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Reductive activation of mitomycins A and C by vitamin C

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ABSTRACT

The anticancer drug mitomycin C produces cytotoxic effects after being converted to a highly reactive bis-electrophile by a reductive activation, a reaction that a number of 1-electron or 2-electron oxidoreductase enzymes can perform in cells. Several reports in the literature indicate that ascorbic acid can modulate the cytotoxic effects of mitomycin C, either potentiating or inhibiting its effects. As ascorbic acid is a reducing agent that is known to be able to reduce quinones, it could be possible that the observed modulatory effects are a consequence of a direct redox reduction between mitomycin C and ascorbate. To determine if this is the case, the reaction between mitomycin C and ascorbate was studied using UV/Vis spectroscopy and LC/MS. We also studied the reaction of ascorbate with mitomycin A, a highly toxic member of the mitomycin family with a higher redox potential than mitomycin C. We found that ascorbate is capable to reduce mitomycin A efficiently, but it reduces mitomycin C rather inefficiently. The mechanisms of activation have been elucidated based on the kinetics of the reduction and on the analysis of the mitosene derivatives formed after the reaction. We found that the activation occurs by the interplay of three different mechanisms that contribute differently, depending on the pH of the reaction. As the reduction of mitomycin C by ascorbate is rather inefficiently at physiologically relevant pH values we conclude that the modulatory effect of ascorbate on the cytotoxicity of mitomycin C is not the result of a direct redox reaction and therefore this modulation must be the consequence of other biochemical mechanisms.

1. Introduction

The mitomycins are a family of highly cytotoxic antibiotics produced by Streptomyces cultures. Mitomycin C (MMC, **1**) is used clinically in chemotherapy for several types of cancer [1] and also as an antifibrotic adjuvant to a number of surgical procedures [2,3]. Another member of the mitomycin family is mitomycin A (MMA, **2**), which differs from MMC in the substituent at the 7-position (7-methoxy instead of 7-amino). MMA is several orders of magnitude more cytotoxic than MMC towards cancer cells, and this cytotoxicity correlates with its higher redox potential [4,5]. MMC is considered the prototype bioreductive drug: it is inert towards nucleophiles in its original structure, but it is converted to an extremely reactive bis-electrophile after a cascade of reactions initiated by reduction of the quinone ring (Fig. 1) [6]. The reductive activation of mitomycin C generates the reactive intermediate **3a,** containing two electrophilic positions -at C1 and C10- that alkylates DNA and other biological molecules [7,8]. The formation of an interstrand cross-link adduct with DNA is consistently considered the cause of its cytotoxic effects [7,9]. In addition to DNA alkylation and cross-linking, other biological modes of action for MMC have been postulated, including redox cycling [10,11], inhibition of ribosomal RNA [12], and inhibition of thioredoxin reductase [13].

A number of 1-electron and 2-electron oxidoreductases have been shown to activate MMC [14], two of which seem to play a key role in cancer cells: DT-diaphorase under normal oxygen conditions [15] and cytochrome P450 reductase under hypoxic conditions [16]. Small-molecule biological reducing agents such as glutathione or dihydrolipoic acid can also activate MMA [5,17], but they do not reduce MMC [18,19] or do so rather inefficiently [20].

Vitamin C (ascorbic acid, **2**, AscH, Fig. 2) is a well-known physiological reducing agent [21]. Ascorbate is a cofactor of a number of oxidase enzymes, playing a key role in a number of hydroxylation reactions [22]. Other biologically relevant roles of AscH include its free radical scavenger activity [23] and the inhibition of the formation of carcinogenic N-nitrosamines by the reaction of nitrite with amines [24]. Quinones are also a substrate for reduction by AscH: several reports have shown that quinones can be reduced by vitamin C to generate the semiquinone or the hydroquinone forms [25–27]. In this context, the formation or reactive oxygen species by redox cycling of quinones using pharmacological concentrations of ascorbate has been proposed as a novel anticancer therapy [28].

Abbreviations: AscH, ascorbic acid; MMA, mitomycin A; MMC, mitomycin C.

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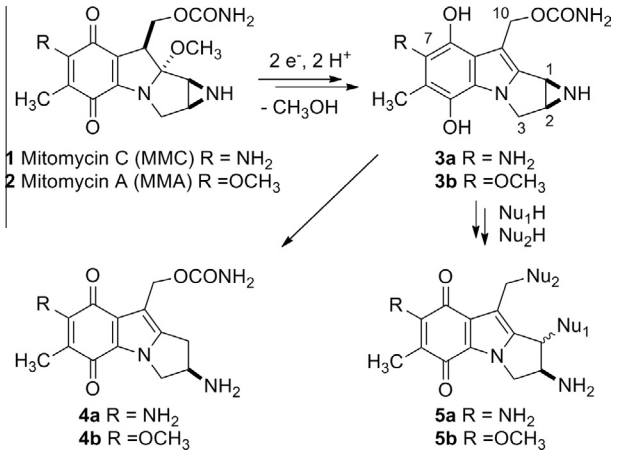

Fig. 1. Mechanism for the reductive activation of mitomycins. Nu_1H and Nu_2H represent two nucleophilic compounds.

