analysed as described above.

Pharmacokinetic analysis

Pharmacokinetic parameters were determined using standard techniques with a one- or two-compartment model as appropriate [22]. Data were fitted to the model by non-linear regression using in-house software on the basis of published procedures [9]. The area under the plasma concentration-time curve ($AUC_{0-\infty}$) was calculated using the expression $AUC_{0-\infty} = A/\alpha + B/\beta$. Alternatively, the plasma and tissue AUC_{0-t} was calculated by the trapezoidal method. The remaining $AUC_{t-\infty}$was calculated from the equation C_t/k, where C_t is the concentration at time t and k is the elimination rate.

Results

In vitro cytotoxicity

Table 1 summarises the cytotoxicity data obtained for DSB-120 in a panel of human colorectal carcinoma cell lines and two murine tumour cell lines. DSB-120 exhibited marked cytotoxicity to all the lines evaluated, with a mean IC_{50} of 42 n*M* being recorded for the colon lines. DSB-120 was approximately 50 times more potent than the bifunctional DNA cross-linking drug cisplatin and showed cytotoxicity comparable with that of Adriamycin. DSB-120 was also shown to be highly cytotoxic to the rodent plasmacytoma ADJ/PC6 as well as to the commonly used L1210 mouseleukaemia cell line.

Table 1 Cytotoxicity of DSB-120 in a panel of human colon cancer cell lines and two murine tumour lines[a]

	Cytotoxicity IC_{50} (μM)[b]		
Cell line	DSB-120	Cisplatin	Adriamycin
Colon Carcinoma:			
LoVo	0.068 ± 0.021	2.78 ± 0.74	0.01 ± 0.0032
BE	0.064 ± 0.024	5.21 ± 2.19	0.03 ± 0.01
Mawi	0.041 ± 0.016	2.57 ± 0.59	0.017 ± 0.0031
HT29	0.033 ± 0.013	1.71 ± 0.07	0.0203 ± 0.0053
WidR	0.05 ± 0.011	2.44 ± 0.14	0.0292 ± 0.0081
SW 480	0.006 ± 0.0024	0.895 ± 0.15	0.0135 ± 0.0036
SW 620	0.032 ± 0.011	0.513 ± 0.14	0.0097 ± 0.0017
Mean	0.042 ± 0.0079	2.30 ± 0.58	0.0185 ± 0.0032
Murine tumours:			
L1210	0.01, 0.07	NA	NA
ADJ/PC6	0.0007, 0.00033	NA	NA

[a]Data represent mean values ± SD for 4 independent determinations except where individual values are shown
[b]Colon-tumour IC_{50} values were determined using a 96-h SRB assay and murine tumour values, from 72-h and 48-h growth-delay experiments
(*NA* not available)

Antitumour activity

Table 2 summarises the antitumour activity of DSB-120 in the murine ADJ/PC6 plasmacytoma. As can be seen in Table 2 this tumour is highly sensitive to bifunctional DNA cross-linking agents such as carboplatin and cisplatin, giving therapeutic indices of 24 and 18, respectively. By contrast, Adriamycin is inactive in this tumour system. DSB-120 showed poor in vivo activity in the ADJ/PC6 tumour with low selectivity for tumour versus critical normal tissues (as indicated by a therapeutic index close to unity). The most effective treatment protocol corresponded to a single i.v.-dose. Notably, DSB-120 was less active following either single or multiple i.p. administration, with no apparent difference being seen with 10% DMSO or ethanol as vehicle. Although the best ED_{90} value for DSB-120 (2.7 mg kg^{-1}) was in the range observed for carboplatin in this tumour (ED_{90} 6 mg kg^{-1}), DSB-120 was markedly more toxic to mice than carboplatin (LD_{50} values 8.4 versus 143 mg kg^{-1}, respectively). In these experiments, acute toxicity to DSB-120 was observed at 3–5 days post drug administration. These studies established the maximum tolerated dose (MTD) as 5 mg kg^{-1} i.v. DSB-120.

