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POTENTIAL ANTITUMOUR MITOSENES: RELATIONSHIP BETWEEN *IN VITRO* DNA INTERSTRAND CROSS-LINK FORMATION AND DNA DAMAGE IN *ESCHERICHIA COLI* K-12 STRAINS

MARC MALIEPAARD,* KARIN A. M. C. SITTERS, NICO J. DE MOL, LAMBERT H. M. JANSSEN, IAN J. STRATFORD,† MIRIAM STEPHENS,† WILLEM VERBOOM‡ and DAVID N. REINHOUDT‡

Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Utrecht University, P.O. Box 80082, 3508 TB Utrecht, The Netherlands; †MRC Radiobiology Unit, Chilton, Didcot OX11 ORD, U.K.; and ‡Laboratory of Organic Chemistry, Faculty of Chemical Technology, University of Twente, P.O. Box 217, 7500 AE Enschede, The Netherlands

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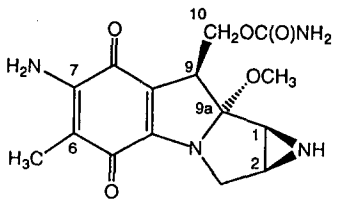
Abstract—This investigation was aimed at determining the possible relationship between DNA interstrand cross-linking and the cytotoxic activity of potential antitumour mitosene compounds. Mitosenes, possessing two good leaving groups at C-1 and C-10, were found to be able to cross-link calf thymus DNA under hypoxic conditions following sodium dithionite ($Na_2S_2O_4$) reduction at pH 7.0 and pH 5.5. DNA interstrand cross-linking was pH dependent for most of the mitosenes used, with a higher amount of cross-links formed at pH 5.5 compared to pH 7.0. Without reduction or under aerobic conditions no cross-link formation was detected. The importance of DNA damage for the toxic effect of these mitosenes was assayed by comparing the survival in a DNA repair deficient and a DNA repair proficient *E. coli* K-12 strain. A correlation between the number of cross-links formed in calf thymus DNA *in vitro* and the IC_{50} values in the DNA repair deficient *E. coli* strain was found. The effect of hypoxia on toxicity of mitosenes was studied in Chinese hamster V79 cells. In these cells, mitosenes appeared to be very active. Under severe hypoxic conditions toxicity of these mitosenes increased, most likely due to the increased lifetime of the activated mitosene species as compared to aerobic conditions. The results suggest that DNA cross-linking following reductive activation is important for the eventual activity of mitosenes in a bacterial system. Increased activity of mitosenes under hypoxic conditions in the V79 cells indicates that these mitosenes may be more active in hypoxic parts of tumours.

Key words: mitosene; mitomycin C; antitumour; reductive activation; DNA cross-linking; toxicity



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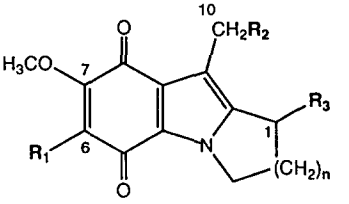
Structure 1.



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Structure 2.

DNA adduct formation is generally considered to be an important event in the cascade of effects leading to cell death induced by a number of antitumour agents: e.g. Adriamycin® [1], cis-platinum [2], MMC§ [3], nitrogen mustards [4] and nitrosoureas [5]. DNA adduct formation by MMC (Structure 1) only takes place upon reductive or acid catalysed activation [6, 7]. Reduction of MMC in the presence of DNA has been shown to yield DNA mono-adducts [8, 9], and DNA interstrand [10, 11] and intrastrand cross-links [12]. DNA interstrand cross-linking is considered to be one of the most important determinants for the eventual antitumour effect of MMC, although DNA mono-adducts may also contribute to the antitumour activity of this

* Corresponding author. FAX (31) 30 516674.

