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Motexafin gadolinium reacts with ascorbate to produce reactive oxygen species†

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Motexafin gadolinium (MGd) oxidizes ascorbate, in neutral buffer and in cell culture, forming reactive oxygen species and a coordination polymer with oxalate.

An important function of metabolism is to maintain cellular systems in a reduced state relative to the aerobic environment. Although reducing metabolites such as glutathione, NADPH, and ascorbate are central to cellular antioxidant defense, these compounds have reduction potentials sufficiently negative for spontaneous electron transfer to oxygen to occur in the presence of an appropriate catalyst. Such catalysts, termed 'redox mediators', have provided important tools with which to examine the impact of oxidative stress in living systems, including its effect in the biological response to ionizing radiation.[1]

The gadolinium(III) complex **1** (motexafin gadolinium, Xcytrin®, MGd) is currently in advanced stage clinical development as an adjuvant to radiation therapy.[2] MGd has previously been reported to enhance the efficacy of radiation in animal tumor models.[3] Recent studies *in vitro*, however, have revealed a surprising sensitivity to the choice of medium.[4] In particular, it was discovered that the amount of ascorbate present correlated with the cytotoxicity of the drug. This finding has led us to consider that MGd can function as a redox mediator. The generation of reactive oxygen species, if occurring, could provide a basis for the mechanism of biological action of this agent. Support for this hypothesis is presented below.

The oxidation of ascorbate in a neutral pH saline solution was quantified by monitoring the decrease of its absorbance at 266 nm.[5] Addition of a catalytic amount of MGd to a solution of ascorbate resulted in no spectral changes in solutions purged with argon. However, under ambient atmospheric conditions, a rapid decrease of the ascorbate absorbance was observed, the rate of which was initially linear and which decreased to background level within *ca*. 30 minutes (Fig. 1A). During the course of *ca*. 60 minutes, the Soret and Q-like absorption bands of the complex at 470 nm and 742 nm, respectively, were converted with clean isosbestic points to new absorbances at 510 nm and 780 nm.

Spectra obtained under an oxygen atmosphere displayed similar features (Fig. 1B), with the exception that the oxidation of ascorbate proceeded with *ca*. 3-fold greater initial rate and to a much greater degree of completion (*Cf*., also, Supplementary Information†). Under ambient conditions, addition of catalase had no significant effect on the rate of ascorbate or oxygen consumption (Table 1).‡ It did, however, lead to a two-fold decrease in the rate at which the species absorbing at 510 and 780 nm, referred to as **2**, was formed. Superoxide dismutase slowed the rate of ascorbate and oxygen consumption by a factor of two, and, moreover, slowed formation of the species absorbing at 510 and 780 nm by at least ten-fold (Table 1).

The identity of the new species **2** was investigated. It was observed to precipitate upon prolonged incubation in buffer, and the resulting solid was catalytically active when added to fresh ascorbate solution. Addition of methanol to this solid resulted in gradual regeneration of the starting complex. Based on these observations, a range of standard chemical analyses, and the finding that identical spectral changes occur upon incubation of complex **1** in buffer with either dehydroascorbate (slow) or disodium oxalate (rapid, *Cf*., Supplementary Information†),§ we propose that species **2** consists of a coordination polymer with oxalate as shown in Fig. 2. Oxalate is a known decomposition product of dehydroascorbate, which is unstable at physiologic pH and in the presence of superoxide.[6]

The effects of oxygen and enzymes outlined above are consistent with the existence of an equilibrium between the single-electron reduced form of MGd and oxygen on the one hand and superoxide anion and MGd **1** on the other (Eqn. 1).¶ [7,8] In accordance with this scheme, perturbations decreasing the

