The Reactions of Mitomycin C with Dithiols

I. Reductive Activation

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Abstract

We report that the clinically used antitumor drug mitomycin C is reductively activated *in vitro* by simple thiols; a mechanism for this activation is proposed based on kinetic data and the identification of mitosene metabolites. The biological implications of these findings are discussed.

Introduction

Mitomycin C (MC, **1**)[1] is a natural antitumour antibiotic used in anticancer therapy[2] that requires reductive activation to exert its biological activity.[3] After bioreduction, MC generates a bifunctional electrophile (**4**, scheme 1) that alkylates cellular nucleophiles, in particular the complementary strands of DNA. [4] A number of enzymes are known to activate MC in mammalian cells.[5] Recent reports indicate that proteins containing a dithiol active site are implicated in the cellular activation of MC.[6] Incongruently, MC was reported to be inert to reductive activation by monothiols (glutathione) and dithiols (DTT),[7,8] We decided to re-examine the reaction of MC with thiols, in particular after recent reports linked the thioredoxin-like domains of GRP58 to the activation of MC in cancer cells, an explicit sign of the involvement of the dithiol group in the cellular activation of MC. The results we present here reconcile this apparent paradox.

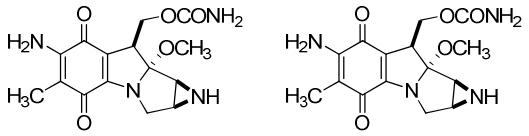


Figure 1. Structure of mitomycin C and mitomycin A.

Results and discussion

The UV analysis of a reaction containing MC and a large excess of DTT (0.1 M) at neutral pH clearly showed the gradual formation of mitosene compounds (**6**, Scheme 1) at the expense of MC (figure 2), the reaction reaching completion in about 120 minutes, as judged by a constant UV spectra. When MC was treated with mercaptoethanol in similar conditions only a marginal conversion of MC to mitosenes was detected during 3 hours. In contrast, MA reacted completely in 2 minutes by treatment using a 500-fold lower DTT concentration.[9a]

A kinetic analysis of the reaction was then performed. The rate of disappearance of MC by treatment with DTT was measured by the decline of the UV absorbance at 360 nm (figure 3). The decay of MC displayed the shape of a sigmoidal curve, characteristic of an autocatalytic reaction. Dissimilarly, the kinetic study of the reaction of MA with thiols showed a pseudo-first order decay. [9a]

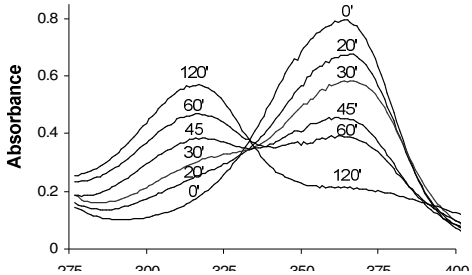


Figure 2. UV assay of the reduction of mitomycin C by DTT. Reaction times are indicated on top of each spectrum.

The autocatalytic activation of MC has been observed before, both during enzymatic[9] and chemical[10] reductions. This mechanism is based on the large redox potential difference between the mitosane (**1**) and mitosene (**4**) structures (scheme 1). Thus, the leucomitosene species, e.g. 5 and 6, transfer their electron(s) to unreduced MC, creating an electron transfer chain reaction, provided the reducing agent is slow to reduce excess MC.[7,10,11]