Table 2 In vivo antitumour activity of DSB-120 against the s.c. implanted ADJ/PC6 plasmacytoma[a]

Vehicle	Route	LD_{50} (mg kg^{-1})	ED_{90} (mg kg^{-1})	Therapeutic index (LD_{50}: ED_{90})
10% DMSO:oil	i.p. × 1	35.5	NA	NA
10% Ethanol:saline	i.p. × 1	8.4	4.9	1.7
10% Ethanol:saline	i.v. × 1	7.1	2.7	2.6
10% Ethanol:saline	i.p. daily × 5	1.7	1.6	1
Cisplatin	i.p. × 1	11	0.6	18
Carboplatin	i.p. × 1	143	6	24
Adriamycin	i.p. × 1	16.5	18.6	0.9
	i.p. daily × 5	3.5	NA	NA

[a]Experiments were carried out on groups of 3 mice using 4 or 5 doses (*NA* not applicable/available)

HPLC analysis

Initially, separations were attempted on octadecylsilane (C18) and octylsilane (C8) reverse-phase packings but the peak shapes were poor. Acceptable peak shape and resolution were achieved on phenyl packings with gradient elution. DSB-120 was well resolved from other peaks and there was no evidence of late eluting peaks (Fig. 2). A peak was detected in the blank and spiked plasma samples at a retention time of 9.8 min (Fig. 2). The efficiency of recovery of the drug from plasma and tissue homogenates was $> 90\%$. Plots of peak area versus drug concentration were linear over the range studied (0.05–50 μg ml^{-1}) and had zero intercepts. The same-day coefficient of variation for six replicate analyses (10 μg ml^{-1} spiked in plasma) was 4.8%. Allowing for a signal-to-noise ratio of 2:1, the lower limit of detection was 0.03 μg ml^{-1} for an injection volume of 20 μl. This represents an on-column detection limit of about 1 ng DSB-120. Thus, the method presented is accurate, precise and has high sensitivity.

Stability

The stability of DSB-120 as determined in whole blood at 37°C and in plasma at 4°, 22° (room temperature) and 37°C over 48 h is shown in Fig. 3. In whole blood there was a rapid degradation of DSB-120 over the initial 4 h at 37°C, with only 18% of the parent drug remaining. During the remaining time there was

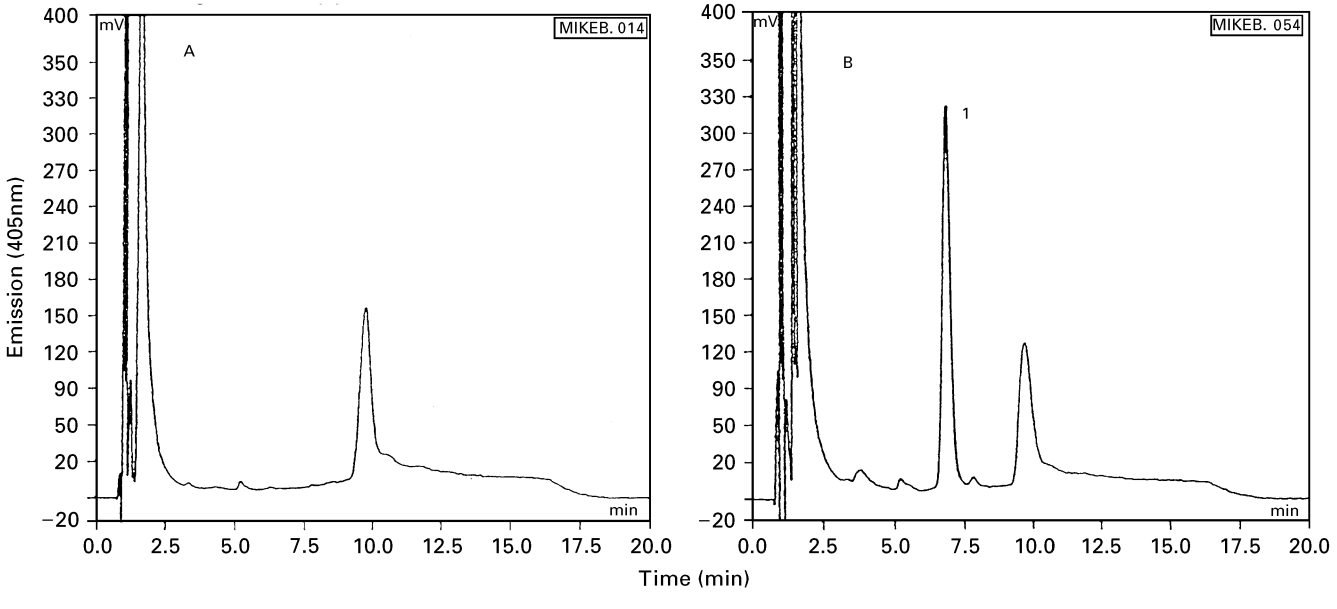

Fig. 2A. Chromatogram of blank plasma and **B** chromatogram of plasma spiked with DSB-120 (5 μg ml^{-1}, *peak 1*). Other experimental conditions are described in Materials and methods