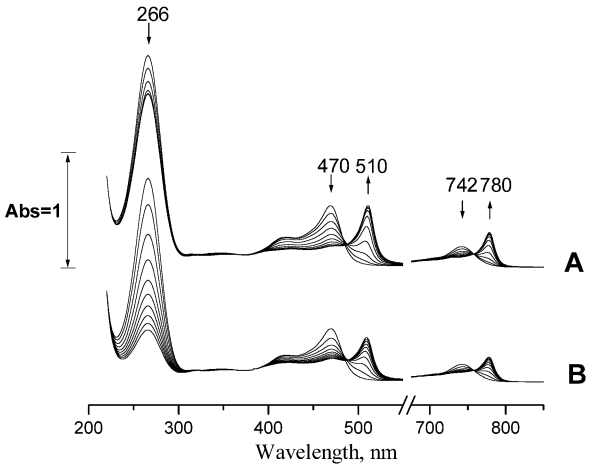

Fig. 1 Spectral changes occurring upon incubation of MGd with ascorbate in buffered solution. A solution of ascorbic acid (1.25 mM) in 50 mM HEPES buffer, pH 7.5, 100 mM NaCl (Chelex-100™ -treated, all concentrations final) was placed in a 1 mm quartz cuvette. The UV-visible spectrum was recorded for 60 minutes following addition of MGd (62 μM). A. Ambient conditions. B. Oxygen atmosphere.

Table 1 Rates of oxygen, ascorbate, and MGd decrease[a]

	$\delta[O_2]/\delta t$	$\delta[Asc]/\delta t$	$\delta[MGd]/\delta t$
No enzyme	5.56 ± 1.13	9.05 ± 0.64	1.22 ± 0.40
Catalase[b]	4.63 ± 0.80	8.30 ± 0.48	0.69 ± 0.07
SOD[c]	1.93 ± 0.15	4.48 ± 0.45	0.09 ± 0.04
Catalase + SOD[bc]	2.38 ± 0.30	3.94 ± 0.34	0.10 ± 0.09

[a] Reaction of ascorbate (1.2 mM) with MGd (62 μM) in 100 mM NaCl, 50 mM HEPES buffer, pH 7.5. Units are μM min^{-1}, mean ± std. dev. of 3 runs. [b] Catalase [EC 1.11.1.6], 2600 units mL^{-1}, or [c] superoxide dismutase [EC 1.15.1.1], 100 units mL^{-1}, added prior to MGd.

† Electronic supplementary information (ESI) available: experimental description and results. See http://www.rsc.org/suppdata/cc/b2/b208760j/

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concentration of reduced MGd, such as an increase in oxygen, would increase the rate of ascorbate oxidation by regeneration of **1**. Superoxide dismutase, on the other hand, would decrease the rate of ascorbate oxidation by inhibiting reduction of superoxide by ascorbate.[5] Removal of superoxide, whether by reduction or disproportionation, would lead to complex regeneration and formation of hydrogen peroxide and dehydroascorbate.‖ [9]

$$MGd + O_2^{\cdot -} \rightleftharpoons MGd^{\cdot -} + O_2 \quad (1)$$

As a specific test as to whether redox cycling by MGd occurs *in vitro*, A549 (human lung cancer line) cells** were incubated with MGd, MGd and ascorbate, or pre-formed oxalate complex **2**. An inhibitor of glutathione synthesis, L-buthionine-[*S*,*R*]-sulfoximine (BSO), was also added, to inhibit hydrogen peroxide removal by glutathione peroxidase. After washing, cultures were treated with dichlorofluorescin acetate (DCFA). Cell suspensions were analyzed by flow cytometry, using an excitation wavelength of 488 nm, and emission filters at 530 nm (FL1) and > 650 nm (FL3). As shown in Fig. 3, an increase in FL1 fluorescence was observed in cells treated with MGd (or **2**) and BSO, indicative of DCFA oxidation to form dichlorofluorecein (DCF).[10] This FL1 signal was increased by co-incubation with ascorbate and also in cells incubated with pre-formed **2**. Moreover, this latter increase correlated with that in the FL3 channel, derived from the fluorescence of MGd at 760 nm. This finding leads us to suggest that the cellular uptake of MGd is facilitated in the presence of ascorbate *via* intermediate formation of the oxalate complex **2**.[11]††

