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Mitomycin C Linked to DNA Minor Groove Binding Agents: Synthesis, Reductive Activation, DNA Binding and Cross-Linking Properties and In Vitro Antitumor Activity

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Abstract—Mitomycin C (MC) is a natural cytotoxic agent used in clinical anticancer chemotherapy. Its antitumor target appears to be DNA. Upon bioreductive activation MC alkylates and cross-links DNA. MC derivatives were synthesized in which MC was linked to DNA minor groove binding agents, analogous to netropsin and distamycin. One, two and three *N*-methylpyrrole carboxamide units were conjugated with MC by a $(CH_2)_5$-tether to the 7-amino group of MC (**11**, **12** and **13**, respectively). In contrast to MC **11**, **12** and **13** displayed non-covalent affinity to DNA. Their bioreductive activation by NADPH-cytochrome *c* reductase proceeded as fast as that of MC. Metabolites arising from reductive and low-pH activation were characterized and found to be analogous to those of MC. DNA cross-linking activities were weak and decreased with an increasing number of *N*-methylpyrrole carboxamide units linked with the mitomycin molecule. No adducts were formed with calf thymus DNA in detectable amounts. In vitro antitumor activities of **11**–**13** were determined using the NCI in vitro antitumor screen. The conjugates **11**–**13** are growth inhibitory; however, their activities are 1.5–2 orders of magnitude lower than that of MC. COMPARE analysis indicates that the mechanism of the action of **11** and **12** correlates moderately with MC but negatively with distamycin. Conjugate **13** correlates neither with MC nor with distamycin. The results suggest that the basic cause of the observed low activity of the MC–minor groove binder conjugates is the fast irreversible decay of the activated MC, competing effectively with the slow drug delivery to CpG sites, required for the alkylation.

Introduction

Mitomycin C (MC; **1**) is a natural antitumor antibiotic, used in the clinic in anticancer chemotherapy. Substantial evidence indicates that its direct molecular target in tumor cells is DNA. Damage to DNA is generated by mono- and bifunctional alkylation of guanine residues by MC, leading to MC–guanine monoadducts (**2**, **3**) and MC–guanine bisadducts; the latter constitute DNA interstrand and intrastrand cross-links (**4, 5**) which are formed specifically at CpG and GpG sequences, respectively (Scheme 1).[1] MC is the only natural antitumor antibiotic known to cross-link DNA in the cell. Both the monofunctional and bifunctional DNA lesions occur only after reductive activation of MC, mediated in vivo by various flavoreductases. This process can be mimicked in cell-free systems by purified reductase or chemical reducing agents.[1] On account of this property, MC is regarded as the prototype bioreductive antitumor agent.[2] Members of this class are hypoxia-selective cytotoxins; i.e. more cytotoxic under hypoxic than anaerobic conditions of drug exposure. Bioreductive drugs are also selectively active against hypoxic regions of solid tumors. In contrast, such regions are relatively resistant to treatment with radiation or common chemotherapeutic agents since these treatments require the presence of oxygen for cell killing.[3] Synthetic bioreductive drugs have been developed for postoperative treatment of solid tumors used in conjunction with treatment with radiation.[4] Furthermore both a combination of MC or porfiromycin (N1a-methyl MC) with radiation are treatment protocols currently undergoing large-scale clinical evaluation.[5,6]

Recent studies of the mechanism of the reductive activation of MC indicated that, in addition to its reaction with DNA, the unstable active form decomposes rapidly

Keywords: Antitumor activity; COMPARE; DNA; mitomycin C; mitomycin-lexitropsin conjugates.

Abbreviations: MC, mitomycin C; dT, 2′-deoxythymidine; MC–Py_1, MC–monopyrrole (**11**); MC–Py_2, MC–bispyrrole (**12**); MC–Py_3, MC–trispyrrole (**13**).

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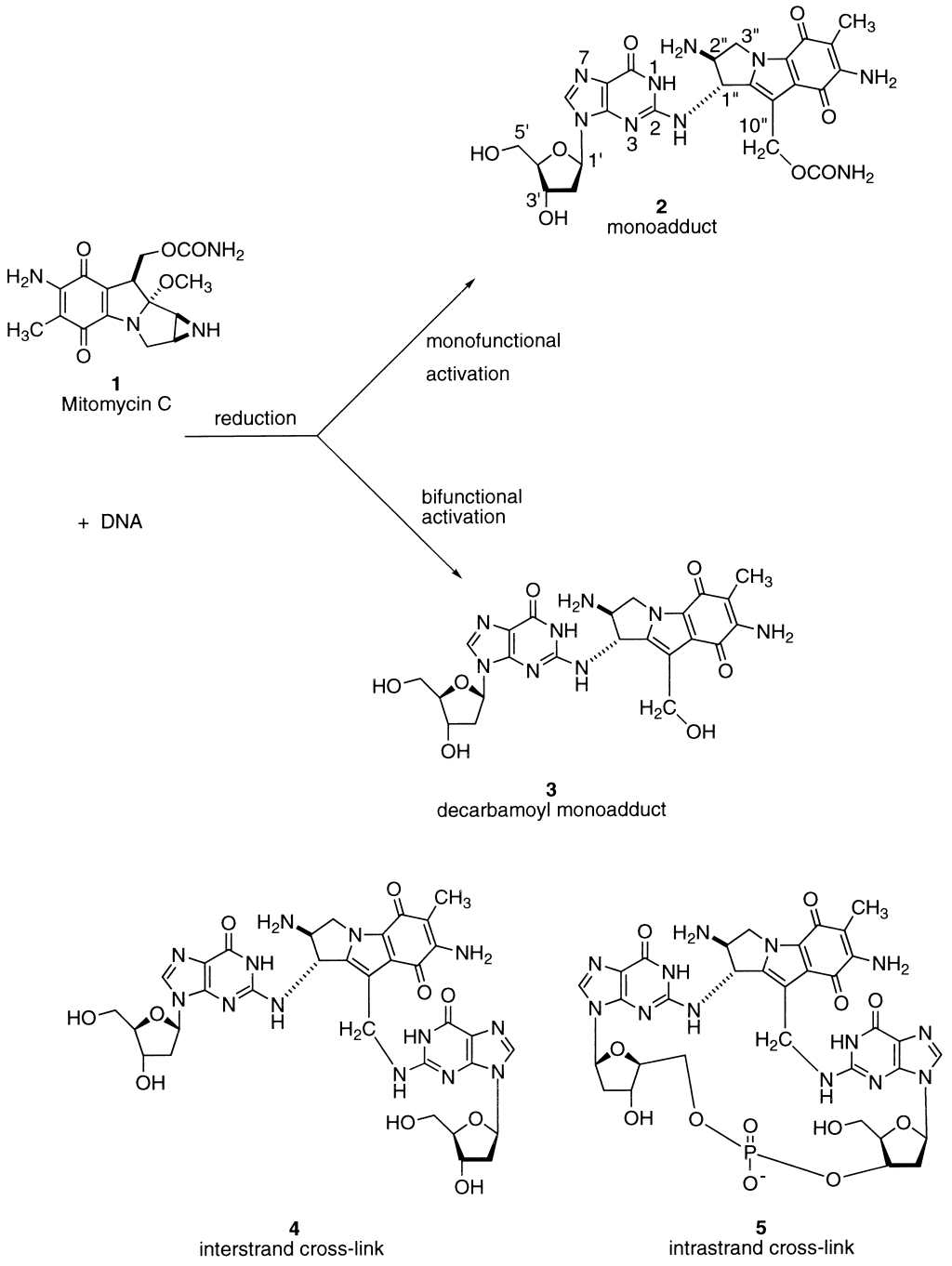
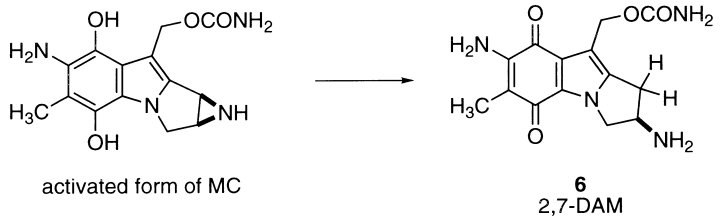


Scheme 1. Mitomycin C–deoxyguanosine adducts.