§ Abbreviations: MMC, mitomycin C; DMF, *N,N*-dimethylformamide; EtBr, ethidium bromide; F_b, fluorescence yield before denaturation; F_a, fluorescence yield after denaturation; log P, logarithm of the partition coefficient between phosphate buffer pH 7.4 and *n*-octanol.

agent [13]. Interstrand DNA cross-links can prevent cell division, and arrest of cells treated with DNA cross-linking agents in G_2 phase has frequently been shown to occur [14].

Mitosenes (Structure 2) are derived from MMC and can follow a similar reductive pathway. Thus, reduction activates the C-10 and C-1 sites of the molecule, leading to loss of leaving groups which can result in covalent binding with DNA and DNA cross-linking [15]. However, a striking difference with MMC is that the relative reactivity of the C-1 and C-10 site of the mitosenes is reversed, i.e. the C-10 site of the mitosene is more prone to react upon reduction than the C-1 site [15]. The presence of two good leaving groups at the C-1 and C-10 positions appears to be a prerequisite for good activity of these mitosenes in *in vitro* tumour models [16], suggesting that, as for MMC, DNA cross-linking is important for antitumour activity. However, *in vivo* activity of a 1,10-dihydroxy-mitosene in a human gastric tumour xenograft was high [17].

This study aims to elucidate the relation between the chemical structure of the mitosenes and the ability to form DNA cross-links. For this purpose cross-linking with calf thymus DNA was assayed *in vitro*. To evaluate the significance of the cross-linking data on a cellular level, these were compared with toxicity data on mitosenes in DNA-repair deficient *E. coli* bacteria. Toxicity in this bacterial system is indicative of the amount of DNA damage [18, 19].

As reductive processes are often strongly influenced by oxygen tension [20], the effect of hypoxia on *in vitro* DNA cross-linking by two 1,10-bisacetoxymitosenes was investigated. The effect of hypoxia on toxicity under well-controlled conditions was further evaluated in Chinese hamster V79 cells.

MATERIALS AND METHODS

Mitosenes were synthesized as described previously [16, 17]. Purity of all mitosenes, as checked with HPLC, was >96%. MMC was from Bristol-Myers Co. DMF p.a. was obtained from Baker (Deventer, The Netherlands). Calf thymus DNA and EtBr p.a. were purchased from Boehringer Mannheim (Almere, The Netherlands). Sodium dithionite ($Na_2S_2O_4$) p.a. was from the Sigma Chemical Co. (St Louis, MO, U.S.A.) and was stored dry. Mitosenes and MMC were kept as 10 mM stock solutions in DMF. These stock solutions were stored in the dark at 4° and were found to be stable under these conditions for at least 1 month. *E. coli* K-12 strains 343/765 and 343/753 were obtained from Prof. G.R. Mohn R.I.V.M. (Bilthoven, The Netherlands). Ingredients for bacterial media were obtained from Difco Laboratories (Detroit, MI, U.S.A.). Water was purified using Milli-Q filtration.

Fluorescence measurements were carried out on a Perkin–Elmer LS-50 spectrofluorimeter. Polarographic measurements were performed at room temperature using an electrochemical system consisting of an EG&G 303 dropping mercury electrode, an Ag/AgCl (saturated KCl) reference electrode, and a platinum wire auxiliary electrode. The potentiostat was computer-controlled, using the GPES 2.2 software package (Eco Chemie, The Netherlands).