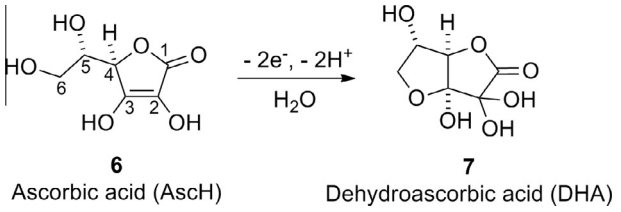

Fig. 2. Oxidation of ascorbic acid to dehydroascorbic acid. The structure of the cyclic hemiacetal monohydrate of dehydroascorbic acid that is present in aqueous solutions is shown [63].

A substantial number of studies have shown that AscH can modulate the biological effects of MMC, either enhancing or inhibiting its effects [29–39]. Considering that quinones can be reduced by AscH, one could hypothesize that the observed modulation of the cytotoxic effects of MMC by ascorbate are the result of a direct redox reaction between MMC and ascorbate. If this reduction occurs, then it would result in either the activation of MMC to cytotoxic alkylating intermediates or in detoxification by conversion of MMC to non-toxic metabolites. To test the likelihood of this hypothesis we studied the reactions between MMC and ascorbate by examining the kinetics of the reduction of MMC to mitosenes and by analyzing the identity of mitosene derivatives formed after reaction of MMC and ascorbate. We also included in our studies the highly toxic parent compound MMA, a more easily reducible member of the mitomycin family of antibiotics.

2. Materials and methods

2.1. General procedures

Mitomycin C was a gift from Kyowa Hakko Kogyo Co. Ltd. Mitomycin A was prepared as reported [40]. Ascorbic acid and sodium ascorbate were from Sigma–Aldrich. HPLC was performed with a Hewlett Packard Series 1100 diode array system. LCMS was performed with a Hewlett Packard Series 1100 diode array HPLC system connected to a Hewlett Packard Series 1100 MSD mass spectrometer. A Macherey Nagel (Nucleodur C18, 5 μm, 4.6 × 125 mm) column was used. The elution system was 5–30% B in 20 min, 30–60% B in 9 min. (*A* = 10 mM ammonium acetate, pH 5.5; *B* = acetonitrile, concentrations expressed in v/v). UV/Vis spectra and kinetic studies were performed with a CARY 300 UV–visible spectrometer. Stock solutions of mitomycins A and C were prepared in H_2O, and their concentrations were determined spectrophotometrically using ϵ_{367} (H_2O) = 21,840 cm^{-1} M^{-1} for MMC and ϵ_{320} (H_2O) = 10,400 cm^{-1} M^{-1} for MMA.

2.2. Kinetics of the reactions between MMC and ascorbate

Reactions were started by adding an aqueous ascorbic acid or sodium ascorbate solution in the appropriate buffer to a buffered aqueous solution of MMC in a UV/Vis cuvette thermostated at 37 °C by a circulating water bath, using a total volume of 0.50 mL. The absorbance at 360 nm was recorded at 1-s intervals. The molar fraction of MMC at time *t* ($\chi_{MMC(t)}$) was calculated from the absorbance readings using the following equation:

$$\chi_{MMC(t)} = (A_t - A_\infty)/(A_0 - A_\infty)$$

where A_0 is the absorbance of the solution at 360 nm before the addition of ascorbate (corrected for the final volume), A_t is the absorbance of the solution at a time *t* after the addition of ascorbate and A_∞ is the absorbance of the solution after all the mitomycin C is consumed.

2.3. Kinetics of the reactions between MMA and ascorbate

Reactions were performed analogously to those of MMC, except that the decrease in the absorbance at 550 nm was recorded.

2.4. Kinetics of the reaction of MMC with ascorbate: dependence on the concentration of ascorbate

The reactions were performed according to the general protocol described above in 500 mM carbonate/bicarbonate buffer pH 10.5. The final concentrations in the reaction mixture were 50 μM MMC and 12–100 mM ascorbate.

2.5. Kinetics of the reaction of MMC with ascorbate: pH-dependence

The reactions were performed according to the general protocol described above. The final concentrations in the reaction mixture were 50 μM MMC, 50 mM sodium ascorbate and 300 mM buffer. Buffers were AcONa/AcOH (for pH 4–5) NaH_2PO_4/Na_2HPO_4 (pH 6–9) and $NaHCO_3/Na_2CO_3$ (pH 10–11). Tris buffer at pH 9.0 inhibited the reaction.

2.6. Kinetics of the reaction of MMA with ascorbate: dependence on the concentration of ascorbate

The reactions were performed according to the general protocol described above in 100 mM phosphate buffer pH 6.7. The final concentrations in the reaction mixture were 30 μM MMA and 2–10 mM ascorbate.

2.7. Kinetics of the reaction of MMA with ascorbate: pH-dependence

The reactions were performed according to the general protocol described above. The final concentrations in the reaction mixture were 30 μM MMA, 2 mM sodium ascorbate and100 mM buffer. Buffers were AcONa/AcOH (for pH 4–5) NaH_2PO_4/Na_2HPO_4 (pH 6–8.5) and $NaHCO_3/Na_2CO_3$ (pH 9.5–10.5).