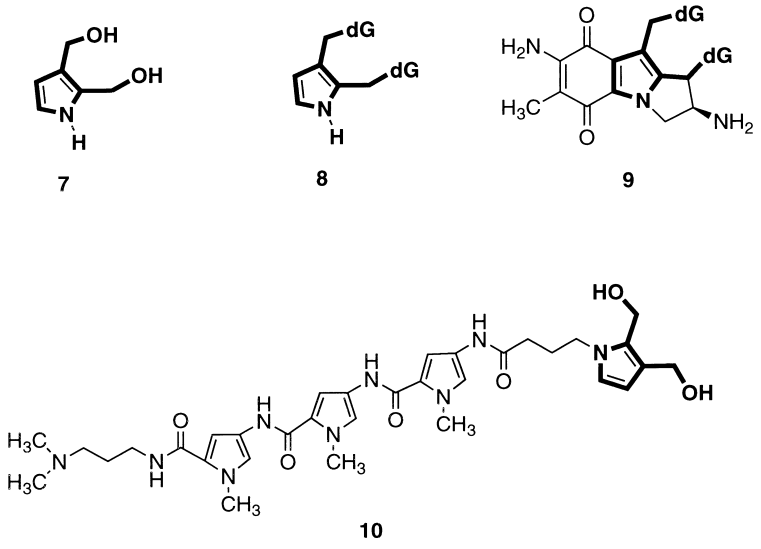
to inactive metabolites, mainly to 2,7-diaminomitosene (**6**; Scheme 2).[7–9] This reductive inactivating pathway also prevails in tumor cells,[10,11] diminishing the in vivo potency of MC in its alkylation and cross-linking of cellular DNA. The partitioning of the active form of MC into reaction with DNA versus conversion to inactive metabolites depends on the kinetic competition of the two processes.[7,8,11] In contrast to most natural products which interact with DNA, MC has no DNA binding affinity until after its conversion to activated mitosene forms.[12,13] Therefore, it is likely that much of the MC that enters the cell is activated at intracellular locations which are not in proximity to DNA. Flavoreductases that activate MC are present in the cytoplasm, microsomal fraction and mitochondria, as well as in the nucleus.[14–16] Consequently in non-nuclear locations the active form is expected to decay mainly to inactive metabolites. As consistent with these notions, MC is approximately 2–3 orders of magnitude less potent than CC-1065 or natural enediynes, both of which bind DNA prior to their activation.[17]

In order to construct more potent mitomycins a rational approach may be to append MC with pre-activation DNA-binding capacity. Accordingly, we designed a series of hybrid drugs in which MC is linked to the oligo



Scheme 2. Formation of reductive metabolite **6** from activated MC.

N-methylpyrrole carboxamide framework, present in netropsin and distamycin, two well-characterized minor groove binders with $(A/T)_n$ sequence specificity.[18] We hypothesized that on account of their affinity to DNA the MC–pyrrole carboxamide hybrids will be rapidly attracted to the cell nucleus, in proximity to DNA. Reductive activation would then occur near their DNA targets, kinetically facilitating the reactions of the activated form with DNA. This would make MC a more efficient DNA damaging agent. A successful model for this scenario in vitro is the cross-linking of DNA by 2,3-bis(hydroxymethyl)pyrrole (**7**; Chart 1). The cross-link (**8**) is structurally closely analogous to that involving MC (**9**; see also **4**) and favors the same sequence, CpG.[19] When this agent was conjugated to a distamycin derivative (**10**) the yield of DNA cross-linking increased 1000-fold. In addition, the conjugate was quite cytotoxic, in contrast to the marginally toxic parent components.[19] Although the selective binding of the distamycin moiety to A/T sequences may have reduced the number of CpG cross-link sites available to the conjugate relative to free 2,3-bis(hydroxymethyl)-pyrrole, the increased efficiency of the cross-linking reaction at those sites apparently over-compensated for the decrease in the number of cross-link target sites. The similarly enhanced cytotoxicity and cross-linking



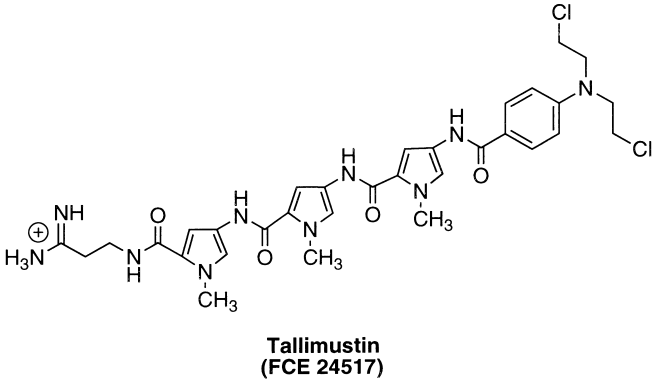


Chart 1. 2,3-Bis(hydroxymethyl)pyrrole and analogous geometry of its DNA cross-link to the geometry of the MC–DNA cross-link.

efficiency of a psoralen–distamycin conjugate as compared to free psoralen further substantiated the success of this type of design.[20]

Several conjugates of mustard-type cross-linking agents and distamycin analogues have been synthesized and investigated for activity.[21] The most notable of these, tallimustin, a benzoic acid mustard–distamycin hybrid, is under clinical trial as an anticancer drug. Structure–activity relationships of such hybrids, however, are not well understood. For example, tallimustin, unlike the parent mustard, does not generate DNA cross-links.[22,23]

An important difference of MC from other DNA cross-linking and alkylating agents is that MC requires enzymatic reductive activation and the active form decomposes very rapidly. Due to lack of appropriate precedent it was not possible to predict whether activation of MC would occur in its conjugate forms, bound or unbound to DNA. In an effort to determine experimentally the merits of the design of MC-oligo-*N*-methylpyrrole carboxamide conjugates as cytotoxic antitumor agents, we synthesized a series of compounds consisting of one, two and three *N*-methylpyrrole carboxamide units linked to the MC moiety by a flexible linker (Chart 2; **11**–**13**). Bioreductive activation and DNA binding and cross-linking properties of the conjugates in comparison with free MC were determined. In addition, the in vitro antitumor activity of each of the new agents **11**–**13** was evaluated against a panel of 60 human tumor cell lines of the National Cancer Institute.[24]

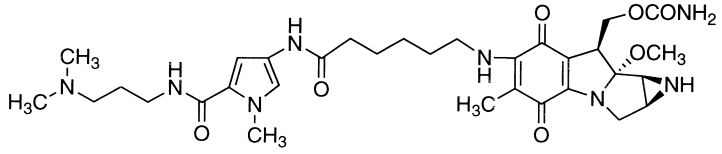
Results

Synthesis of mitomycin C–*N*-methylpyrrole carboxamide conjugates 11, 12 and 13 (Scheme 4)

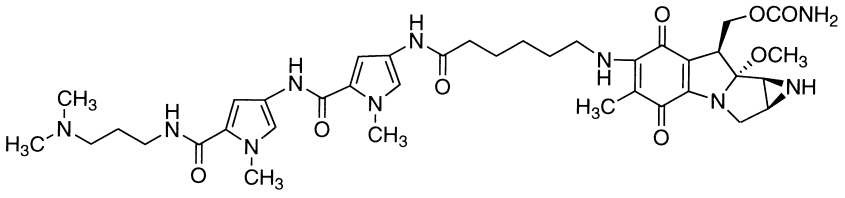
The key step of linking the chemically fragile mitosane moiety[25] to the *N*-methylpyrrole carboxamides was based on the well-precedented facile displacement of the 7-methoxy group of mitomycin A (**14**) by primary amines (Scheme 3).[26] The requisite primary amine derivatives of the mono-, bis- and tris-*N*-methylpyrrole carboxamides (**21**, **22** and **23**) were synthesized starting from the known[27] nitropyrroles **15**, **16** and **17**, respectively. The nitro group was reduced to the amino group by catalytic hydrogenation. This was followed by in situ acylation of the primary amine by the *N*-t-BOC-protected 6-aminocaproic acid, activated by carbonyldiimidazole, to give **18**, **19** and **20**. Subsequent removal of the t-BOC groups by TFA yielded **21**, **22** and **23**. Incubation of each with mitomycin A (**14**) in methanol for 16 h at room temperature yielded the desired MC–mono-, bis- and tris-*N*-methylpyrrole carboxamide conjugates **11**, **12** and **13**, respectively, in excellent yields. All new structures were rigorously verified by HRMS or MS, and ^{1}H NMR.