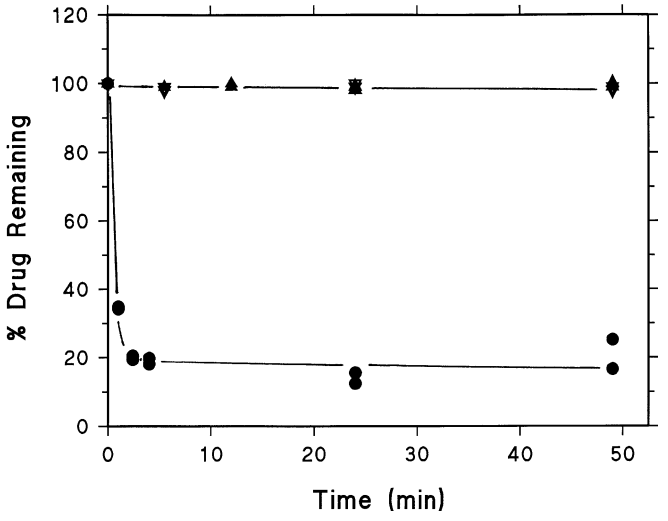

Fig. 3 Stability of DSB-120 in mouse whole blood and plasma ex vivo. DSB-120 (10 μg ml^{-1}) was incubated in whole blood at 37°C (*black circles*) and in plasma at 4° (*white inverted triangles*), 22° (*white triangles*, room temperature) and 37°C (*black triangles*). At the indicated times, aliquots were removed and drug concentrations were determined by reverse-phase HPLC. For other details see Materials and methods

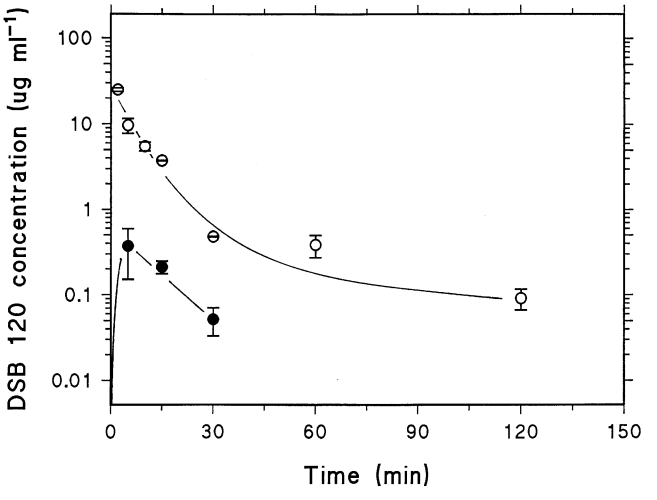

Fig. 4 Pharmacokinetics of DSB-120 as determined in BALB/c mice bearing s.c. ADJ/PC6 plasmacytoma tumours following an i.v. dose of 5 mg kg^{-1}. *Open symbols* represent plasma DSB-120 concentrations and *closed symbols*, ADJ/PC6 tumour drug concentrations. Data represent mean values ± SD ($n = 3$ mice per time point). Similar results were obtained in non-tumour bearing DBA-2 mice

minimal additional parent-drug loss. DSB-120 was remarkably stable in mouse plasma at all three temperatures studied, with < 10% parent-drug loss being observed after 48 h incubation. These data necessitate that blood and tissue samples be handled carefully and processed rapidly to minimise ex vivo degradation. The procedures used were chosen so as to maximise ex vivo stability.