2.8. LC/MS analysis of the reactions of mitomycins with ascorbate

A solution of MMC or MMA in water (100 μL of a 3 mM solution) was treated with a solution of ascorbate (15 μL of a 10 mM solution in the corresponding buffer). The buffers used were AcOH/AcONa for pH 4 and pH 5, NaH_2PO_4/Na_2HPO_4 for pH

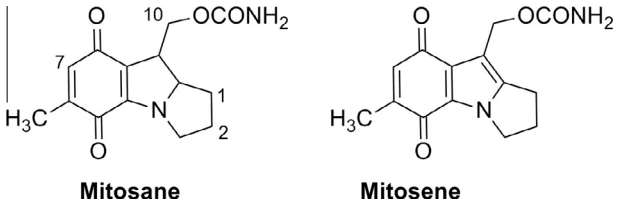

Fig. 3. Structures of the mitosane and mitosene core.

6–pH 8 and $NaHCO_3/Na_2CO_3$ for pH 9.The resulting solutions were maintained at room temperature for 2 h and analyzed directly by LCMS.

2.9. Spectral data

8: EI-MS (ES^+) *m/z*: 260 ($M-OCONH_2^-$), 343 ($M+Na^+$), 359 ($M+K^+$). UV/Vis (λ_{max}, nm): 252, 310. **9**: EI-MS (ES^+) *m/z*: 418 ($M-OCONH_2^-$), 501 ($M+Na^+$), 517 ($M+K^+$). UV/Vis (λ_{max}, nm): 250, 312. **10**: EI-MS (ES^+) *m/z*: 302 ($M-OCONH_2^-$), 385 ($M+Na^+$), 401 ($M+K^+$). UV/Vis (λ_{max}, nm): 252, 310. **11**: EI-MS (ES^+) *m/z*: 275 ($M-OCONH_2^-$), 358 ($M+Na^+$), 374 ($M+K^+$). UV/Vis (λ_{max}, nm): 234, 287, 345. **12**: EI-MS (ES^+) *m/z*: 433 ($M-OCONH_2^-$), 516 ($M+Na^+$), 532 ($M+K^+$). UV/Vis (λ_{max}, nm): 234, 286, 352. **13**: EI-MS (ES^+) *m/z*: 317 ($M-OCONH_2^-$), 400 ($M+Na^+$), 416 ($M+K^+$). UV/Vis (λ_{max}, nm): 236, 284, 342. **4a**: EI-MS (ES^+) *m/z*: 244 ($M-OCONH_2^-$), 327 ($M+Na^+$), 343 ($M+K^+$). UV/Vis (λ_{max}, nm): 250, 312 360 (sh). **14**: EI-MS (ES^+) *m/z*: 420 ($M-OCONH_2^-$), 442 ($M+Na^+$), 458 ($M+K^+$). UV/Vis (λ_{max}, nm): 244, 316, 360 (sh) **4b**: EI-MS (ES^+) *m/z*: 259 ($M-OCONH_2^-$), 342 ($M+Na^+$), 358 ($M+K^+$). UV/Vis (λ_{max}, nm): 230, 287, 350. **15**: EI-MS (ES^+) *m/z*: 435 ($M+H^+$), 457 ($M+Na^+$), 473 ($M+K^+$). UV/Vis (λ_{max}, nm): 231, 288, 360. **16**: EI-MS (ES^+) *m/z*: 286 ($M-OCONH_2^-$), 347 ($M+H^+$), 369 ($M+Na^+$), 385 ($M+K^+$). UV/Vis (λ_{max}, nm): 252, 310.

2.10. Comparison with authentic samples

Samples of 1-hydroxy-7-aminomitosenes (**8**) were prepared by acid hydrolysis of MMC in water according to the procedure described by Taylor and Remers [41]. Samples of 1-hydroxy-7-methoxymitosenes (**11**) were prepared similarly by acid hydrolysis of MMA. 2-amino-7-methoxymitosene (**4b**) was prepared by reductive activation of MMA with DTT [17]. 2,7-DAM (**4a**) was prepared by catalytic hydrogenation of MMC in methanol [42].

3. Results

The reduction of the quinone ring in mitomycins A or C triggers the elimination of methanol, resulting in the irreversible conversion of the mitosane chromophore to the mitosene chromophore (Fig. 3), two structures with significantly different absorbance spectra [43]. This irreversible structural change allows to study of the reductive activation of mitomycins using UV/Vis spectroscopy [17,20,44,45]. The first experiments to determine if MMC could be reduced by AscH were performed by tracking the conversion of the 7-aminomitosane chromophore (λ_{max} 360 nm) to the 7-aminomitosene chromophore (λ_{max} 315 nm). The time-dependent UV/Vis spectra of a reaction containing MMC and a large excess of AscH (50 mM) at pH 5.0 clearly showed the gradual decrease of the mitosane chromophore and the appearance of the mitosene chromophore (Fig. 4A), indicating the formation of mitosene derivatives at the expense of MMC. The reaction reached completion in about 90 min, as judged by constant UV/Vis spectra. The reductive activation of MMA by ascorbate was analyzed similarly by monitoring the conversion of the 7-methoxymitosane chromophore