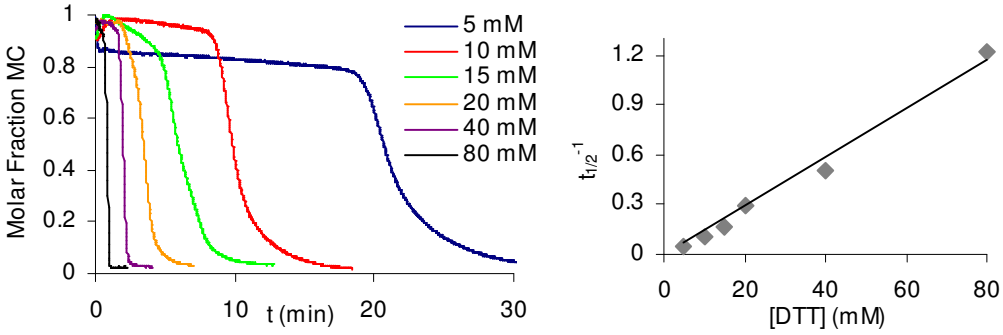
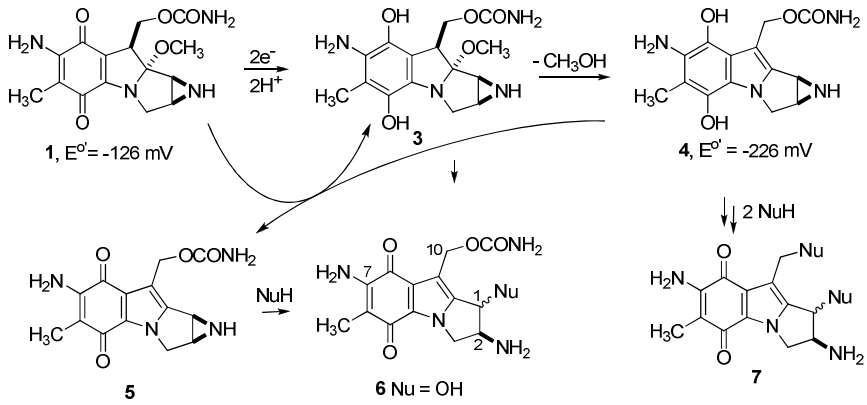


Figure 3. (a) Dependence on the concentration of DTT. All reactions contain 30 µM. MC at pH 9.0. Concentrations of DTT were as indicated in the chart. (b) Relationship between t1/2-1 and DTT concentration at pH 9.0.; [MC] = 5

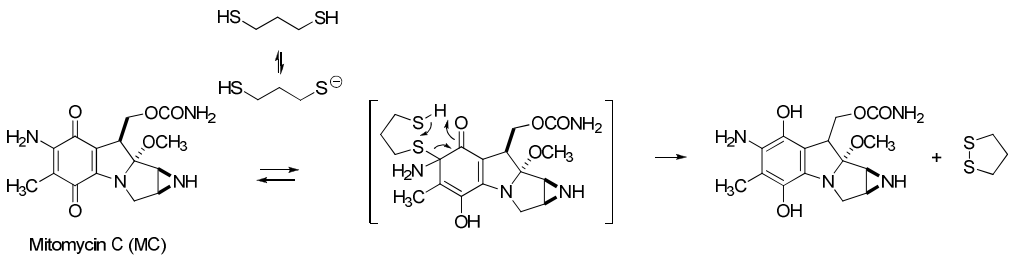
The identity of the mitosenes formed in the reaction of MC with DTT was studied by LCMS. MC was activated with substoichometric DTT at pH 9.5 until all MC is consumed, then quenched with diluted aqueous buffer at neutral pH. LCMS analysis showed the formation of the expected diastereomeric pair of hydroxymitosenes **6**.[11]



Scheme 1. Reductive activation of MC. NuH represents a nucleophile.

The kinetics of activation of MC were analyzed in detail, varying the thiol (DTT, 1,3-propanedithiol, dihydrolipoic acid and mercaptoethanol), the concentration of thiol, the concentration of MC and the pH. The rate of reaction (plotted as $t_{1/2}^{-1}$) was linearly proportional to the concentration of both thiol and MC, concordantly with the proposed mechanism.

Based on these findings we propose that the activation of MC by thiols occurs by a mechanism analogous to the one we previously proposed for the activation of MA by thiols:[9a] an initial conjugate addition of thiolate to the quinone of MC, followed by an internal redox reaction to give the hydroquinone of MC and the corresponding disulfide (scheme 2).



Scheme 2. Hypothesis for the mechanism of activation of MC by thiols.