DNA interstrand cross-linking of MMC and mitosenes. One mililitre of a solution containing 150 nmol of MMC or mitosene and 350 μg calf thymus DNA in 150 mM Tris–acetate buffer with pH 5.5 or 7.0 was purged with nitrogen for 10 min. In case of aerobic experiments solutions were purged with oxygen. Reduction was started by the addition of $Na_2S_2O_4$ (final concentration 300 μM). The $Na_2S_2O_4$ stock solution was prepared in nitrogen purged water directly before use. After 30 min of reduction the reaction was stopped by flushing with air. Unbound mitosene was removed by spun column chromatography (centrifugal molecular sieve filtration) over Sephadex G-25 coarse, followed by ethanol precipitation of DNA. The precipitated DNA was rehydrated overnight in water (200 μL) at 4°. From this solution 80 μL were mixed in a cuvette with ethidium bromide measuring solution (3 mL, containing 3 μg/mL ethidium bromide and 0.4 mM EDTA in 20 mM phosphate buffer pH 12), after which fluorescence was measured at 600 nm (excitation wavelength 525 nm). The DNA was then denaturated at 95° for 3 min and allowed to cool at 20° for exactly 10 min. Immediately, after, the fluorescence was measured once again. A blank solution containing only DMF instead of mitosene or MMC stock solution was treated the same way. The ratios between the fluorescence after (F_a) and before (F_b) denaturation of the samples [$(F_a/F_b)_s$] and the blank DNA sample [$(F_a/F_b)_b$] were used to calculate the extent of cross-link formation by means of the following formula [21]:

$$\% \text{ Cross-linking} = \frac{(F_a/F_b)_s - (F_a/F_b)_b}{1 - (F_a/F_b)_b} \times 100\%.$$

DNA repair test. Two *E. coli* K-12 strains were used in this assay: the DNA repair proficient 343/765 strain ("wild-type", lac^-) and the DNA repair deficient 343/753 strain ($recA^-$, $uvrB^-$, lac^+) [22]. Bacteria were maintained using NR-S agar and PEP-S media as described by Mohn *et al.* [19, 22]. The two strains can be distinguished at the same NR-S agar plates due to their different lactose metabolism, leading to different colouring by the neutral red indicator, which was added to the agar plates. After *E. coli* bacteria were grown overnight in PEP-S media they were mixed in a ratio of 1 to 9 (343/765 vs 343/753) to obtain comparable amounts of both *E. coli* strains in the incubation mixture. The *E. coli* bacteria were spun down at 3000 rpm for 10 min and the medium removed. Bacteria were resuspended in 60 mM phosphate buffer, pH 7.1, containing 140 mM glucose. This resuspension step was repeated and bacteria solutions diluted to obtain approx. 7×10^7 bacteria/mL. Bacterial mixtures (2 mL) were incubated with various concentrations of mitosenes under air. Samples were taken at various time intervals and after appropriate dilution, plated on NR-S agar plates and grown for 36 hr at 37°. The number of colonies of each strain after several incubation periods were counted and the surviving fraction (N/N_0) calculated.

Table 1. Structure of mitosenes

Compound	Code	N	R_1	R_2	R_3
3	WV15	1	$-CH_3$	$-OC(O)CH_3$	$-OC(O)CH_3$
4	WV14	1	$-CH_3$	$-OH$	$-OH$
5	WV21	1	$-CH_3$	$-H$	$-H$
6	WV16	1	$-CH_3$	$-OC(O)N(H)C_2H_5$	$-OH$
7	WV18*	1	$-CH_3$	$-OC(O)CH_3$	$-OC(O)CH_3$
8	WV28	1	$-CH_3$	$-OC(O)C_3H_7$	$-OC(O)C_3H_7$
9	WV2	2	$-H$	$-OC(O)CH_3$	$-OC(O)CH_3$
10	WV7	1	$-H$	$-OC(O)CH_3$	$-OC(O)CH_3$

* Aziridinyl group at C-7.

Table 2. DNA interstrand cross-linking by 150 μM mitosenes or MMC upon $Na_2S_2O_4$ activation (300 μM) in 150 mM Tris–acetate buffer, pH 5.5 and 7.0 under hypoxic conditions