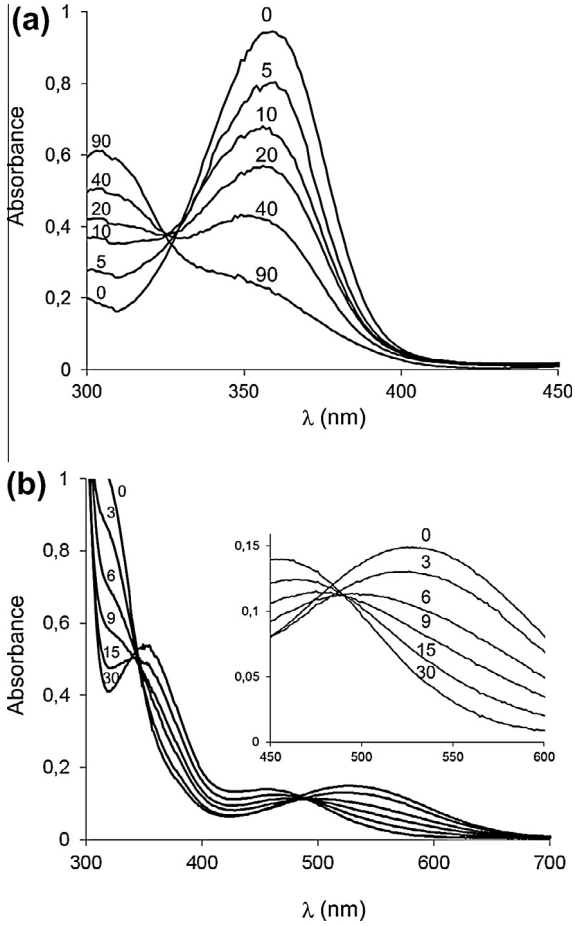

Fig. 4. (a) UV/Vis assay of the reduction of 50 μM mitomycin C by 50 mM sodium ascorbate in 300 mM phosphate buffer, pH 5.0 at 37 °C. (b) UV/Vis assay of the reduction of 30 μM mitomycin A by 2 mM sodium ascorbate in 200 mM phosphate buffer, pH 7.5 at 37 °C. Reaction times (min) are indicated on top of each spectrum.

(λ_{max} 550 nm) to the 7-methoxymitosene chromophore (λ_{max} 350 nm) (Fig. 4B). The reduction of MMA can also be monitored by the decrease of an absorbance maximum at 317 nm [17], but this wavelength could not be used in this case due to interferences caused by the presence of ascorbate/dehydroascorbate. MMA was reduced in 30 min using 2 mM ascorbate at pH 7.5 indicating, as expected, a much higher reactivity for MMA compared to MMC.

The rate of reaction showed a marked pH-dependence for both mitomycins (Fig. 5). In the case of MMC the rate decreased when the pH was increased from pH 4 to pH 5, and then increased the range pH 5–11 with increasing pH values (Fig. 5a and c). For MMA the rate of reduction increased with increasing pH values in the studied pH range (Fig. 5b and d). The rate of reduction of both mitomycins was proportional to the concentration of added ascorbate (Fig. S1, Supporting information).

The formation of mitosene compounds formed after the activation of both mitomycins by AscH was analyzed using LC/MS (Fig. 6). In addition to the known metabolites **8**, **10**, **11**, **13** [41], **4a** [46], **4b** [17] and **16** [47] (Fig. 7), several metabolites that incorporate ascorbate as nucleophile were identified based in their UV and ESI-MS.[1] For MMC, two of them were assigned as the isomeric C1 ascorbate adducts **9** (Fig. 7): their UV spectra is characteristic of 1-substituted mitosenes, and the MS shows molecular ions

[1] The characterization of the novel adducts is based solely in their UV and ESI-MS, and no further attempts were made to isolate and rigorously characterize the ascorbate-mitomycin adducts due to limited availability of mitomycins and their low proportion in a complex mixture of mitosenes.

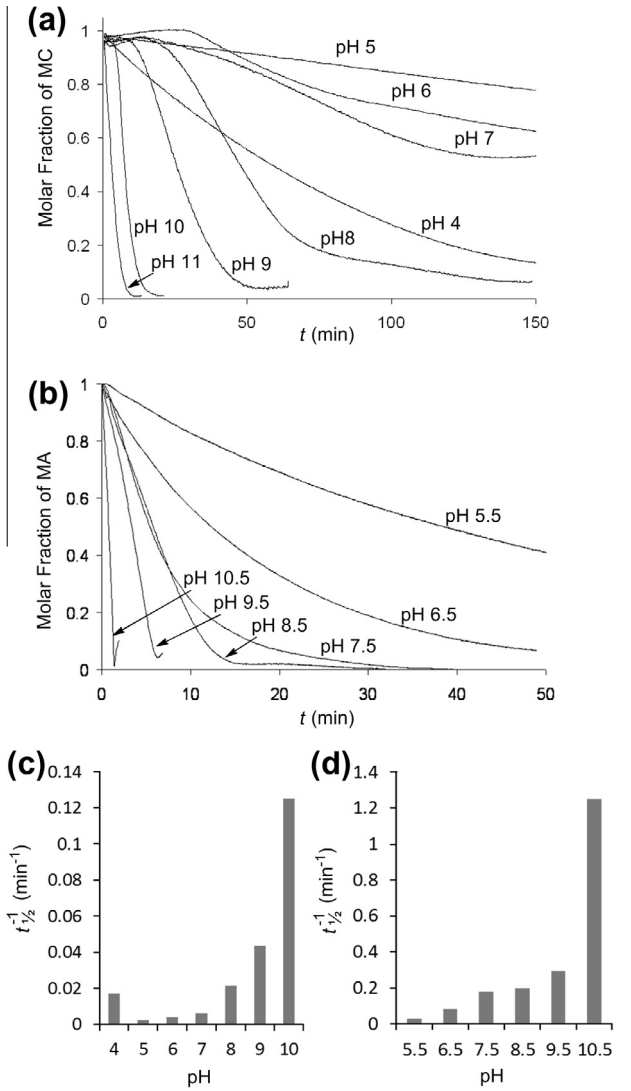

Fig. 5. pH-dependence of the reaction of mitomycins A and C with ascorbate. (a) Reaction of 50 mM MMC with 50 mM ascorbate at 37 °C. (b) Reaction of 30 mM MMA with 2 mM ascorbate. (c) and (d) Half-time for the reduction of MMC and MMA by ascorbate respectively, determined from the data shown in (a) and (b). See materials and methods for details.