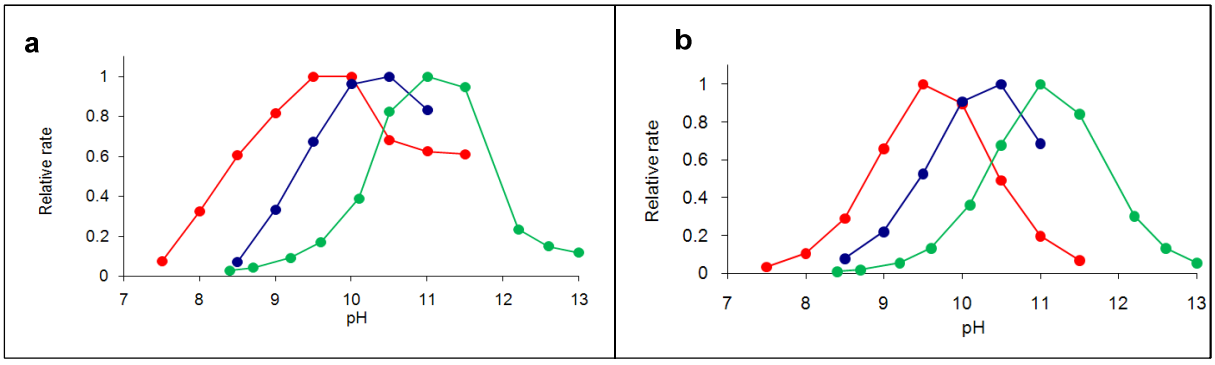


Figure 4. (a) pH dependence of the rates of reaction of MC with the three thiols evaluated in this work: DTT (red); 1,3-propanedithiol (blue); MER (green); DHLA (black). The relative rates are defined as the ratio $t_{1/2}^{-1}/(t_{1/2}^{-1})_{max}$, where $t_{1/2}$, is the time required for 50% conversion of MC at a given pH determined from the curves of decay of MC, and $(t_{1/2}^{-1})_{max}$ is the maximum rate observed at the pH values assayed for each thiol. (b) Theoretical pH dependence derived from equation 1 for the dithols used in this work; color codes are the same as in figure 5a.

When the pH dependence was studied, it was found that the reaction rate follows initially a linear relationship until a certain pH, reaches a maximum value and then decreases. The optimum pH values for the activation of MC with the three thiols used in this work were all around the sulphydriyl pK_a value of each thiol (figure 4). The graphic data of pH-dependence (figure 4) clearly illustrates the reason why earlier attempts to activate MC at pH around neutral failed: extrapolation of the linear data points (below the optimum pH) indicates that as the pH of the reaction approaches 8 (for mercaptoethanol and propanedithiol) or 7 (for DTT), the half-time for the conversion of MC tends to be infinite, particularly with the relatively low concentrations of thiols that were employed in the earlier assays.[6,9b] The pH dependence data concords with a mechanism analogous to the one we previously proposed for the reduction of MA by dithiols. The formation of the intermediate requires a thiolate anion, while its conversion to hydroquinone requires a protonated thiol (scheme 3). The fact that reductions of MC by dithiols are favored at a pH bounded by the pKa1 and pKa2 of the dithiol lends support to the proposed mechanism, as it is precisely at those pH values where higher concentrations of both thiol and thiolate will be present. Additionally, the observed pH dependence could be fitted to equation I where the rate of reaction is dependent on the pH and the pKa values of the two sulfhydryl groups (figure 4b).

$$k = k_{max}^{-1} \times [1 + ([H^+] / K_{a1}) + (K_{a2} / [H^+])]^{-1}$$

Equation 1 . k is the relative rate of activation of MC; k_{max} is the maximum rate; K_{a1} and K_{a2} are the equilibrium constants for deprotonation of the thiol groups in the dithiol. The pKa values shown in figure 5 were used, except for DHLA, where a pK_{a2} of 11.5 was used, as only an average value of pK_a for DHLA is reported in the literature (*22*).

Are the results presented here relevant in vivo? In principle, it would appear not to be so, as the pH required for an efficient reaction is distant from regular physiological values. We consider improbable the involvement of glutathione (a cellular monothiol) in the activation of MC in vivo, as the concentrations of thiolate required for activation are several orders of magnitude higher than those present in cells. Likewise, free lipoate coenzymes are unlikely to contribute significantly to the cellular activation of MC, as the optimum pH for the activation with DHLA is 4 units above physiological pH values. However, the rationale changes if we consider the exceptional acid-base properties of other biological dithiols. Proteins of the thioredoxin superfamily (thioredoxin, glutaredoxin, protein disulphide-isomerases, disulfide bond formation protein A) contain a dithiol in the active site, where the pKa of the N-terminal cysteines in the -Cys-X-X-Cys- motif is abnormally lo[12] while thioredoxin reductases contain an even more acidic selenol group. At physiological pH, both the thiol from Trx and the selenol from TrxR are deprotonated and in the proposed mechanisms for their reactions they behave as strong nucleophiles[13]