Compound	No. of good leaving groups	% DNA cross-linking pH 5.5	pH 7.0	IC_{50} (μM)
3	2	44.0 ± 9.1	14.0 ± 4.1	0.12 ± 0.01
4	0	4.5 ± 2.1	−4.0 ± 3.0	≫ 50
5	0	−1.3 ± 2.0	0.4 ± 0.1	≫ 100
6	1	9.8 ± 0.1	3.9 ± 3.9	7.9 ± 1.3
7	2	36.3 ± 1.6	15.5 ± 3.6	0.15 ± 0.02
8	2	39.7 ± 4.0	16.8 ± 0.4	ND*
9	2	12.4 ± 0.8	14.2 ± 2.1	0.95 ± 0.06
10	2	11.7 ± 1.3	11.4 ± 2.3	0.60 ± 0.05
MMC		55.3 ± 7.7	72.7 ± 4.8	0.039 ± 0.006
MMC†		36.5 ± 4.9	23.5 ± 1.3	ND
3 (oxic)		4.9 ± 1.4	ND	ND
7 (oxic)		2.8 ± 0.6	ND	ND

Also included are IC_{50} values from the DNA repair deficient *E. coli* 353/753 strain. Finally cross-linking data using 150 μM mitosenes **3** and **7** under aerobic conditions are included. % DNA cross-linking and IC_{50} values are means ± SD obtained from at least three experiments.

* ND, not determined.

† 30 μM.

Polarographic assay of mitosene reduction in E. coli *K*-12 343/765 *and* 343/753. Bacteria were grown as described above. A bacteria suspension was prepared containing 7×10^7 bacteria of both 343/765 and 343/753 strains/mL in glucose-phosphate buffer pH 7.1. In an electrochemical cell, a volume of 5 mL was purged with nitrogen for 10 min, and compound **3** or **4** was then added to a concentration of 10 μM. Polarographic scans were performed at regular time intervals as described [18]. A blank solution, in which the mitosene was omitted, was measured under the same conditions.

Toxicity of mitosenes in Chinese hamster V79 cells under aerobic and hypoxic conditions. Toxicity in Chinese hamster V79 cells was determined as described previously [23]. In these experiments, the Chinese hamster cells are exposed to various concentrations of mitosenes under aerobic and hypoxic conditions for 3 hr at 37°, and toxic effects are assessed using the MTT assay.

RESULTS AND DISCUSSION

DNA interstrand cross-link formation

DNA interstrand cross-link formation was assayed using $Na_2S_2O_4$ reduction of the mitosenes (for structures see Table 1). For MMC, reduction with $Na_2S_2O_4$ results in relatively high yields of DNA interstrand cross-links, as compared to other types of reductive activation [10, 24]. Results of the fluorescence DNA cross-link assay are summarized in Table 2. Mitosenes, possessing two good leaving groups at C-1 and C-10, are able to cross-link DNA upon reductive activation with a 2-fold excess of $Na_2S_2O_4$. Compound **4**, with two poor leaving hydroxygroups at C-1 and C-10, and compound **5**, without any leaving groups at these sites, produced hardly any DNA cross-link at both pH-values studied. Previously we described the stability of these mitosenes upon reduction [15], and the results obtained in the DNA cross-link assay for compounds **4**, and **5** are consistent with that finding. Unexpectedly, compound **6**, with only one good leaving group (at C-10), was able to produce some cross-links at lower pH. For all compounds, reductive activation was a necessary event for cross-link formation to occur both at pH 5.5 and 7.0 (for data on **3**, see Fig. 1). Reduction of the active bisacetoxymitosenes **3** and **7** at pH 5.5 under aerobic conditions strongly decreased the amount of cross-linking, probably due to the decreased lifetime of the alkylating species caused by increased redox-

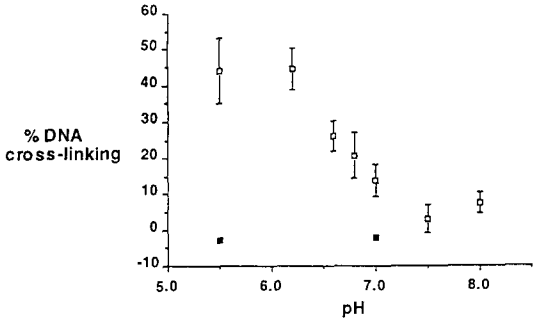

Fig. 1. pH profile of interstrand cross-linking of calf thymus DNA (350 μg/mL) by 150 μM of mitosene **3** in 150 mM Tris–acetate buffer upon reductive activation (30 min) with a 2-fold excess of $Na_2S_2O_4$ (□) or without reduction (■) under nitrogen. After the reaction was stopped samples were purified and ethidium bromide fluorescence was measured as described in Materials and Methods. Values are means ± SD from at least three experiments.