Plasma and tumour pharmacokinetics

The plasma pharmacokinetics of DSB-120 (5 mg kg^{-1} i.v.) are summarised in Table 3. Figure 4 shows the plasma time course for DSB-120 in BALB/c mice bearing ADJ/PC6 tumours following i.v. administration of 5 mg kg^{-1} DSB-120 (MTD). Plasma pharmacokinetics were biexponential, with a short initial distribution phase ($t_{1/2\alpha}$ 3.97 min; 3.5–4.6 min, SE) and a longer terminal elimination phase ($t_{1/2\beta}$ 38 min; 32–47 min, SE). The C_{max} was 25 μg ml^{-1} and the volume of distribution was 1.19 ml g^{-1}. Plasma clearance was 1.3 ml g^{-1} h^{-1} and the corresponding $AUC_{0-\infty}$ was 230 μg ml^{-1} min.

Figure 4 shows the concentrations of DSB-120 detected in s.c. ADJ/PC6 tumours following an i.v. dose of 5 mg kg^{-1} i.v. As can be seen, peak tumour concentrations were very low at 0.4 μg g^{-1} and occurred at 5 min. The total AUC for tumour tissue was 5.8 μg g^{-1} min, representing 2.5% of that occurring in the plasma. Tumour-tissue drug concentrations appeared to reach steady-state after the initial 5 min, and the mean tumour/plasma steady-state ratios as determined from 5 to 30 min varied between 5% and 10%.

Urinary recovery

Table 4 shows there was very little parent drug recovered (< 1%) unchanged in DBA-2 mouse urine over the interval of 0–24 h following an i.v. dose of 5 mg kg^{-1} DSB-120. Figure 5 shows there were several peaks present in the urine from drug-treated but not control mice, which may indicate the presence of novel metabolites. Treatment of mouse urine with glucuronidase did not markedly increase the parent-drug recovery, suggesting that glucuronidation was not a major route of drug elimination (< 0.5%).

Table 3 Summary of DSB-120 pharmacokinetics in BALB/c mice following an i.v. dose of 5 mg kg^{-1} [a]

	C_{max} (μg ml^{-1})	$t_{1/2\alpha}$ (min)	$t_{1/2\beta}$ (min)	Cl (ml g^{-1} h^{-1})	$AUC_{0-\infty}$ (μg ml^{-1} min)
Plasma	25 (23.8–26.2)	3.97 (3.5–4.6)	38.0 (31.9–46.9)	1.3 (1.2–1.4)	230 (211–249)
Tumour	0.37 (0.14–0.47)	NA	NA	NA	NA

[a]Data represent mean values, with ranges in parentheses (C_{max}, AUC, Cl) or SE (*NA* Not applicable)

Table 4 Summary of the 0 to 24-h urinary recovery of DSB-120 following an i.v. dose of 5 mg kg^{-1}[a]

	Urinary excretion (% administered dose)		
	0–8 h	8-24 h	Total
DSB-120	0.82	0.2	1.0
Glucuronide	0.5	0.04	0.5

[a]Data represent mean values for 3 separate determinations; errors are $< 1.0\%$ of the values shown (SE)

Plasma binding

Drug binding was determined after incubation of DSB-120 at 10 μg ml^{-1} in fresh whole plasma from BALB/c mice for 15 min at 37°C. Plasma binding was extensive at 96.6 ± 0.12% (mean ± SD, $n = 6$).

Discussion

DSB-120 represents the lead compound in a novel class of highly potent, sequence-selective, DNA interstrand cross-linking agents. Previous studies have shown that DSB-120 is highly cytotoxic to a panel of human ovarian cancer cell lines, with mean IC_{50} values being 93 n*M* [19], a value comparable with that found for the colon tumour panel presented herein (IC_{50} 42 n*M*). Our results also confirm that in vitro, DSB-120 is about 50 times more active than cisplatin under identical conditions. Marked cytotoxicity was also shown in two murine tumour cell lines (L1210 and ADJ/PC6) in vitro.

In view of these encouraging in vitro observations, antitumour studies were carried out using the bifunctional alkylating-agent sensitive ADJ/PC6 plasmacytoma. DSB-120 exhibited poor antitumour activity in this model using a variety of administration protocols, probably due to low selectivity for tumour versus normal tissue (therapeutic indices ~ 1). A comparison of LD_{50} data shows that DSB-120 was more toxic than cisplatin, carboplatin and Adriamycin in tumour-bearing BALB/c mice. The time of death (3–5 days post-treatment) is consistent with a haematological type of dose-limiting toxicity for DSB-120, and similar toxicities have been observed with other reactive DNA cross-linking agents (e.g. see Honess and Bleehen [7]).