Proof of purity of 11, 12 and 13

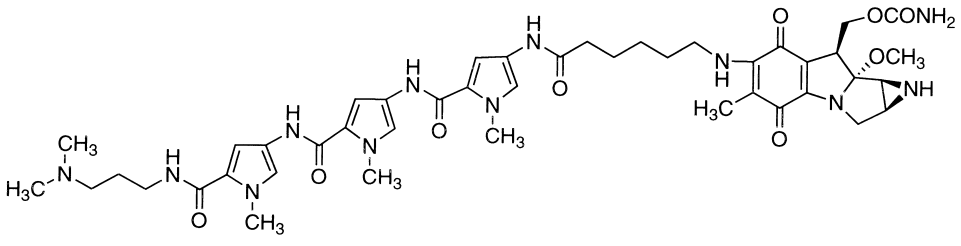
HPLC of the samples showed single peaks as detected by UV absorbance at 254 nm (elution times: **11**, 22 min; **12**, 24 min; **13**, 26 min). ^{1}H NMR spectra indicated no impurities in any of the compounds. Rigorous proof



11
MC-monopyrrole conjugate

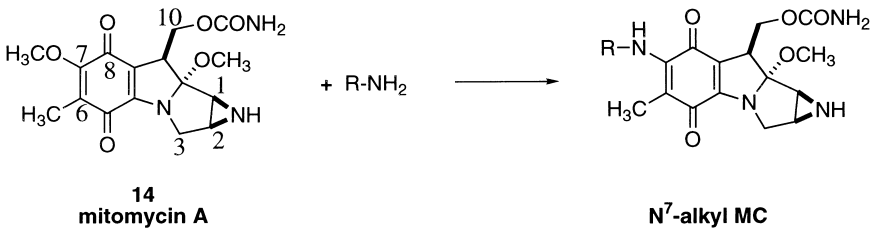


12
MC-bispyrrole conjugate

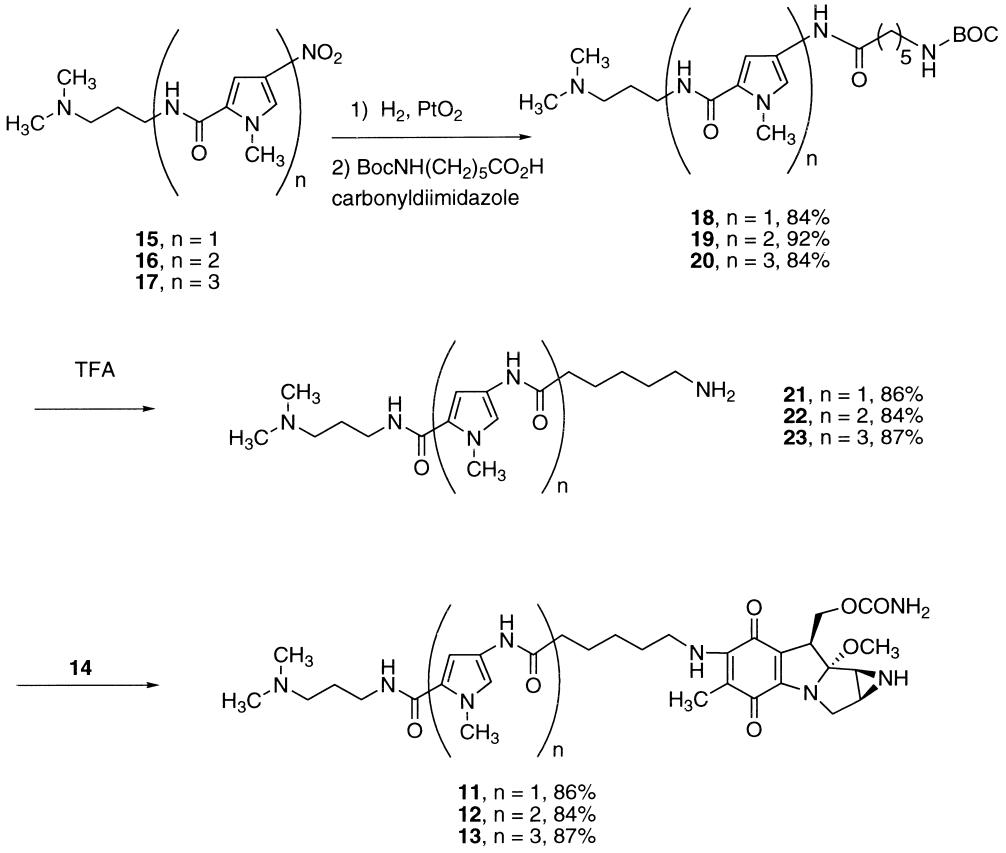


13
MC-trispyrrole conjugate

Chart 2. Mono-, bis- and tris-*N*-methylpyrrole carboxamide conjugates of MC.



Scheme 3. Reaction of mitomycin A with primary amines.



Scheme 4. Synthesis of MC–*N*-methylpyrrole carboxamide conjugates **11**, **12** and **13**.

was provided by determining the molar extinction coefficients (ε) of **11**–**13** at 374 nm, corresponding to a λ_{max} of the MC chromophore. At this wavelength the methylpyrrole carboxamide moiety has negligible absorption, as seen in the UV spectra of **21**, **22** and **23** (data not shown). The ε_{374} values of **11**–**13** were very similar to that reported for MC (Table 1). Purity in HPLC, the ε_{374} values and the ^{1}H NMR spectra together constitute reliable proof of purity of **11**–**13**.

Non-covalent binding affinity of 11–13 to calf thymus DNA

Using the EtBr displacement assay[28] as in previous DNA binding studies of drug–lexitropsin conjugates,[29] it is shown that the bis- and tris-pyrrole MC conjugates **12** and **13** bound to DNA in the micromolar range; binding by the mono-pyrrole **11** was approximately 100-fold weaker. The unconjugated MC showed no binding, as expected[12] (Table 1).

Reductive enzymatic activation of 11–13 in the absence or presence of DNA

In order to assess whether the modified mitomycin moieties of **11**, **12** and **13** were substrates of a MC-activating enzyme the rate of reduction of **11**, **12** and **13** and MC by NADH-cytochrome *c* reductase in neutral aqueous buffer was measured under identical assay conditions. The rate of reduction of MC can be assayed conveniently by the disappearance of MC from the reaction mixture followed by HPLC at various times.[30] The same method was employed here for **11**–**13**. The results show that all three conjugates **11**–**13** were reduced by NADH-cytochrome *c* reductase and NADH at rates similar to that observed for MC itself (Fig. 1a).

The same method was employed for assaying the reduction rate when DNA was present in the reaction mixture. The procedure was more complex, however, since the DNA had to be enzymatically hydrolyzed

Table 1. Molar extinction coefficients at 374 nm (ε_{274}) and apparent binding constants (K_{app}) to calf thymus DNA of **11**, **12**, **13**, MC and distamycin

	ε_{374} (M^{-1} cm^{-1}) (in H_2O)	K_{app} (μM^{-1})
11	18,900	0.036 (±0.009)
12	17,500	1.29 (±0.07)
13	18,100	3.87 (±0.68)
MC	21,840 (365 nm)	No binding[12]
Distamycin		0.77[44]

before the HPLC analysis at each time point, in order to release all remaining starting material (**11**–**13**) from its DNA-bound form. The results indicated that the reduction proceeds with equal efficiency both in the presence and absence of DNA (Fig. 1b).

Products of acidic and reductive activation of the conjugates 11–13 (Chart 3)

Diode-array HPLC and LCMS were used to study the formation of metabolites after acid treatment of conjugates **11**–**13** or after their chemical or enzymatic reduction by $Na_2S_2O_4$ and NADH-cytochrome *c* reductase, respectively. All activation conditions resulted in the disappearance of the starting materials and in the formation of several metabolites. Characterization of the metabolites by their UV and mass spectra (data not shown) indicated a similar chemistry to that of MC:[7,32,33] the acidic treatment or the enzymatic reduction produced metabolites **24**–**26**, containing the inactive mitosene moiety; enzymatic activation (NADH-cytochrome *c* reductase) produced, in addition, the 2,7-diaminomitosene derivatives **27**–**29**. The chemical reduction with sodium dithionite gave additional metabolites resulting from bifunctional activation, i.e. activation of both the C-1 and C-10 functions of the MC residue (**30**–**35**). In addition to the expected metabolites, the activation of **11**, **12** and **13** produced **21**, **22** and **23**, respectively, under all activation conditions indicating dissociation of the pyrrole–mitomycin linkage. These compounds were identified by their spectroscopic data and by comparison to authentic samples.

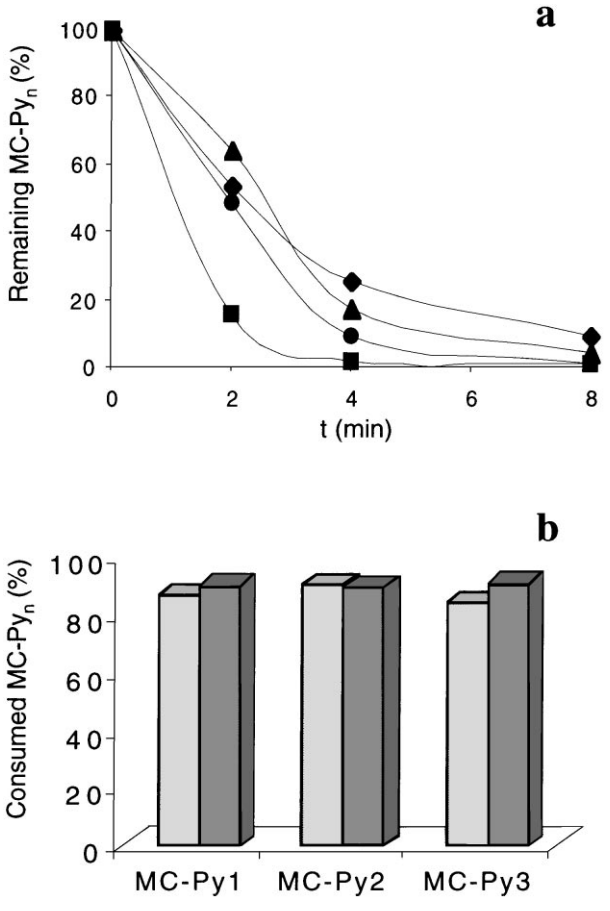


Figure 1. Reduction of MC and MC–Py_n conjugates **11**–**13** by NADPH-cytochrome *c* reductase. (a) Time course of the reduction in the absence of DNA: circles, MC; squares, MC–Py_1 (**11**); triangles, MC–Py_2 (**12**); rhombus, MC–Py_3 (**13**). (b) Comparison of the extent of reductions in the absence (light bars) and presence (dark bars) of DNA at 8 min [data from (a)].