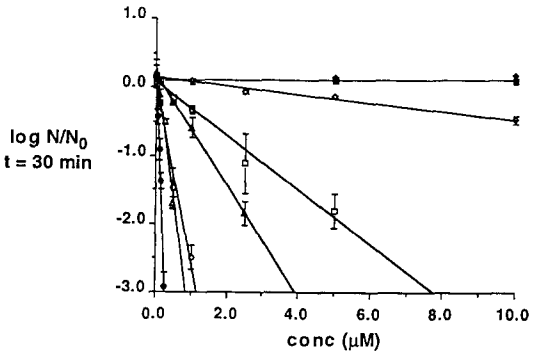

Fig. 2. Colony formation of DNA repair deficient strain 343/753 after 30 min of incubation with various concentrations of MMC (□) and mitosenes **3** (△), **4** (◇), **5** (○), **6** (■), **7** (▲), **9** (◆), and **10** (●). Incubations (2 mL, 7×10^7 bacteria/mL) were carried out at 37° in glucose-phosphate buffer, pH 7.1. N/N_0 is the fraction of colony forming bacteria. The DNA repair proficient 343/765 strain is not affected at all concentrations of mitosenes or MMC used. Values are means from at least three experiments.

cycling of the quinones under these oxic conditions (Table 2).

For most mitosenes DNA interstrand cross-link formation was pH dependent, with a larger number of cross-links formed at lower pH. A similar pH-dependency of DNA cross-linking by the bisacetoxy-mitosene **3** has been observed independently using the method developed by Hartley *et al.* [25,*]. Earlier studies in our laboratory showed that the conversion rate of these mitosenes upon reduction was increased at lower pH [15], and the increase in DNA interstrand cross-linking at lower pH is consistent with that finding. However, when the pH-dependency of DNA cross-linking by mitosene **3** was investigated in detail (see Fig. 1), maximal DNA interstrand cross-link formation occurred at pH 6.2, with no further increase being noted when the pH was decreased to 5.5.

A pH-dependency of MMC induced DNA cross-linking in both cell-free [26, 27] and cellular systems [28, 29] has been reported. The absence of this pH effect with 150 μM MMC under the conditions used in the present study was shown to be caused by saturation of the MMC cross-linkable sites in the DNA. Using a concentration of 30 μM instead of 150 μM of MMC, pH-dependency of MMC induced DNA cross-linking was observed (Table 2), in accordance with the literature [26, 27].

DNA damage test

To study the possible relationship between *in vitro* cross-linking ability and DNA damage on a cellular level, the effect of mitosenes in a bacterial DNA damage test system was investigated [18, 19]. The advantage of this system is that the cell wall is permeable for small molecules [19] and thus intracellular transport will not be discriminative for

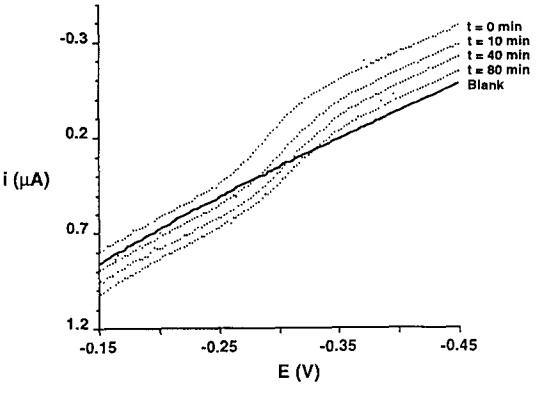

Fig. 3. Polarograms of an incubation mixture of 10 μM of mitosene **4** and 7×10^7 *E. coli* 343/765 and 343/753 bacteria/mL in glucose-phosphate buffer pH 7.1, recorded at several time intervals.

the various compounds. A time- and dose-dependent effect of the mitosenes on the viability of the DNA repair deficient *E. coli* 343/753 strain was observed. The colony formation (as log N/N_0 values) of this strain after 30 min of incubation with various concentrations of mitosenes or MMC is shown in Fig. 2. From these plots, IC_{50} values were obtained and are presented in Table 2.