Pharmacology studies were undertaken in an attempt to understand the discrepancy between the in vitro and the in vivo data and to aid further drug-development strategies. Stability studies showed that DSB-120 remained remarkably intact in mouse plasma at various temperatures but was readily degraded in whole blood at 37°C. This may be a consequence of DSB-120 reacting with glutathione released from haemolysed whole blood, a source of reduced glutathione (GSH) [18]. DSB-120 in vitro cytotoxicity has previously been shown to correlate with cellular GSH levels in a panel of ovarian tumour cell lines, and BSO pretreatment can partially reverse this resistance, producing a corresponding increase in DNA

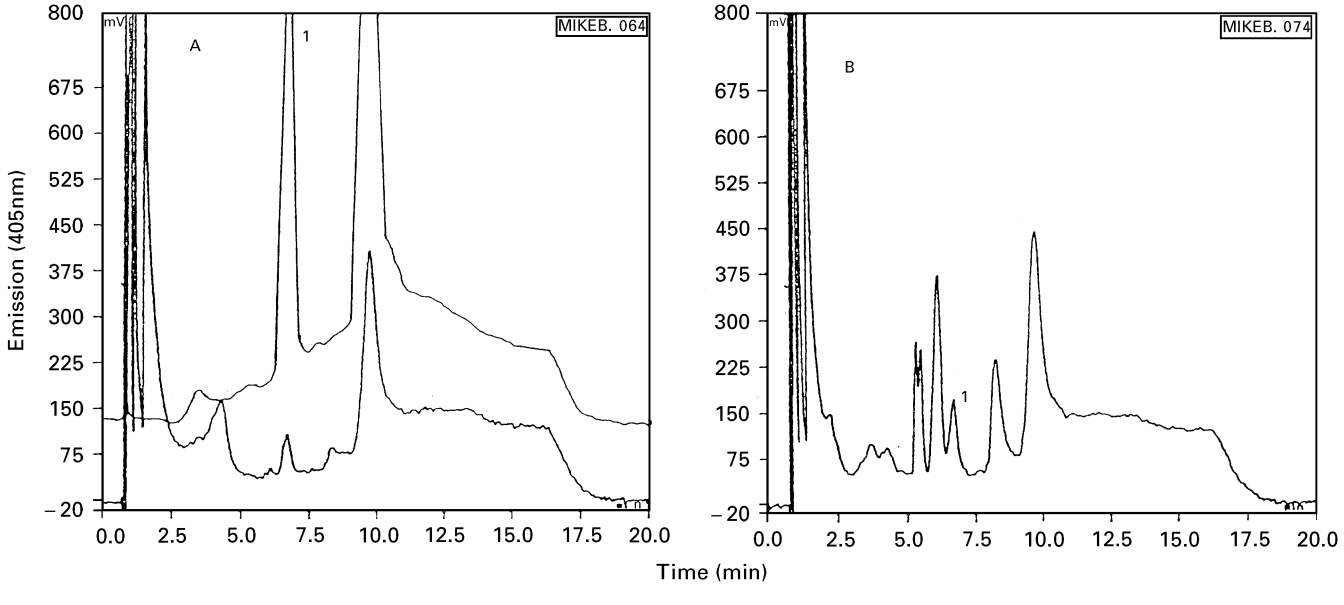

Fig. 5A, B Typical chromatograms obtained from mouse urine following DSB-120 drug administration (5 mg kg^{-1} i.v.). **A** *Upper trace* Water spiked with DSB-120 (5 μg ml^{-1}, *peak 1*). *Lower trace* 1/10 diluted blank mouse urine. **B** Urine (1/10 diluted) from mice given 5 mg kg^{-1} DSB-120 i.v., showing low concentrations of DSB-120 (0.28 μg ml^{-1}, *peak 1*) and several metabolites. For experimental details see Materials and methods

interstrand cross-linking [19]. In addition, PBDs have also been shown to react readily with thiol-containing nucleophiles such as thiophenol [21]. Drug-binding studies showed that DSB-120 was highly bound to plasma proteins (96.6%). Similarly high plasma protein binding has been reported for several active bifunctional alkylating agents such as lomustine (CCNU) and chlorambucil [12, 13].