Cross-linking of DNA by the conjugates 11–13 in a cell-free system

pBR322 plasmid DNA linearized by cleavage by the restriction enzyme *Eco*RI served as the substrate for testing the cross-linking activity of the conjugates and MC. The assay method of Hartley and co-workers[31] was employed. According to this method, linearized DNA is 3′-end-labeled by ^{32}P-phosphate, treated with the cross-linking agent, and subsequently denatured by heating. On cooling, the cross-linked DNA undergoes quick renaturation on account of the covalent linkage(s) between the two strands, while non-cross-linked DNA remains single stranded. The renatured and single-stranded fractions are separated by non-denaturing agarose gel electrophoresis and quantitated by phosphorimagery. The method was used for mitomycin and its derivatives previously.[30] DNA cross-linking by MC requires a reducing agent for activation of the drug; $Na_2S_2O_4$ was employed under anaerobic conditions in the present case. The results (Fig. 2) show that all three conjugates **11**–**13** cross-link pBR322 DNA. The monopyrrole conjugate **11** is more active than MC while **12**, and especially **13**, are less so. At 100 μM drug concentration MC and **11** cause 100% cross-linking, while **12** and **13** cross-link 60 and 18% of the DNA, respectively. At >10 μM concentration the efficiency of **12** and especially of **13** decline relative to that of MC.

Lack of formation of DNA adducts

MC–pyrrole conjugates **11**–**13** were activated with either sodium dithionite, NADH-cytochrome *c* reductase/NADH or catalytic hydrogenation in the presence of calf thymus DNA. Non-covalently bound drug metabolites were removed by dialysis or successive extractions with *n*-butanol,[34] until the absorbance at 320 nm remained constant. The DNA was then enzymatically digested and analyzed by HPLC. The only significant peak, in addition to the expected nucleosides, corresponded to the metabolites **21**, **22** and **23** (Fig. 3b and c). In positive control experiments MC was employed

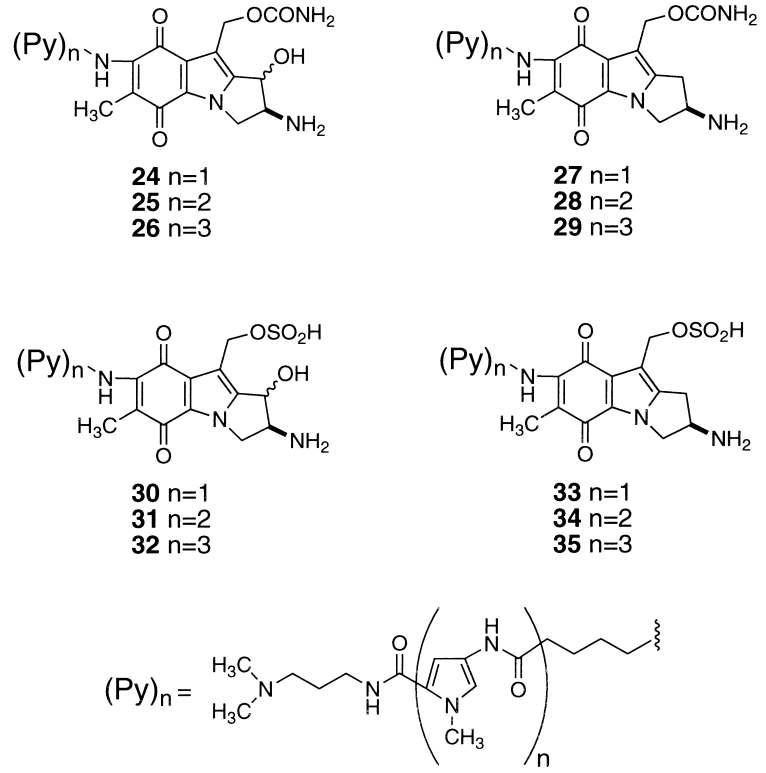


Chart 3. Metabolites formed after chemical or enzymatic activation of **11**–**13**.

under identical activating conditions. Deoxyguanosine–MC adducts were readily detectable in the HPLC assay[1] (e.g. Fig. 3a). Three oligodeoxynucleotides having sequences that are methylpyrrole carboxamide binding sites flanked by CpG[19] (MC target) were also tested as substrates. These were the following: [d-(ATATACGTATAT)]$_2$, [d-(GATCGAATATTCGATC)]$_2$ and [d-(GATCGAATTCGATC)]$_2$ (Chart 4). Reactions were run with MC as positive controls. The reaction mixtures were analyzed by DPAGE. Monoalkylated and cross-linked oligonucleotides are known to migrate slower than unmodified controls under such conditions in general. However, only the reactions with MC and, to a lesser extent, with **11** led to the detection by UV shadowing of slower migrating bands (data not shown).

In vitro antitumor activities of the mitomycin C–*N*-methylpyrrole carboxamide conjugates 11, 12 and 13

Each substance was evaluated for in vitro antitumor activities using a panel of over 60 different human cancer cell lines derived from 9 different tissues. This panel of cell lines, known as the in vitro antitumor screen of the National Cancer Institute, has been developed and operated by the NCI and has been used to test over 10,000 substances per year for growth inhibition and cytotoxicity.[24] The mean of the 50% growth inhibitory activities (GI_{50}) in individual cell lines observed for **11**–**13** are given in Table 2, together with those of mitomycin C and distamycin, determined previously in the same screen. Netropsin has not been tested. The data show that **11**, **12** and **13** are cytotoxic; however, they are less active overall than MC, by approximately two orders of magnitude. The same conclusion can also be drawn when data are compared for individual cell lines (data not shown). The measured cytotoxicity of the substances (LC_{50}) (data not shown) also leads to the same conclusion. The activities of **11**, **12** and **13** have the same order of magnitude as those of distamycin (Table 2).

In order to discern the mechanism of action of the MC–minor groove binder hybrid agents **11**–**13** the results of the tests were evaluated by the COMPARE algorithm.[35] This program is used to calculate the pattern of variation of the inhibitory activity of a drug within a series of cell lines. Patterns generated by different drugs are then compared with one another. The extent of similarity is expressed by a correlation value, the Pearson coefficient, which is the measure of the extent of a correlation with the "seed" compound for which the Pearson coefficient, is taken as 1. Such correlation values have been shown to be indicators of the degree of similarity of the mechanism of action between two drugs. In the present work, correlations were computed between (i) the GI_{50} of MC and the hybrid agents, (ii) the GI_{50} of distamycin and the hybrid agents, and (iii) the GI_{50} of the hybrid agents within the series **11**–**13**. The results are shown in Table 3. They indicate, first of all, that there is no correlation between the mechanism of action of MC and distamycin, as expected. The hybrid drugs **11** and **12**, on the other hand, show a moderate correlation with MC (0.490 and 0.589) but no correlation with distamycin. Hybrid **13** does not correlate with MC (0.163) and distamycin (0.203) even though the 3-pyrrole DNA binding unit of **13** is essentially identical structurally to