The presumed reduction of mitosenes by endogenous bacterial reductases was tested polarographically. Using this polarographic technique, only mitosenes which are reduced and subsequently transported out of the bacteria are measured. By using compounds **3** and **4** with their two acetoxygroups and two hydroxygroups at positions C-1 and C-10, respectively, the effect of lengthening the C-1 and

* Tomasz M and He Q-Y, Personal communication, Hunter College of the City University of New York, U.S.A.

C-10 substituents was evaluated. The results on mitosene **4** are shown in Fig. 3. For mitosene **3**, analogous results were obtained (data not shown). Both mitosenes were reduced at a comparable rate by the bacteria, as indicated by the decrease in the cathodic wave, and the increase in the anodic wave as a function of time. From the polarographical data a half-life of 35 min was obtained for reduction of both mitosenes at an initial mitosene concentration of 10 μM. Because no differences in reduction velocity between mitosenes **3** and **4** were detected and half-wave reduction potentials of the mitosenes used in this study all fall within a relatively small range [16], we have assumed that reduction in the *E. coli* bacteria occurred for every mitosene used in this study.

No effect on the DNA repair proficient 343/765 strain was noted with all concentrations of mitosenes or MMC used. The insensitivity of the DNA repair proficient strain to all mitosenes and MMC indicates that no irreparable DNA damage occured under the conditions applied here.

Because of the pH-dependency of DNA cross-linking and the accelerated conversion of mitosenes upon reduction at lower pH [15], we checked the pH in the glucose–phosphate incubation mixture containing 7×10^7 bacteria of each strain. No difference in pH between the two *E. coli* suspensions was detected, leading us to conclude that pH effects are not involved in the increased sensitivity of the DNA repair deficient *E. coli* 343/753 strain to mitosenes. The effect of the mitosenes on the viability of the DNA repair deficient *E. coli* can therefore be regarded as a measurement for DNA damage caused by the reductively activated mitosenes.

In the DNA damage test, mitosenes lacking good leaving groups at C-1 and/or C-10 (i.e. compounds **4, 5** and **6**) proved to be much less active than the other mitosenes. A correlation was observed between the number of DNA interstrand cross-links formed by mitosenes upon $Na_2S_2O_4$ reduction and the activity in the 343/753 strain, with the mitosenes yielding the highest number of cross-links (e.g. mitosenes **3** and **7**) being the most active compounds in the DNA damage test. The fact that bifunctional mitosenes are the most active compounds in the test strongly suggests that DNA cross-linking is the major cause of this effect. The lower toxicity of mono-functional **6** in the 343/753 strain suggests that DNA mono-adducts in the *E. coli* strains are much less important for activity than DNA cross-linking.

We previously observed that mitosene activity in *in vitro* tumour models was strongly dependent on lipophilicity [16]. However, no such correlation was found in this bacterial system. This lack of correlation is likely due to the highly permeable membranes of these bacteria [19]. In these bacteria, differences in transport characteristics of mitosenes (as determined partly by their log P value) are thus expected to be less important than in eucaryotic cells [16].