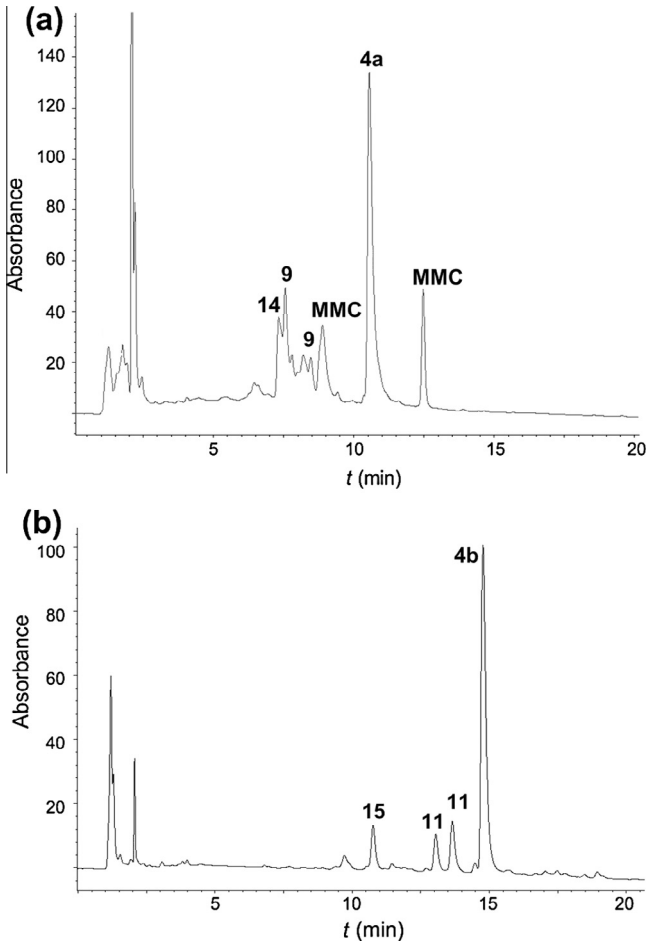

Fig. 6. HPLC analysis of the reaction of MMC and MMA with ascorbate in phosphate buffer (pH 6), showing the formation of C1-reduced mitosenes as major products (a) Chromatogram of a reaction of MMC (0.5 mM) with ascorbate (20 mM) at 20 °C for 18 h (λ = 315 nm). (b) Chromatogram of a reaction of MMA (0.5 mM) with ascorbate (5 mM) at 20 °C for 3 h (λ = 280 nm).

corresponding to Na^+ and K^+ adducts of **9**, and a major peak corresponding to the loss of the carbamoyloxy group (Supporting information, Figs. S2A and S2B). We propose structure **14** (Fig. 7), the adduct at C10 of 2,7-diaminomitosene **4a**, for the other ascorbate-MMC adduct observed in the HPLC chromatograms: the UV spectra shows the characteristic bands of the mitosene chromophore and the MS shows as major peaks the molecular ions for the H^+, Na^+ and K^+ adducts of structure **14** (Supporting information, Figs. S2G and S2H). For MMA, the analogous structures **12** and **15** (Fig. 7) are proposed using a similar rationale. The precise alkylation site of ascorbate in these adducts is uncertain. It is known that the alkylation of AscH provides a mixture of 3-O-alkyl, 2-O-alkyl, 2,3-di-O-alkyl and/or 2-C-alkyl derivatives, and the predominant product depends on the specific reaction conditions [48–50]. The alkylation of AscH with quinone methides derived from 4-hydroxybenzyl alcohols in water proceeded predominantly through the C-2 position [51]. At pH 10.5 or higher ascorbate reacts predominantly through the O-2 position [52]. In our case, ascorbate adducts are observed at pH 8 or below, and therefore the most likely positions for alkylation are C-2 or O-3. In some reactions between MMC and ascorbate at pH 7 we could identify three peaks in the HPLC trace that showed an MS corresponding to structure **9** (Fig. S3, Supporting information), indicating that ascorbate is alkylated at two sites, probably C-2 and O-3.

The identity of the mitosene compounds formed and their relative proportions depended markedly on the pH of the reaction (Fig. 8). At pH 6, C1-reduced mitosenes were the most abundant species observed in the chromatogram. When the pH was either increased or decreased from pH 6, the relative amounts of C1-reduced mitosenes diminished in favor of mitosenes derived from monofunctional activation.