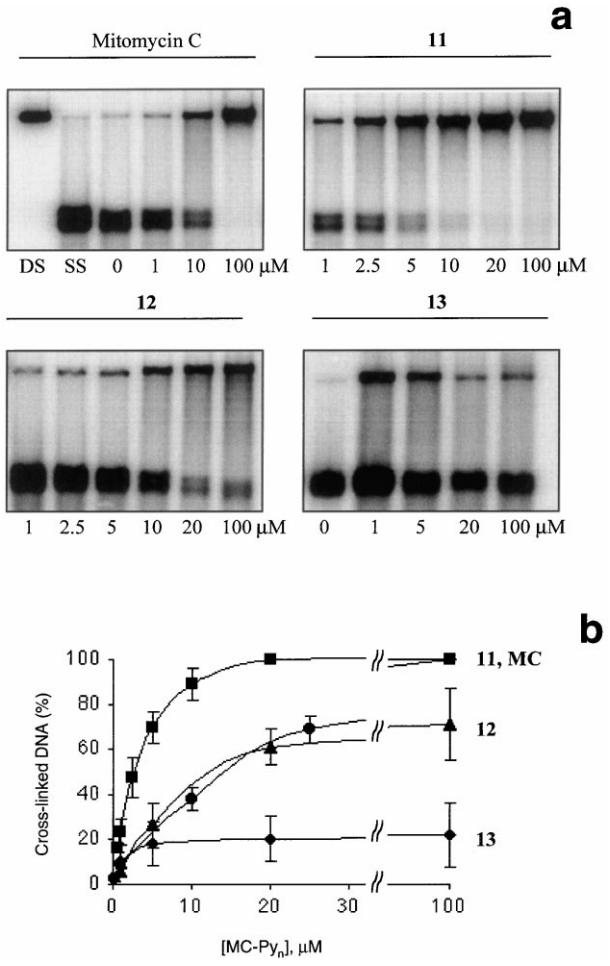


Figure 2. Comparison of the cross-linking efficiency of pBR322 DNA by MC and **11**–**13** under activation by $Na_2S_2O_4$. (a) Phosphorimaging of agarose gels showing cross-linking of 3′-^{32}P-labeled pBR322 DNA. Concentration of drug is noted at the bottom of each lane (see Experimental for details). (b) Percentage cross-link as a function of the concentration, as measured by phosphorimagery. Results shown are the average of at least three different experiments. Key: circles, MC; squares, **11**; triangles, **12**; rhombus, **13**.

distamycin. The mechanisms of the three hybrid agents moderately correlate with one another (Table 3, last 3 columns).

To augment the results of the NCI testing, agents **11**–**13** were tested for cytotoxicity in a mouse mammary tumor cell line, under both hypoxic and aerobic conditions. No cytotoxicity was observed upon treatment with up to 40 μM (4×10^{-5} M) drug (2 h, 37°C) under either condition in this cell line.[36]

Discussion

It has been a general experience that cytotoxic DNA-reactive agents become more cytotoxic when conjugated to minor groove binders. Examples[21] are conjugates of the bifunctional alkylator 2,3-bis(hydroxymethyl)pyrrole (**7**)[19], CPI-type minor groove-alkylating cytotoxins, duocarmycin, anthramycin, DNA cleaving enediynes,

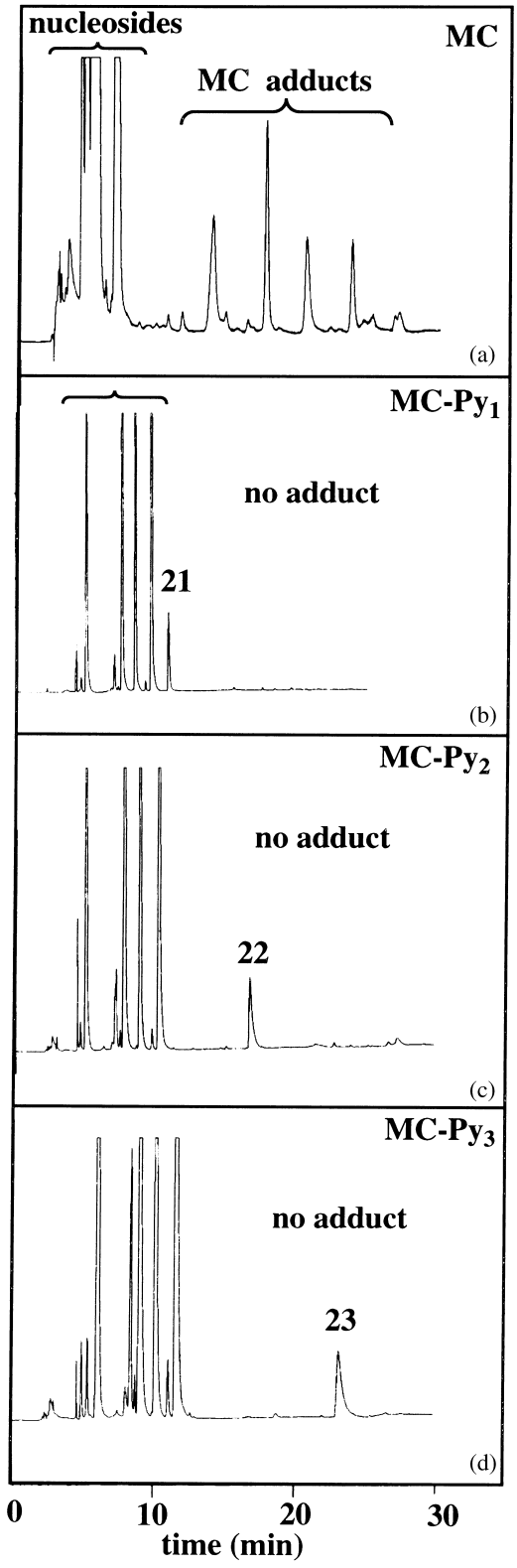


Figure 3. HPLC of enzymatic digests of calf thymus DNA after reactions with MC, **11**, **12** or **13** under activation by $Na_2S_2O_4$. A mixture of calf thymus DNA (5 mM) and drug (1 mM) was treated with $Na_2S_2O_4$ (1.5 mM) under anaerobic conditions. Following purification (see text for details) the DNA was digested with P1 nuclease/phosphodiesterase I/alkaline phosphatase and analyzed by reverse-phase HPLC. (a), MC; (b), **11**; (c), **12**; (d), **13**.

5'-d(ATATACGTATAT)

5'-d(GATCGAATTCGATC)

5'-d(GATCGAATATTCGATC)

5'-d(ATTACGCGCGTAAT)

Chart 4. Synthetic oligodeoxyribonucleotides tested as substrates of alkylation and cross-linking by **11**–**13**.

and even psoralen, which reacts with DNA in the major groove. Similarly, positive results were obtained in the benzoic acid N-mustard (BAM) and chlorambucil mustard (CHL) series.[34,37] In most cases the cytotoxicity increased with the increasing number of pyrrole[37] or imidazole units.[38] In light of this experience, the low biological activity of the MC–oligopyrrole carboxamide conjugates **11**–**13** in the human tumor cell panel of the NCI was unexpected. The results showed clearly that the attachment of minor groove binders to MC failed to increase the cytotoxicity of the conjugates relative to MC. On the contrary, the cytotoxicities decreased 50- to 100-fold and even the most active conjugate (**13**) was only 2-fold more cytotoxic than the minor groove binder distamycin itself (Table 2).

We attempted to determine why this popular and generally successful design aiming at increased cytotoxicity was apparently ineffective in the case of MC. First, the MC conjugates **11**–**13** were examined for their intended increased binding affinity relative to MC. The results verified that even a single pyrrole carboxamide unit (in **11**) confers affinity to DNA, and the affinity increases with addditional pyrrole units (Table 1), in common with the binding properties of other alkylator conjugates.[39] Enzymatic reduction of the MC quinone of the conjugates, required for the DNA alkylation and cross-linking activities, proceeded smoothly (Fig. 1). Apparently, the presence of the large substituents in the 7-amino group of the MC moiety did not inhibit reduction by NADPH-cytochrome *c* reductase, a flavoenzyme implicated in the activation of MC in tumor cells in vivo.[15] This has been a general experience, even with potent N^7-substituted MC analogues.[40] Therefore a lack of activation is not likely to account for the low biological activity. The products resulting from acidic and reductive activation (**24**–**35**) formed in the absence of DNA were analogous to those formed from MC under the same conditions, indicating that the pyrrole carboxamide moiety did not influence the metabolic transformations of MC to stable, inactive mitosene type substances. The conjugate linkage showed some instability upon acidic or reductive activation, as evidenced by the isolation of **21**–**23** from the appropriate reaction mixtures. However, this dissociation occurred only to the extent of 10–15% under either condition. In neutral aqueous solution at room temperature in the absence of activation the conjugates **11**–**13** were stable for 16 h. Thus instability of the conjugate linkage cannot account for the low biological activity of **11**–**13**.

A conspicuous difference in behavior from MC, the parent cytotoxin, however, is a lack of ability of **11–13** to form DNA adducts. MC generates covalent calf thymus DNA adducts after acidic or reductive activation, readily detectable by HPLC of the digest of the DNA. In contrast, the conjugates **11**–**13** yielded no detectable adducts of DNA under a variety of conditions. The digests contained unmodified nucleosides, and only one additional component, corresponding to **21**, **22** or **23**, respectively; that is the activation-induced decomposition products from **11**, **12** and **13**. The presence of **21**–**23** in the digests is probably due to their tenacious noncovalent binding to DNA, resistant even to extraction with n-butanol. The lack of covalent adduct formation with the conjugates was confirmed by testing a variety of self-complementary oligonucleotides as substrates, including those known to be excellent substrates of **10**.[19]

These results suggest that the origin of the low cytotoxic activity of **11**–**13** relative to MC lies in their lower

Table 2. Growth inhibitory properties of the MC–mono-, bis- and tris-*N*-methylpyrrole carboxamide conjugates **11**, **12** and **13** in comparison with MC and distamycin as determined in the National Cancer Institute tumor cell line panel[24]

Compound (NCI test reference number)	GI_{50}[a] (mean graph midpoint), M
Mitomycin C (S26980A)	8.32×10^{-7}
11 (S702522A)	8.71×10^{-5}
12 (S702523A	6.71×10^{-5}
13 (S702524A)	4.27×10^{-5}
Distamycin (S82150A)	8.91×10^{-5}

[a] GI_{50}: drug concentration (M) causing 50% inhibition of growth. ''Mean graph midpoint'' was calculated from experimental values of GI_{50} of the approximately 60 individual cell lines constituting the test panel and it corresponds closely to their mean.