Pharmacokinetics studies were carried out using the i.v. route, as this gave the highest antitumour activity. Following i.v. administration the plasma clearance of DSB-120 was biexponential, and significant errors (357%) would have been introduced by treating the data as monoexponential [5]. Peak plasma concentrations were 25 $\mu g\,ml^{-1}$ and were well above those shown to be active in a panel of colon and ovarian tumour lines in vitro (96-h IC_{50} values ranged from 0.006 to 0.33 μM, equivalent to 3.19–176 $ng\,ml^{-1}$) and in the murine ADJ/PC6 tumour cell line (72-h IC_{50} value 0.5 nM equivalent to 270 $pg\,ml^{-1}$). Moreover, if it is assumed that the drug was intact throughout the period of in vitro exposure then the overall exposure for the human cell lines at the IC_{50} ranged from 18.4 to 101 $\mu g\,ml^{-1}$ min and was 1.18 $\mu g\,ml^{-1}$ min for ADJ/PC6, a value well within the plasma $AUC_{0\rightarrow\infty}$ of 230 $\mu g\,ml^{-1}$ min. However, it should be noted that the amount of free drug in vivo may be limited by extensive plasma protein binding of DSB-120 and/or degradation.

Concentrations of DSB-120 measured in ADJ/PC6 tumours were markedly lower than those detected in plasma, reaching a peak of only 0.4 $ug\,g^{-1}$ (equivalent to 0.75 μM) with correspondingly low tumour/plasma ratios (5–10%) and tumour AUC values (5.8 $\mu g\,g^{-1}$ min, equivalent to 10.9 μM min). However, tumour drug concentrations were very similar to the IC_{50} values occurring in the human tumour cell lines and were above the corresponding IC_{50} values recorded for the ADJ/PC6 tumour cell line. Nevertheless, it is probable that not all the measured drug is freely available for binding to DNA, and a substantial fraction may be bound to cellular proteins or thiols. The low therapeutic index also suggests that the therapeutic efficacy may have been compromised by normal tissue toxicity. This may result from an inability of normal tissue to repair the novel, minor-groove DSB-120 DNA cross-link. Perhaps surprisingly, the use of multiple daily i.p. doses produced negligible antitumour activity in vivo although drug delivery to the tumour may be suboptimal under these conditions, possibly due to extensive first-pass metabolism. Urinary recovery experiments showed that 98% of the administered-dose was unaccounted for, possibly as a result of metabolism or tissue binding. It is possible that some of the metabolic products include ring-opened species.

In conclusion, DSB-120 showed potent in vitro cytotoxicity against both human and rodent tumour cell lines. The lack of in vivo antitumour activity observed for DSB-120 may have been due to low concentrations in tumour tissue and excessive normal tissue toxicity. This is consistent with the high plasma protein binding and extensive metabolism of this compound in mice. These pharmacokinetic data suggest that improved antitumour activity may be achieved by decreasing the electrophilicity of the imine species to create a less reactive drug. This may allow greater tumour drug penetration and the realisation of the full potential of this novel class of antitumour compounds.

Acknowledgements We thank Melanie Valenti and Paul Rogers for their excellent technical assistance during this investigation. This work was supported by funding from the UK Cancer Research Campaign.