4. Discussion

4.1. On the mechanisms of activation of mitomycins by ascorbate

Both the pH-dependence of the rate of reduction of mitomycins and the pH-dependent distribution of mitosene compounds can be rationalized considering that the three known mechanisms for the activation of mitomycins operate in the reactions of MMC and MMA with ascorbate. Before discussing our results on the reduction of mitomycins by ascorbate we present in the following paragraphs a brief review of the mechanisms for the activation of mitomycins (Fig. 9): the acidic (non-reductive) activation [45], the autocatalytic reductive activation mechanism [44] and the

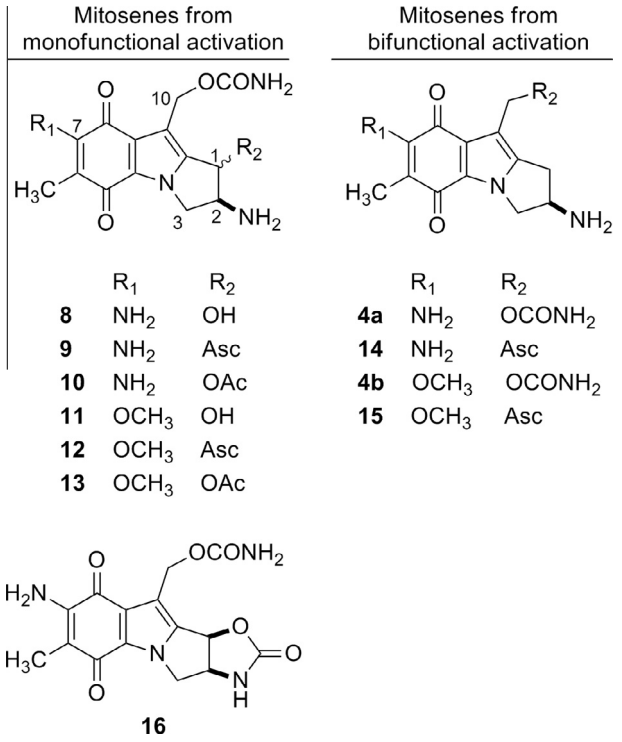

Fig. 7. Structure of the mitosene derivatives detected after the reactions of MMC or MMA with AscH.

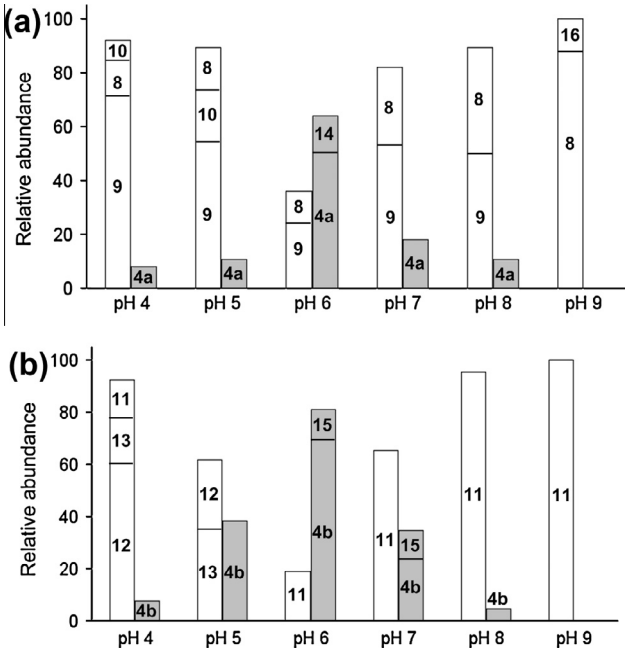

Fig. 8. Distribution of mitosene compounds detected by HPLC after the reaction of MMC (a) or MMA (b) with ascorbate at different pH values. White bars represent compounds derived from monofunctional activation (acid activation of autocatalytic reductive activation). Grey bars represent mitosenes derived from bifunctional activation.

bifunctional reductive activation mechanism [53]. The acidic activation occurs by an acid-catalyzed elimination of methanol in MMC or MMA to form **18** without the need of a reduction [45]. The mitosenes formed by this mechanism are exclusively C-1 substituted mitosenes retaining the C-10 carbamoyloxy functional