Table 3. Pearson correlation of the pattern of growth inhibitory properties (GI_{50}) of MC–*N*-methylpyrrole carboxamide conjugates **11**–**13** with those of MC and distamycin[a]

Compound (NCI test reference number)	Pearson correlation coefficient comparing GI_{50}				
	MC	Distamycin	**11**	**12**	**13**
Mitomycin C (S26980A)	1.000	0.007	0.490	0.589	0.163
11 (S702522A)	0.490	0.160	1.000	0.676	0.582
12 (S702523A)	0.589	−0.103	0.676	1.000	0.404
13 (S702524A)	0.163	0.203	0.582	0.404	1.000
Distamycin (S82150A)	0.007	1.000	0.160	−0.103	0.206

[a] Calculated by the COMPARE program using the log GI_{50} patterns.[24]

efficiency of covalent alkylation and cross-linking of DNA. This, in turn, may be due to a unique difference in the mechanism of MC from that of the other alkylating agents discussed above: the extremely short life of the active alkylating species.[41] The non-covalent binding of the activated MC conjugate to DNA at non-cross-linkable sites may present a kinetic disadvantage for the covalent reactions by inhibiting the movement of this species toward CpG sequences, required for alkylation and cross-linking. Consequently, the fast irreversible MC inactivation processes, e.g. hydrolysis and conversion to 2,7-diaminomitosene (Scheme 2),[8] will compete effectively with the site-specific guanine alkylation. Furthermore, the inactivated conjugate will inhibit binding of the active conjugate. No such inhibitory effects are expected in the case of the simpler, successful model **10**, since its alkylating function (**7**) is stable in contrast to the MC conjugates. Indeed, **10** has enhanced alkylation and cytotoxic activity compared to the unconjugated drug, **7**. Enhancement may also be expected in cases where the covalent reaction is affinity driven rather than base- and sequence-specific, as was found to be the case of enediyne–distamycin conjugates.[21]

An alternative cause of the lack of appreciable reactivity of the MC conjugates with DNA may be considered, namely that the MC–pyrrole tether of the minor groove-bound conjugate is not flexible enough to allow MC to orient properly for the reaction with guanine at the CpG site. Nevertheless, quantitative inspection of the NMR-derived structure of an oligonucleotide duplex cross-linked by **10** at the 5′-CGAATT sequence[42] suggests that the $(CH_2)_5$ tethers used in our investigation should be long enough to allow sufficient flexibility for the analogous alignment of the MC cross-link formed by the conjugates **11**–**13**.

Employing a sensitive assay of cross-link formation using pBR322 DNA as the substrate[31] we obtained evidence for DNA cross-linking capability of the three conjugates. This result is reconcilable with the negative results observed using calf thymus DNA or short oligonucleotides when one considers the much greater sensitivity of the pBR322 DNA assay: an average of one cross-link per 5000 basepairs scores as 100% cross-linked DNA, compared to one per only 10 basepairs in a 10-mer duplex oligonucleotide substrate. The positive results with pBR322 DNA demonstrate that the MC moiety of the conjugates is capable of cross-linking DNA, albeit at low efficiency. This suggests that the cytotoxicity of **11**–**13** is related to this activity. The cross-linking activity declines with increasing number of methylpyrrole units (Fig. 3) supporting the above hypothesis that the stronger the AT-selective binding is to DNA, the less likely it is that the activated MC finds its CpG target before it gets inactivated. It is hard to explain why the parent MC was less active in this assay than the monopyrrole conjugate **11**. It is possible that some of the CpG-containing sequences of pBR322 DNA are uniquely reactive with the conjugated mitomycins, in analogy to the high reactivity found of a unique short sequence of pBR322 DNA with tallimustin.[22]

Further indication that the mode of action of the MC–methylpyrrole carboxamide conjugates **11** and **12** is related to that of MC rather than distamycin is given by the results of COMPARE, which gave moderate Pearson coefficients in relation with MC (0.490 and 0.589 respectively) (Table 3). No correlation was obtained with **13**, however, which may be related to the observed poor cross-linking activity of **13** relative to **11** and **12**.

In analogy to the present findings, recently another group of bioreductive antitumor agents, the nitrodeazaflavins, were reported to show decreased cytotoxicity upon their conjugation to methylpyrrole carboxamide minor groove binders.[43] Like in the case of the MC conjugates, their cytotoxicity to tumor cells remained low or decreased with increasing number of pyrrole units conjugated to the nitrodeazaflavin moiety. However, the DNA cleaving efficiency in the in vitro pBR322 plasmid test system increased in the conjugated forms, in further analogy to the present case. These agents, like MC, are reductively activated to form DNA-reactive species, which, alternatively, decay to inactive metabolites irreversibly. Although the mechanism involves enzymatic reduction of the nitro group details of these processes are not yet known. It is tempting to speculate that the negative effect of the minor groove binding moieties on the cytotoxicity of nitrodeazaflavin is caused by a similar kinetic inhibition, proposed above for the MC–minor groove conjugates **11**–**13**.

Summary and Conclusion

Conjugation of MC to mono-, bis- and tris-methylpyrrole carboxamide units resulted in hybrid drugs which were less cytotoxic than MC. In vitro studies identified a likely basis for the reduced cytotoxicity of these agents: a lower efficiency of DNA alkylation and cross-linking. As a cause, we propose that binding of the hybrid agents at non-alkylatable sites of DNA slows down the approach of the short-lived reductively activated MC to its required sequence-specific (CpG) target sites. Furthermore, conjugate molecules bearing an irreversibly inactivated MC residue may themselves compete with the active conjugates for the target sites. Our results and analysis of analogous precedents[43] suggest that conjugation of A/T-specific minor groove binders with prodrugs which are enzymatically activated to an irreversibly short-lived site-specific reactive agent constitutes an ineffective design of DNA-targeted cytotoxic drugs. However, conjugation of MC to non-specific DNA binding groups may lead to enhanced potency as desired. This possibility is under investigation in our laboratory.

Experimental

Materials and methods

Mitomycin C and mitomycin A were received from Kyowa Hokko Kogyo Co., Ltd., Tokyo, Japan. Other materials and their sources were as follows: synthesis

reagents, Aldrich. EcoRI, pBR322 and Klenow fragment of DNA Polymerase I (exo-), New England Biolabs. α-^{32}P-Labeled nucleotide triphosphates, NEN; agarose (DNA grade) and HPLC solvents, Fisher; calf thymus DNA and NADPH-cytochrome *c* reductase, Sigma; DNase I (code D), phosphodiesterase I, and alkaline phosphatase, Worthington. ^{1}H NMR spectra were recorded in GE 300 MHz or Jeol JNM GX400 spectrometers. UV spectra were recorded in a Cary 3 UV–Visible spectrophotometer. Absorbance readings were performed in a Gilford 250 spectrophotometer. LCMS was performed with a Hewlett–Packard Series 1100 diode array HPLC system connected to a Hewlett–Packard Series 1100 MSD mass spectrometer. HRMS was provided by Richard Milberg, Chemical Sciences, University of Illinois, Urbana-Champaign. HPLC was performed in a Beckman System Gold 125 instrument, equipped with a diode array detector System Gold 168, fitted with a Rainin C-18, 5×250 mm column. A linear gradient of 6–50% CH_3CN in 30 mM NaH_2PO_4, pH 5.8, was used. Agarose gels were run on a DNA Sub Cell apparatus (Biorad). Agarose gels were prepared by dissolving 1 g of agarose in 100 mL of buffer containing 40 mM Tris–AcOH, 2 mM EDTA, pH 8.2. After electrophoresis, gels were transferred onto sequencing filter paper (Biorad), covered with Saran-Wrap, and dried at 80°C in a Biorad model 583 gel dryer. Phosphorimagery was performed with a Storage Phosphor Screen (Applied Biosystems), using Image Quant software (Applied Biosystems). Ethanol precipitations were performed by admixing the aqueous solution of DNA with 0.1 vol of 3 M NaOAc, pH 5, and 7 vol of cold ethanol, followed by centrifugation (12,000 rpm, 4°C, 15 min). The supernatant was removed and the pellet was dried in a speed-vac. Calf thymus DNA was sonicated and dialyzed before use.