Differential mitosene toxicity under oxic and hypoxic conditions

The reductive activation/alkylation process, essential for DNA cross-linking by mitosenes, can be precluded by redox-cycling of the quinone in the presence of oxygen [20]. The DNA damage test was performed under aerobic conditions, and therefore redox-cycling of mitosenes may compete with their DNA alkylation in the *E. coli* strains. Assaying a DNA damage test using mitosene **3** under nitrogen (as in the DNA cross-link assay) did not significantly influence the toxicity of the compound in the *E. coli*. However, nitrogen flushing does not exclude the presence of oxygen completely. To study toxicity of mitosenes **3** and **7** under stricter conditions, experiments were performed with V79 Chinese hamster cells under aerobic and severely hypoxic (<1 μmol/dm^3 O_2 in solution) conditions [23]. Under hypoxic conditions toxicity in the V79 cells of **3** and **7** increased 2- and 9-fold, respectively, relative to aerobic conditions (Table 3). The lesser activity of these mitosenes under aerobic conditions is likely to be caused by redox-cycling of the quinones, leading to a shorter lifetime of reduced mitosene species and thus diminished DNA cross-linking. This hypothesis is supported by the diminished cross-linking ability of mitosenes **3** and **7** upon $Na_2S_2O_4$ reduction under aerobic conditions (Table 2). Both mitosenes **3** and **7** were shown to be highly active in the V79 cell line. The absence of mitosene **5** toxicity in the V79 cells quite clearly indicates the importance of the leaving groups at the C-1 and C-10 position of the mitosene, thereby indicating that in these V79 cells as well DNA cross-linking may be crucial for toxicity of these mitosenes.

Interestingly, addition of 200 μM of the DT-diaphorase inhibitor dicoumarol in the V79 cell incubation mixture under aerobic conditions led to a 62 and 28-fold decrease in toxicity of mitosene **3** and **7**, respectively (Table 3). This marked effect of dicoumarol suggests that the two-electron donating enzyme DT-diaphorase may play a role in the reductive activation of these mitosenes and is another indication of the importance of reduction as a prerequisite for activity. The importance of DT-diaphorase for toxicity of mitosenes in V79 cells probably also explains why MMC is much less active in these cells. MMC has been shown to be a poor substrate for DT-diaphorase at physiological conditions [as is also apparent by the lack of effect of dicoumarol on MMC toxicity in V79 cells (Table 3)] [27]. The role of DT-diaphorase, together with the role of one-electron reducing enzymes in the reductive activation and DNA adduct formation by these mitosenes, is currently being explored further.

Conclusions

This study demonstrates that DNA interstrand cross-linking occurs after reductive activation of bifunctional mitosene compounds under hypoxic conditions. The number of DNA cross-links formed increases at lower pH, in accordance with the higher conversion rates found upon reduction at lower pH [15], and diminishes under aerobic conditions. The correlation between the *in vitro* cross-linking ability of mitosenes and the toxicity in the DNA repair deficient *E. coli* 343/753 strain suggests that DNA interstrand cross-link formation plays a prominent role in toxicity in this bacterial system. The low activity of the mono-functional mitosene **6** in the *E.*

Table 3. IC_{50} values of mitosenes and MMC for toxicity in Chinese hamster V79 cells under aerobic and extreme hypoxic conditions, using the MTT assay

Compound	IC_{50} (nM)		IC_{50} (nM)		
	Air	Air + dicoumarol*	Protection factor	N_2	Differential toxicity air/N_2
3	1.2	75	62	0.6	2.0
5	180,000	ND	ND	180,000	1.0
7	36	1000	28	4.0	9.0
MMC	800	800	1.0	400	2.0

Values are obtained from up to four individual experiments.
* 200 μM dicoumarol added.

coli 343/753 strain probably indicates that DNA mono-adduct formation by these mitosenes is of less importance than DNA cross-linking in the bacterial system.

Also in Chinese hamster V79 cells, DNA cross-linking by mitosenes is assumed to be important for activity. In this mammalian system, the mitosenes appear to be highly active, with pronounced activity under hypoxic conditions. Therefore, these mitosenes may exert selective activity in the hypoxic parts of a tumour, as in the core of solid tumours [30]. Moreover, the increased number of DNA cross-links formed upon reduction at lower pH suggests that artificially lowering the internal pH of tumour cells [29, 31] may render the cells more sensitive to these mitosenes.

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