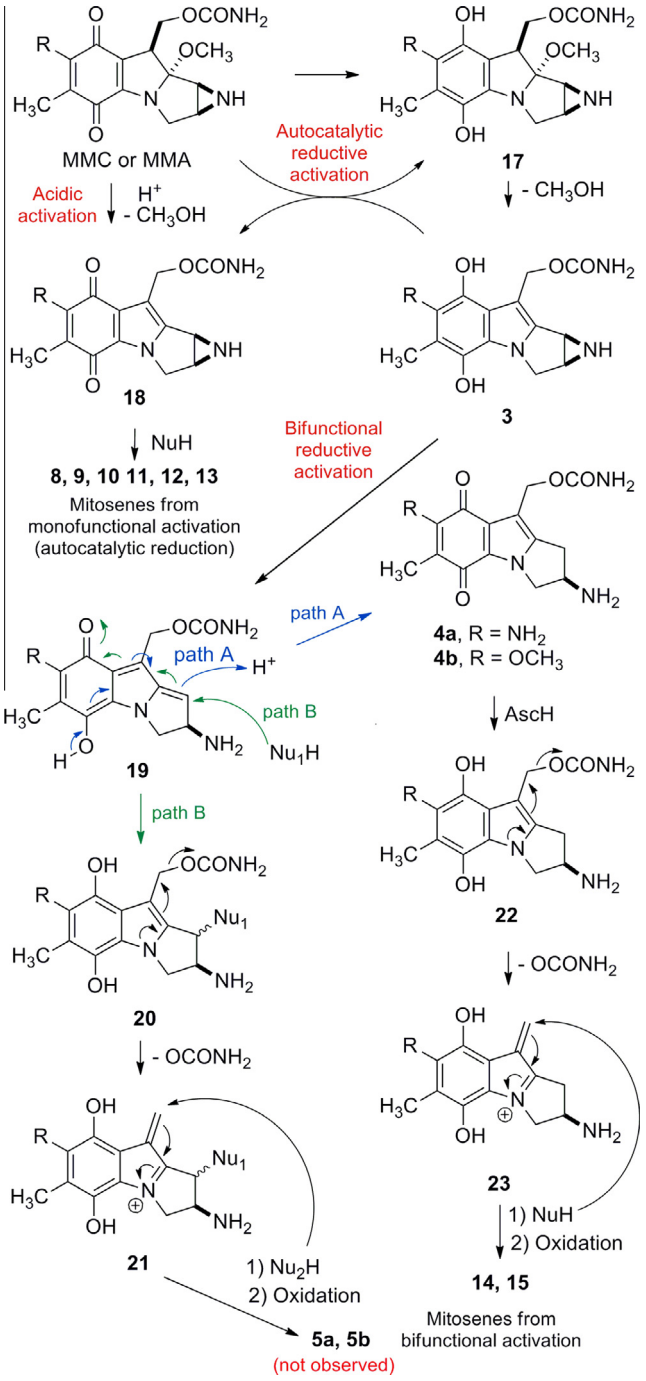

Fig. 9. Mechanisms for the reductive activation of mitomycins A and C by ascorbate.

group intact (e.g. **8**–**13**) [41,45]. Addition of nucleophiles to **18** occurs by a S_N1 mechanism to form two isomers at C1.

The autocatalytic mechanism and the bifunctional activation mechanism share in common the first two mechanistic steps: a reduction of the quinone ring to form hydroquinone **17**, followed by elimination of methanol to give **3** [53]. The leucoaziridinomitosene **3** constitutes the branch point for the competing autocatalytic activation mechanism and the bifunctional activation mechanism. In the autocatalytic path, **3** reduces mitomycin to form aziridinomitosene **18** and hydroquinone **17,** that eliminates methanol to form leucoaziridinomitosene **3**, triggering the autocatalytic reaction. The reduction of mitomycins by **3** is driven by the large redox

potential difference between mitosenes and mitosanes [44]. The autocatalytic reduction of mitomycins is favored by slow reducing agents and by alkaline pH values, and it is kinetically characterized by a sigmoid reaction curve [20,44]. The metabolites formed by this pathway are C-1 substituted mitosenes, analogous to those formed in the acidic activation [20,44]. The acidic activation only occurs significantly at pH values below 5, therefore the formation of mitosenes containing the C10 carbamoyloxy group in reactions at pH above 5 indicates that the autocatalytic mechanism is operating.

The bifunctional activation pathway for the activation of mitomycins involves an intramolecular aziridine-opening reaction in leucoaziridinomitosene **3** to form **19** (Fig. 9). The vinylogous quinone methide **19** constitutes another branch point in the mechanism of activation of mitomycins, as a consequence of its dual reactivity: The C1 carbon in **19** can function as an electrophile due to its vinylogous quinone methide structure, or as a nucleophile as a consequence of its vinylogous enol nature. The reaction of **19** as an electrophile (path B in Fig. 9) results eventually in the addition of two nucleophiles at C1 and C10 to give mitosenes with structures **5a** (from MMC) and **5b** (from MMA) (Fig. 1). The formation of interstrand cross-links in the reaction of reductively activated mitomycins with duplex DNA is the result of this type of reactivity. Alternatively, **19** can react as a vinylogous enol by tautomerizing to the corresponding keto form (path A in Fig. 9), which results in the formation of C1-reduced mitosenes **4a** (from MMC) or **4b** (from MMA). 2,7-diaminomitosene **4a** is the major in vivo metabolite of MMC [54], and it functions as a DNA alkylating agent, forming adducts at C10 [55]. The formation of covalent DNA adducts by **4a** requires an additional reductive activation step that converts the C10 carbon into an electrophilic position, as shown in Fig. 9.

The data obtained in the pH-dependence experiments shown in Figs. 5 and 8 illustrates the interplay of the three mechanisms reviewed above in the reduction of mitomycins with ascorbate. The observed pH-dependent distribution of mitosene derivatives (Fig. 8) serves to determine the contribution of the three mechanisms at a given pH value. Both MMC and MMA showed a similar pH-dependent distribution of products, reflecting similar overall reactivity for both mitomycins. At pH 4 both mitomycins react predominantly by the acidic activation mechanism. At pH 5, MMC reacted mostly by acidic activation, while the bifunctional activation mechanism starts to contribute significantly to the activation of MMA. The bifunctional activation becomes the major pathway for both mitomycins only at pH 6. From this point forward, its contribution starts to decrease in favor of the autocatalytic pathway, a mechanism that becomes the only one operating at pH 9 both for MMC and MMA. The data at from pH 6 to pH 9 explains how the pH determines the partition of **19** between the monofunctional activation pathway (conversion to **4**) and the bifunctional activation pathway (conversion to **20**). The distribution of mitosenes shown in Fig. 8 can be traced back in the activation mechanisms (Fig. 9) to the partition of **19** between **4** and **20** (the acidic activation of MMC that also produces adducts **8**–**13** is not relevant at pH $\geqslant$ 6). A singular feature of the HPLC traces of the reactions of mitomycins and ascorbate is that adducts with structures **5a** (for MMC) or **5b** (for MMA) were not observed, as it is usually the case in the reactions of MMC under bifunctional activation conditions [56–58].