Molar extinction coefficients (ε_λ) of **11**–**13** were determined by weighing 1–2 mg of the dry sample on a microbalance in an aluminum boat. The boat with the sample was transferred into water. The solution was diluted to volume. ε_{274} was calculated from the measured absorbance of the solution at 274 nm.

Synthesis of compounds 18–20. A solution of nitropyrrole **15**[24] (254 mg, 1.0 mmol) in CH_3OH (5 mL) containing 5% Pd/C (50 mg) was hydrogenated (1 atm) for 6 h at rt, filtered through silica gel, and concentrated under vacuum. The residue was dissolved in toluene, concentrated, and dried under vacuum to give the crude aminopyrrole that was used in the next step without further purification: A solution of carbonyldiimidazole (225 mg, 1.4 mmol) in DMF (1 mL) was added to a solution of *N*-Boc-6-aminocaproic acid (350 mg, 1.5 mmol) in DMF (2 mL), stirred for 4 h at rt, then treated with a solution of crude aminopyrrole in DMF (1 mL). The mixture was stirred at rt for 16 h, then concentrated under vacuum. The residue was dissolved in Cl_3CH (50 mL), washed with 0.5 M K_2HPO_4, dried over $MgSO_4$ and concentrated under vacuum. The residue was purified by flash chromatography (Cl_3CH/CH_3OH 4:1, containing 1% Et_3N) to give **18** (370 mg, 84%). Compounds **19** and **20** were synthesized following an analogous procedure. **18**: ^{1}H NMR (Cl_3CD) δ (ppm) 1.27–1.50 (m, 4H), 1.43 (s, 9H), 1.61–1.76 (m, 4H), 2.21–2.26 (m, 2H), 2.29 (s, 6H), 2.46 (t, 2H, J = 6.3 Hz), 3.06 (q, 2H, J = 6.4 Hz), 3.40 (q, 2H, J = 5.8 Hz), 3.85 (s, 3H), 4.78 (br s, 1H), 6.47 (d, 1H, J = 1.7 Hz), 7.11 (d, 1H, J = 1.6 Hz), 7.73 (t, 1H, J = 4.8 Hz), 8.34 (s, 1H). HRMS calcd for $C_{22}H_{40}N_5O_4$ (**18**·H$^+$) 438.3080, found 438.3080. **19**: ^{1}H NMR (Cl_3CD) δ (ppm) 1.22–1.38 (m, 4H), 1.43 (s, 9H), 1.59–1.75 (m, 4H), 2.25–2.30 (m, 2H), 2.26 (s, 6H), 2.42 (t, 2H, J = 6.4 Hz), 3.05 (q, 2H, J = 6.6 Hz), 3.41 (q, 2H, J = 5.9 Hz), 3.86 (s, 6H), 4.77 (br s, 1H), 6.51 (d, 1H, J = 1.4 Hz), 6.67 (d, 1H, J = 1.2 Hz), 7.03 (d, 1H, J = 1.5 Hz), 7.17 (d, 1H, J = 1.5 Hz), 7.66 (t, 1H, J = 4.8 Hz), 8.19 (s, 1H), 8.29 (s, 1H). HRMS calcd for $C_{28}H_{47}N_7O_5$ (**19**·H$^+$) 560.3560, found 560.3569. **20**: ^{1}H NMR (Cl_3CD) δ (ppm) 1.23–1.31 (m, 2H), 1.40–1.45 (m, 2H), 1.41 (s, 9H), 1.58–1.73 (m, 4H), 2.23 (t, 2H, J = 8.1 Hz), 2.29 (s, 6H), 2.47 (t, 2H, J = 6.1 Hz), 3.00 (q, 2H, J = 6.0 Hz), 3.31–3.35 (m, 2H), 3.79 (s, 6H), 3.80 (s, 3H), 4.88 (t, 1H, J = 5.6 Hz), 6.69 (s, 2H), 6.73 (s, 1H), 7.03 (d, 1H, J = 1.5 Hz), 7.20 (s, 1H), 7.22 (s, 1H), 7, 28 (s, 1H), 7.60 (br s, 1H), 8.51 (br s, 1H), 8.77 (br s, 1H), 8.86 (br s, 1H). HRMS calcd for $C_{34}H_{52}N_9O_6$ (**20**·H$^+$) 682.4040, found 682.4039.

Conversion of 18–20 to the respective free amines 21–23. A solution of **18** (58 mg, 0.13 mmol) in CH_2Cl_2 (1.5 mL) was treated with TFA (0.5 mL), stirred for 2 h at rt, then concentrated in vacuo. The residue was dissolved in CH_2Cl_2 (25 mL), treated with anhydrous K_2CO_3, filtered, and concentrated in vacuo to give crude **21**. Compounds **22** and **23** were prepared from **19** and **20** in an analogous manner. **21**: ^{1}H NMR (D_2O) δ (ppm) 1.37–1.45 (m, 2H), 1.63–1.70 (m, 4H), 1.95–2.04 (m, 2H), 2.36 (t, 2H, J = 7.3 Hz), 2.89 (s, 6H), 2.98 (t, 2H, J = 7.0 Hz), 3.18 (t, 2H, J = 7.9 Hz), 3.39 (t, 2H, J = 6.6 Hz), 3.78 (s, 3H), 6.70 (s, 1H), 7.48 (s, 1H). HRMS calcd for $C_{17}H_{32}N_5O_2$ (**21**·H +) 338.2556, found 338.2559. **22**: ^{1}H NMR (D_2O) δ (ppm) 1.35–1.43 (m, 2H), 1.61–1.72 (m, 4H), 1.95–2.01 (m, 2H), 2.29 (t, 2H, J = 7.4 Hz), 2.89 (s, 6H), 2.99 (t, 2H, J = 7.4 Hz), 3.17 (t, 2H, J = 8.0 Hz), 3.35 (t, 2H, J = 6.7 Hz), 3.72 (s, 6H), 6.65 (s, 1H), 6.68 (s, 1H), 7.47 (s, 1H), 7.91 (s, 1H). HRMS calcd for $C_{23}H_{38}N_7O_3$ (**22**·H +) 460.3036, found 460.3035. **23**: ^{1}H NMR (D_2O) δ (ppm) 1.29–1.34 (m, 2H), 1.51–1.62 (m, 4H), 1.84–1.88 (m, 2H), 2.23 (t, 2H, J = 7.4 Hz), 2.81 (s, 6H), 2.75–2.88 (m, 2H), 3.04 (t, 2H, J = 7.5 Hz), 3.27 (t, 2H, J = 6.4 Hz), 3.75 (s, 3H), 3.76 (s, 3H), 3.80 (s, 3H), 6.70 (d, 1H, J = 1.7 Hz), 6.75 (d, 1H, J = 1.7 Hz), 6.81 (d, 1H, J = 1.6 Hz), 7.03 (d, 1H, J = 1.7 Hz), 7.06 (d, 1H, J = 1.6 Hz), 7.43 (s, 1H). HRMS calcd for $C_{29}H_{44}N_9O_4$ (**23**·H +) 582.3516, found 582.3515.

Synthesis of 11–13 by coupling of mitomycin A (14) with 21–23. Crude **21** was dissolved in CH_3OH (1 mL), treated with pyridine (0.2 mL) and mitomycin A (**14**) (58 mg, 0.13 mmol). The mixture was stirred for 16 h at rt protected from light, then concentrated in vacuo and purified by flash chromatography ($CHCl_3$/CH_3OH 5:1 containing 1% Et_3N) to give **11** (73 mg, 86%). Conjugates **12** and **13** were synthesized following an analogous procedure. **11**: ^{1}H NMR (D_2O) δ (ppm)