In the mechanism we postulate (scheme 3), the optimum pH for the activation of MC by dithiols is predicted to present a value flanked by the pKa values of their sulfhydryl residues. At such pH values, the most acidic thiol (or selenol) group will be deprotonated, and the nucleophilic addition (first step in scheme 3) will be favored. The second sulfhydryl group of the dithiol presents pKa values above 8 both for Trx and TrxR, and will be mostly protonated at neutral or acidic pH (figure 5), therefore favoring the second step in the proposed mechanism (scheme 3). As a result, our mechanistic model predicts that the Trx (pKa1 = 7.1) might activate MC at pH around neutral, and TrxR (pKa1 = 5.8) could be capable of activating MC even at acidic pH values. The fact that reductions of MC by dithiols are favoured at pH values bounded by the pK_{a1} and pK_{a2} of the dithiol also concords with the proposed mechanism. The menchanism involves thiol groups in both the protonated and deprotonated states, and it is precisely at those pH values where the rates should be maximized, since higher concentrations of both species must be present.

Conclusion

We conclude that the activation of MC with DTT can possibly be reproduced in living organisms by biological thiols.[14] The results presented here serve and a chemical foundation to explain the reported involvement of biological dithiols in the modulation of mitomycin C cytotoxicity: dithiols could detoxify MC by activating MC in the cytosol, where it would be hydrolized to inactive metabolites, and could activate MC to form toxic metabolites by reducing MC in close proximity to the biological target. Additional research is currently being performed be determine if Trx and other dithiol-containing proteins are indeed capable of reducing MC.

References

[1] Abbreviations: DTT,D,L-dithiothreitol ; MA, mitomycin A; MC mitomycin C; Trx, thioredoxin; TrxR, thioredoxin reductase; mitosene: structure **6**, without substituents in the 2- and 7-positions;

[2] Paz, M. M. in *Anticancer Therapeutics* (Ed.: S. Missailidis), John Wiley and Sons, Oxford, **2008**, pp. 112–115.

[3] Suresh Kumar, G. Lipman, R. Cummings, J. Tomasz, M. *Biochemistry* **1997**,*36*, 14128–14136.

[4] Tomasz, M. Lipman, R. Chowdray, D. Pawlak, J. Verdine, G. L. Nakanishi, K. *Science* **198**, *235*, 1204–1209.

[5] a) Sartorelli, A. C. Hodnick, W. F. Belcourt, M. F. Tomasz, M. Haffty, B. Fischer, J. J. Rockwell, S. *Oncology Res*. **1994**, 10/11, 501-508. b) Cummings, J. Spanswick, V. J. Tomasz, M. Smyth, J. F. *Biochem. Pharmacol.* **1998**, *56*, 405–414.

[6] a) Kontou, M. Adelfalk, C. Ramirez, M. H. Ruppitsch, W., Hirsch-Kauffmann, M. Schweiger, M. *Oncogene* **2002**, *21*, 2406–2412. b) Adikesavan, A. K. Jaiswal, A. K. *Mol. Cancer Ther*. **2007**, *6* 2719-2727.

[7] While thiols themselves did not reduce MC, they reacted as nucleophiles with MC activated by other reducing agents: Sharma, M. Tomasz, M. *Chem. Res. Tox.* **1994**, *6*, 390-400.

[8] He, Q.-Y. Maruenda, H. Tomasz, M. *J. Am. Chem. Soc*. **1994**, *116*, 9349-9350.

[9] Peterson, D. M. Fisher, J. *Biochemistry* **1986**, *25*, 4077-4084.

[10] Hoey, B. M. Butler, J. Swallow, A. J. *Biochemistry* **1988**, *27*, 2608- 2614.

[11] Tomasz, M. Lipman, R. Biochemistry **1981**, *20*, 5056-5061

[12] Holmgren, A. Structure **1995**, 3, 239-243.

[13] Johansson, L. Gafvelin, G. Arnér, E. S. J. *Biochim Biophys Acta*, **2005**, *1726*, 1–13.

[14] Other antitumour quinones have been shown to be reduced *in vitro* by TrxR with an efficacy similar to that of Trx, the natural substrate. See: Cenas, N. Nivinskas, H. Anusevicius, Z. Sarlauskas, J. Lederer, F. Arnér, E. S. J. *J. Biol. Chem*. **2004**, *279*, 2583–2592.