References

1. Bose DS, Jones GB, Thurston DE (1992) New approaches to pyrrolo [2, 1-*c*] [1, 4] benzodiazepines: synthesis, DNA-binding and cytotoxicity of DC-81. Tetrahedron 48:751
2. Bose DS, Thompson AS, Ching J, Hartley JA, Berardini MD, Jenkins TC, Neidle S, Hurley LH, Thurston DE (1992) Rational design of a highly efficient irreversible DNA interstrand cross-linking agent based on the pyrrolobenzodiazepine ring system. J Am Chem Soc 114:4939
3. Bose DS, Thompson AS, Smellie M, Beradini MD, Hartley JA, Jenkins TC, Neidle S, Thurston DE (1992) Effect of linker length on DNA-binding affinity, cross-linking efficiency and cytotoxicity of C8-linked pyrrolobenzodiazepine dimers. J Chem Soc [D] 14:1518
4. D'Incalci M, Broggini M, Hartley JA (1992) Sequence and gene-specific drugs. In: Workman P (ed) New approaches in cancer pharmacology: drug design and development. Springer, New York Berlin Heidelberg, pp 5-11
5. Dvorchik BH, Vesell ES (1978) Significance of error associated with use of the one-compartment formula to calculate clearance of thirty-eight drugs. Clin Cancer Ther 23:617
6. Goddard PM, Valenti MR, Harrap KR (1991) The role of murine tumour models and their acquired platinum-resistant counterparts in the evolution of novel platinum antitumour agents: a cautionary note. Ann Oncol 2:535
7. Honess DJ, Bleehen NM (1982) Sensitivity of normal mouse marrow and RIF-1 tumour to hyperthermia combined with cyclophosphamide or BCNU: lack of therapeutic gain. Br J Cancer 46:236
8. Jenkins TC, Hurley LH, Neidle S, Thurston DE (1994) Structure of a covalent DNA minor-groove adduct with a pyrrolobenzodiazepine dimer: evidence for sequence-specific interstrand cross-linking. J Med Chem 37:4529
9. Jennrich RI, Sampson PF (1968) Application of a stepwise regression to non-linear estimation. Technometrics 10:63
10. Jones GB, Davey CL, Jenkins TC, Kamal A, Kneale GG, Neidle S, Webster GD, Thurston DE (1990) The non-covalent interaction of pyrrolo[2, 1-c] [1, 4]benzodiazepine-5, 11-diones with DNA. Anticancer Drug Des 5:249
11. Kelland LR, Abel G, McKeage MJ, Jones M, Goddard PM, Vallenti M, Murrer B, Harrap KR (1993) Preclinical antitumour evaluation of bis-acetato-ammine-dichloro-cyclohexylamine platinum (IV): an orally active platinum compound. Cancer Res 53:2581
12. Lee FYF, Workman P (1983) Modification of CCNU pharmacokinetics by misonidazole – a major mechanism of chemosensitisation in mice. Br J Cancer 47:659

13. Lee FYF, Coe P, Workman P (1986) Pharmacokinetic basis for the comparative antitumour activity and toxicity of chlorambucil, phenylacetic acid mustard and ββ-difluorochlorambucil (CB 7103) in mice. Cancer Chemother Pharmacol 17:21
14. Moutzouris JA, Wang JJ, Thurston DE, Hurley LH (1994) Comparison of a DSB-120 DNA interstrand cross-linked adduct with the corresponding bis-tomaymycin adduct: an example of a successful template-directed approach to drug design based upon the monoalkylating compound tomaymycin. J Med Chem 37:3132
15. Neidle S, Puvvada MS, Thurston DE (1994) The relevance of drug-DNA sequence-specificity to antitumour activity. Eur J Cancer 30:567
16. Potter M, Robertson CL (1960) Development of plasma-cell neoplasms in BALB/c mice after intraperitoneal injection of paraffin-oil adjuvant, heat-killed staphylococcus mixtures. J Natl Cancer Inst 25:847
17. Puvvada MS, Hartley JA, Jenkins TC, Thurston DE (1993) A quantitative assay to measure the relative DNA-binding affinity of pyrrolo[2, 1-c][1, 4]benzodiazepine (PBD) anti-tumour antibiotics based on the inhibition of restriction endonuclease BamH1. Nucleic Acids Res 21:3671
18. Shaw LM, Bonner HS, Brown DQ (1994) Metabolic pathways of WR-2721 (ethyol, amifostine) in the BALB/c mouse. Drug Metab Dispos 22:895
19. Smellie M, Kelland LR, Thurston DE, Souhami RL, Hartley JA (1994) Cellular pharmacology of novel C8-linked anthramycin-based sequence-selective DNA minor groove cross-linking agents. Br J Cancer 70:48
20. Thurston DE (1993) Advances in the study of pyrrolo[2, 1-c][1, 4]benzodiazepine (PBD) antitumour antibiotics. In: Neidle S, Waring M (eds) Molecular aspects of anticancer drug-DNA interactions. Macmillan, London, pp 54–88
21. Thurston DE, Morris SJ, Nevell TG (1990) Evaluation of the electrophilicity of DNA-binding pyrrolo[2, 1-c][1, 4]benzodiazepines by HPLC. J Antibiot (Tokyo) 43:1286
22. Wagner JG (1975) Fundamentals of clinical pharmacokinetics. Drug Intelligence, Chicago, Illinois
23. Walton MI, Workman P (1993) Pharmacokinetics and bioreductive metabolism of the novel benzotriazine di-*N*-oxide hypoxic cytotoxin tirapazamine (WIN 59075; SR 4233: NSC 130181) in mice. J Pharmacol Exp Ther 265:938