Two features are evident from the experiments studying the influence of pH on the rate of reduction of mitomycins (Fig. 5). One of them is that the reaction curves take different shapes depending on the pH: The curves for MMC at pH 4 and the curves for MMA at pH values from 5.5 to 7.5 follow a quasi-exponential decay, while reactions at higher pH values displayed a sigmoid curve for both mitomycins. This result reflects a changing contribution of the three operating mechanisms depending on the reaction pH. Leucoaziridinomitosene **3**, the species whose accumulation triggers the autocatalytic mechanism (Fig. 9) is more stable with increasing pH, as its conversion to **19** is triggered by protonation of the aziridine ring. As a consequence, the autocatalytic mechanism becomes increasingly preponderant at higher pH values at the expense of the bifunctional activation mechanism. The other feature of these experiments is that the overall rate of reduction increases with increasing pH values (with the exception of the acidic activation of MMC at pH 4). This pH-influence correlates well with the pH-dependence reported for the reduction of other quinones with AscH. The rate of reduction of p-benzoquinone with AscH increased when the pH was increased from pH 2 to pH 5 [27]. Mukai et al. reported that the reduction of tocopheroxyl radical with vitamin C was markedly pH-dependent, with a maximum rate at pH 8, suggesting that ascorbate monoanion is the actual reducing species [59]. In the reduction of mitomycins with ascorbate, the rate of reduction continues to increase after pH 8 (Fig. 5), but this effect should not be attributed to a faster reduction of the quinone ring by ascorbate. Instead, the faster reaction rates at alkaline pH are most likely the result of an increasing contribution of the autocatalytic mechanism (white bars in Fig. 8) as a consequence of the greater stability of **3** at higher pH values.

4.2. Biological significance

A number of studies have shown that the biological effects caused by MMC exposure can be altered by AscH. For example, Marshall and Rauth reported that the presence of AscH increased the aerobic but not the hypoxic cytotoxicity of MMC towards Chinese hamster ovary cells and a repair-deficient mutant [30]. Other studies showed a slight inhibition by AscH of the clastogenic effects of MMC in mice [34] and a potentiation of the antitumor activity of MMC by ascorbate [33]. Several reports from Getoff et al. showed that the activity of MMC was modified by AscH under irradiation [35–39]. In some cases differing results were obtained depending on the model studied: when monitoring the effects of AscH on MMC-induced sister chromatid exchanges (SCEs), Krishnaja and Sharma found that pretreatment with ascorbate enhanced MMC-induced SCEs in human lymphocytes in vitro [29], while other researchers found that AscH ascorbic inhibited SCEs induced by MMC in bone marrow and spleen cells in mice [31,32].

The work discussed here tried to ascertain if a direct redox reaction between MMC and ascorbate could be responsible for the reported effects of ascorbate on MMC activity. If such an reduction takes place it could result in either the activation of MMC to cytotoxic alkylating species or, alternatively, in detoxification by conversion of MMC to the non-cytotoxic derivative 2,7-diaminomitosene metabolite **4a** [60]. Our results indicate that, at physiologically relevant pH values, the reduction of MMC requires concentrations of ascorbate larger than 50 mM, much greater than those present in cells (micromolar-low millimolar range) [61]. Also, at pH 5–7 the reduction takes place at a very slow rate (Fig. 5a and c), with 60–80% of MMC still unreacted after 2.5 h. At more acidic or more basic pH values the reaction progresses faster, but these reactions take place by mechanisms that are not relevant physiologically: an acidic activation mechanism that requires pH < 5 or an autocatalytic reaction that requires large concentrations of MMC.

In contrast, the highly cytotoxic drug MMA is reduced efficiently using concentrations of ascorbate in the low mM range. The differential reactivity of MMA and MMC with ascorbate concords well with the higher redox potential of MMA compared to MMC ($E_{½}$ of MMA and MMC are −0.19 and −0.40 V, respectively) [4]. The efficient reduction of MMA by ascorbate was not unexpected, as the ability of ascorbate to reduce other

methoxyquinones has been reported earlier [26]. This efficient reduction suggests that the direct reaction of ascorbate with MMA may play a role in the high cytotoxicity of this drug.

Our results agree with the results reported by Marshall and Rauth in their investigation on the effects of ascorbate in the cytotoxicity of MMC towards CHO cells, where they found evidence that ascorbate was not acting by directly reducing MMC [30]. They proposed that the increased toxicity of MMC in the presence of ascorbate that they observed might be due to an increased production of bioreductive enzymes or of compounds capable of modulating the reduction process. An additional mechanism by which ascorbate may potentiate the effects of MMC is by inhibiting multidrug resistance glycoprotein (MDR1) [62].

5. Conclusions

In summary, our results indicate that the modulatory activity on the effects of MMC by AscH is unlikely to be the result of a direct redox reaction between MMC and ascorbate and, therefore, alternative biochemical mechanisms must be responsible for this modulation. In contrast, a reductive activation by ascorbate may contribute to the potent cytotoxic activity of MMA.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bioorg.2013.03.002.

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