1.29–1.40 (m, 2H), 1.59–1.70 (m, 4H), 1.88 (s, 3H), 1.85–1.95 (m, 2H), 2.29 (t, 2H, $J=7.5$ Hz), 2.63 (s, 6H), 2.80–2.92 (m, 2H), 3.10–3.18 (m, 2H), 3.18 (s, 3H), 3.33 (t, 2H, $J=6.5$ Hz), 3.49–3.58 (m, 4H), 3.82 (s, 3H), 4.15 (d, 2H, $J=10.5$ Hz), 4.63 (dd, 1H, $J=10.6$ Hz, 4.3 Hz), 6.70 (d, 1H, $J=1.9$ Hz), 7.10 (d, 1H, $J=1.9$ Hz). ESIMS: *m/e* 654 ($M+H^+$). UV (H_2O) λ_{max} (ε) 220, 374 (18,900). **12**: ^1H NMR (D_2O) δ (ppm) 1.40–1.46 (m, 2H), 1.62–1.74 (m, 4H), 1.90–1.95 (m, 2H), 1.98 (s, 3H), 2.33 (t, 2H, $J=7.2$ Hz), 270 (s, 6H), 2.86–2.99 (m, 4H), 3.21 (s, 3H), 3.31–3.40 (m, 2H), 3.52–3.60 (m, 4H), 3.81 (s, 3H), 3.88 (s, 3H), 4.16–4.25 (m, 2H), 4.66 (dd, 1H, $J=10.6$ Hz, 4.3 Hz), 6.82 (d, 1H, $J=1.4$ Hz), 6.86 (d, 1H, $J=1.4$ Hz), 7.13 (s 1H), 7.17 (s, 1H). ESIMS: *m/e* 777.32 ($M+H^+$). UV (H_2O) λ_{max} (ε) 224, 302, 374 (17,500). **13**: ^1H NMR (CD_3OD) δ (ppm) 1.40–1.44 (m, 2H), 1.63–1.74 (m, 4H), 1.90–1.97 (m, 2H), 1.99 (s, 3H), 2.33 (t, 2H, $J=7.2$ Hz), 2.70 (s, 6H), 2.82–2.94 (m, 4H), 3.21 (s, 3H), 3.31–3.40 (m, 2H), 3.52–3.74 (m, 4H), 3.88 (s, 3H), 3.89 (s, 3H), 3.90 (s, 3H), 3.95–4.20 (m, 2H), 4.66 (dd, 1H, $J=10.5$ Hz, 4.3 Hz), 6.84 (d, 1H, $J=1.6$ Hz), 6.86 (s, 1H), 6.95 (d, 1H, $J=1.4$ Hz), 7.13 (d 1H, $J=7.5$ Hz), 7.18 (d, 1H, $J=1.5$ Hz), 7.19 (d, 1H, $J=1.5$ Hz). ESIMS: *m/e* 899.4 ($M+H^+$). UV (H_2O) λ_{max} (ε) 227, 309, 374 (18,100).

Reduction kinetics of MC–pyrrole conjugates. (a) In the absence of DNA NADPH-cytochrome *c* reductase (final concentration 2.5 units/mL) was added to a deaerated solution containing 1 mM **11**, 2 mM NADPH and 2 mM dT in 10 mM NaH_2PO_4–Na_2HPO_4; 1 mM EDTA, pH 7.5. Aliquots were removed at several time intervals, quenched with 10 vol of cold ethanol and stored at −20°C until analyzed. The consumption of **11**, **12** or **13** was monitored by HPLC using the following conditions: solvent A, 30 mM sodium phosphate, pH 5.8; solvent B, 60% acetonitrile in A; linear gradient 6–80% B in 45 min. The percentage consumption of starting material was calculated from the percentage decrease of the HPLC peak area ratio of starting material to dT present as a marker. (b) In the presence of DNA cytochrome *c* reductase (final concentration 1.2 units/mL) was added to a deaerated solution containing 15 mM calf thymus DNA, 0.10 mM **11**, 0.5 mM NADH and 0.1 mM dT in 10 mM NaH_2PO_4–Na_2HPO_4; 1 mM EDTA, pH 7.5. Aliquots were removed at several time intervals, quenched with 10 vol of 10 mM NaH_2PO_4–Na_2HPO_4; 1 mM EDTA, pH 7.5, previously heated to 90°C. The mixture was heated for 5 min at 90°C to ensure the inactivation of the enzyme. The solutions were cooled to room temperature and admixed with DNase I (25 units), phosphodiesterase I (2 units) and alkaline phosphatase (5 units). The enzymatic digestion ensures the release of DNA-bound drug. For comparison, reactions in which DNA was omitted were performed in parallel. The consumption of **11** was determined by HPLC as above, except that bromophenol blue, instead of dT, was added as marker. The rate of disappearance of **12** and **13** was determined following an analogous procedure.

Formation of metabolites after activation of 11–13. (i) Enzymatic activation: The formation of metabolites by reduction of **11**–**13** by NADH-cytochrome *c* reductase and NADH was performed as described above. (ii) Acidic activation: 1 mM solutions of **11**–**13** were incubated in 0.02 M HCl for 2 h, then stored at −20°C for later analysis. (iii) Sodium dithionite activation: A solution of the conjugate (**11**–**13**) (1 mM, 0.50 mL; 0.5 μmol total) in 10 mM NaH_2PO_4–Na_2HPO_4, pH 7.5, was deaerated by argon bubbling. A solution of freshly prepared 15 mM sodium dithionite in the same reaction buffer were added in five 10 μL portions at 10 min intervals. The mixtures were analyzed by diode array HPLC and LCMS using the following conditions: solvent A, 10 mM NH_4OAc, pH 7; solvent B, 60% acetonitrile in A; linear gradient 6–100% B in 45 min.

pBR322 DNA cross-linking assay.[31] A mixture of pBR322 DNA (5 μg, 5 μL), *Eco*RI restriction endonuclease (5 μL, 50 units), 10 μL *Eco*RI buffer (10 μL, supplied with the *Eco*RI enzyme) and 10 μL sterile H_2O was incubated for 60 min at 37°C. The linear DNA was labeled at the 3′ end by adding α-^{32}P-dATP (2 μL, 20 μCi) and Klenow polymerase (1 μL, 5 units). The mixture was incubated for 20 min at 37°C and the DNA was purified by ethanol precipitation. A mixture of labeled pBR322 DNA (approximately 100 ng) and MC–pyrrole conjugate (at various concentrations) in 90 μL 10 mM NaH_2PO_4–Na_2HPO_4–1 mM EDTA (pH 7.5) was deaerated by bubbling argon for 1 min, and 10 μL of a freshly prepared 10 mM solution of sodium dithionite in deaerated buffer was added. The samples were incubated for 45 min at room temperature, and the reactions were stopped by opening the tubes to air and gently stirring. The DNA was purified by ethanol precipitation, dissolved in strand separation buffer (30% DMSO, 1 mM EDTA, pH 9, 0.25% xylene cyanol, 0.25% bromophenol blue), heated at 90°C for 5 min, then immediately ice-cooled. Samples were loaded on a 1% agarose gel and run at 40 V for 16 h. The gels were dried and the bands were visualized and quantified by phosphorimagery.

Reaction of calf thymus DNA with reductively activated 11–13. A solution of calf thymus DNA in 0.01 M Tris–HCl, pH 7.4, containing 1 μmol DNA was admixed with a solution of **11** in methanol (0.2 μmol total), the mixture was lyophilized, redissolved in 0.1 M Tris–HCl, pH 7.5 (200 μL), and deaerated by bubbling argon for 10 min. Five portions of a freshly prepared 10 mM solution of sodium dithionite (6 μL each; 0.3 μmol total) in deaerated buffer were added at 10 min intervals, and the reactions were stopped by opening the tubes to air and gently stirring. The DNA was purified by 5 extractions with *n*-BuOH, followed by ethanol precipitation, then it was dissolved in 10 mM NH_4OAc (900 μL), admixed with 3 units of P1 nuclease and incubated for 3 h at 37°C. After this period, the solution was admixed with 100 μL of 500 mM Tris–HCl–5 mM $MgCl_2$ (pH 8.3), phosphodiesterase I (5 units) and bacterial alkaline phosphatase (10 units). The mixture was incubated 5 h at 37°C, then analyzed by HPLC, using the following conditions: solvent A, 30 mM sodium phosphate, pH 5.8; B, 60% acetonitrile in A; linear gradient 6–80% B in 45 min.

Determination of DNA binding constants of conjugates 11–13 by the ethidium displacement assay.[28] Concentration of ethidium bromide (EtBr) was measured by UV spectrometry by using the molar extinction coefficient 5850 $(M.cm)^{-1}$ at 480 nm in water. Calf thymus DNA mononucleotide concentration was measured by using the molar extinction coefficient 6600 at 260 nm. Concentrations of MC analogues were measured by using the molar extinction coefficients at 374 nm, listed in Table 1. A 3 mL cuvette was used and the titration mixture was constantly stirred with the help of a magnetic stirrer. The buffer was 10 mM Tris, 1 mM EDTA, pH 7.1. Ethidium bromide concentration was maintained between 1.0 and 3.0 μM. A constant ratio of EtBr and DNA concentration was maintained as 1:30 for maximum fluorescence (excitation wavelength, 546 nm; emission wavelength, 595 nm). Aliquots of a stock drug solution in 10% MeOH (concentration in the range of 2–8 mM) were added to the fluorescence solution and the fluorescence measured after each addition until a 50% reduction of fluorescence occurred. Fluorescence was plotted against drug concentration after correcting for the dilution factor. Drug concentration at 50% quenching was calculated from the plots. The apparent binding constant (K_b) of drug to CT DNA was then calculated from the equation, K_{EtBr} [EtBr] = K_b $[drug]_{50}$, where K_{EtBr} is binding constant of EtBr to CT DNA, [EtBr] is concentration of ethidium bromide and $[drug]_{50}$ is concentration of the drug required to reduce the fluorescence of the EtBr–DNA complex to half of its original value.

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