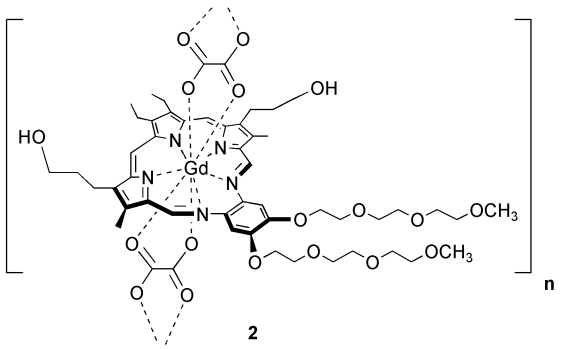

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Fig. 2 Structure proposed for motexafin gadolinium oxalate complex **2**, formed from the corresponding monomeric diacetate complex **1**.

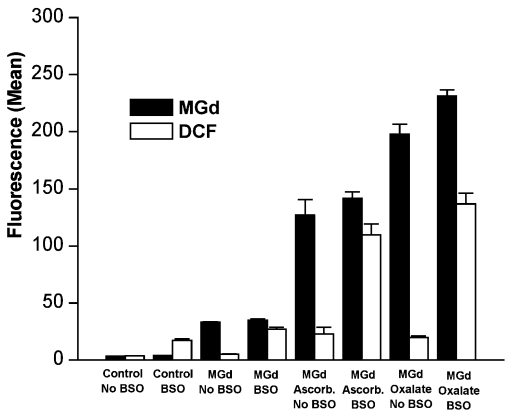

Fig. 3 Analysis of A549 lung cancer cells by flow cytometry. A549 cells (4×10^5 cells) were treated with MGd (50 μM) with or without ascorbate (100 μM) or MGd oxalate complex **2** (50 μM) in RPMI 1640 medium supplemented with 10% dialyzed fetal bovine serum for 22 h. BSO (100 μM) was also added where indicated. Cultures were washed with Dulbecco's phosphate buffered saline (PBS), and treated with DCFA (0.25 μg mL^{-1}) in Hank's sterile saline for 10 minutes. Cultures were washed with PBS, treated with trypsin, and the resulting suspensions subjected to analysis by flow cytometry. Mean fluorescence at 530 nm (DCF) and > 650 nm (MGd) is shown. Ascorbate treatment alone had no effect on fluorescence (see Supplementary Information†). Error bars indicate standard deviation (n = 3).

The above experiments, considered in concert, provide support for the proposal that reactive oxygen species are formed intracellularly in the presence of MGd, especially in ascorbate-containing media. Reactive oxygen species generation could contribute to the increased radiation response observed in preclinical models.[3] *tert*-Butyl hydroperoxide, for example, has been shown to enhance radiation response *in vitro*.[12] Interestingly, texaphyrins can display considerable (as much as 10-fold) selective accumulation in tumor tissue.[2,3] To our knowledge, the development of a redox cycling agent which localizes selectively to neoplasia *in vivo* would represent a new approach to enhancing the therapeutic response to ionizing radiation or chemotherapy.[13] Studies to evaluate the consequences of electron transfer to MGd from ascorbate and other reducing metabolites (*e.g.*, NADPH) present in biological systems are currently underway.

Notes and references

‡ Oxygen was measured using a ruthenium bipyridine tipped fiber optic probe (Ocean Optics, Inc.) calibrated with a Clark electrode.

§ Presence of oxalate in compound **2** was confirmed using ^{13}C NMR, IR, and ion chromatographic analyses (Supplementary Information†).

¶ The equilibrium proposed was examined recently using pulse radiolytic techniques.[7] The equilibrium constant was reported to be *ca.* 3 in favor of reduced MGd, underscoring the fact that MGd is more electron affinic than oxygen.[8]

‖ Non-catalyzed superoxide disproportionation is estimated to occur with a rate constant of 1×10^5 $M^{-1}s^{-1}$ at pH 7.4.[9]

** Human lung cancer cell line A549 was obtained from the American Type Culture Collection.

†† Complex **2** was found to be significantly less fluorescent than MGd[11] The FL3 signal observed in cells treated with complex **2** may derive in part from intracellular re-formation of MGd.

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