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MAMMALIAN CELL TOXICITY AND BACTERIAL MUTAGENICITY OF NITROSOIMIDAZOLES

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Abstract—It is currently believed that the biological activity of such therapeutic 5-nitroimidazoles as metronidazole is mediated by a short-lived, highly toxic species that arises from nitro group reduction. We found that the 5-nitroimidazole, 1-methyl-4-phenyl-5-nitroimidazole (5-NO_2), is at least 1000-fold less cytotoxic for CHO cells and mutagenic for Ames tester strain TA100 than its homologous nitroso compound, 1-methyl-4-phenyl-5-nitrosoimidazole (5-NO). Such evidence, along with previous work showing a similar relative bactericidal potency of these compounds, is consistent with the labile nitrosoimidazole being a biologically active species of the nitroimidazole, and indicates that mammalian cells are very susceptible to such an active form. The high potency of both 5-NO and 1-methyl-4-nitroso-5-phenylimidazole (4-NO), in contrast to the lack of potency of 1-methyl-4-nitro-5-phenylimidazole (4-NO_2) relative to 5-NO_2, is additional evidence to support the suggestion that the activity of a nitroimidazole is determined mainly by the ease with which it is reduced.

Activation of such 5-nitroimidazole drugs as metronidazole proceeds by reduction of the nitro group, yielding a labile bactericidal intermediate [1, 2], which may be reduced further to the biologically inactive 5-aminoimidazole [3]. Recently, it has been found that 1-methyl-4-phenyl-5-nitrosoimidazole (5-NO)† has much greater bactericidal potency than its homolog, 1-methyl-4-phenyl-5-nitroimidazole (5-NO_2), which itself shows activity similar to those of such therapeutic nitroimidazoles as metronidazole (1-[2′-hydroxy]ethyl-2-methyl-5-nitroimidazole) [4]. Other nitrosoimidazoles, in particular 1-methyl-4-nitroso-5-phenylimidazole (4-NO), have also been found to be more bactericidal than their homologous nitroimidazoles [4], whereas, in accord with previous experience [5, 6], the 4-nitroimidazole, 1-methyl-4-nitro-5-phenylimidazole (4-NO_2), has little bactericidal potency. The nitrosoimidazoles also have other properties consistent with those postulated for an activated form of the nitroimidazole drugs [4].

Although the 5-nitroimidazoles apparently react readily with the DNA of susceptible bacteria [7], they have no detectable toxicity on either other bacteria [4] or mammalian cells under normal conditions [8, 9]; and they apparently pose little risk for human patients [10, 11]. Oxygenated mammalian cells may not be susceptible to the nitroimidazoles either because such cells do not reduce the nitroimidazoles to their active form [12] or because they are not susceptible to that active form.

To distinguish between these possibilities we have compared the cytotoxicities of the nitroimidazoles and their homologous nitrosoimidazoles for Chinese hamster ovary (CHO) cells and have also examined the relative mutagenicities of these compounds for the Ames TA100 histidine auxotroph [13].

EXPERIMENTAL PROCEDURE

Metronidazole was a gift of G. D. Searle & Co. (San Juan, PR). 4(5)-Nitroso-5(4)-phenylimidazole (4/5-NO), 4(5)-nitro-5(4)-phenylimidazole (4/5-NO_2), 4-NO, 4-NO_2, 5-NO, and 5-NO_2 were prepared as described previously [4].

CHO cells, provided by H. Nagasawa (Department of Cancer Biology, Harvard School of Public Health, Boston, MA) were cultured and maintained at 35° in minimal essential medium (Gibco Laboratories, Grand Island, NY) supplemented with 25 μg/ml of gentamicin and 10% fetal bovine serum (Hazleton Laboratories, Lenexa, KS), in an atmosphere of humid air with 5% carbon dioxide. Experiments were performed on cells in exponential growth; cell numbers were determined with an electronic particle counter (Coulter Electronics, Inc., Hialeah, FL).

Approximately 50 CHO cells were incubated in 5 ml of growth medium in a 60 mm diameter petri dish (Falcon Plastics, Oxnard, CA). After 24 hr (to allow the cells to adhere), the test compound in 50 μl of dimethyl sulfoxide (DMSO) was added and the incubation continued for an additional 10 days. Plates were then stained with methylene blue, and colonies of greater than 100 cells were enumerated in comparison to control plates treated only with

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† Abbreviations: 5-NO, 1-methyl-4-phenyl-5-nitrosoimidazole; 5-NO_2, 1-methyl-4-phenyl-5-nitroimidazole; 4-NO, 1-methyl-4-nitroso-5-phenylimidazole; 4-NO_2, 1-methyl-4-nitro-5-phenylimidazole; 4/5-NO, 4(5)-nitroso-5(4)-phenylimidazole; 4/5-NO_2, 4(5)-nitro-5(4)-phenylimidazole; and CHO cells, Chinese hamster ovary cells.

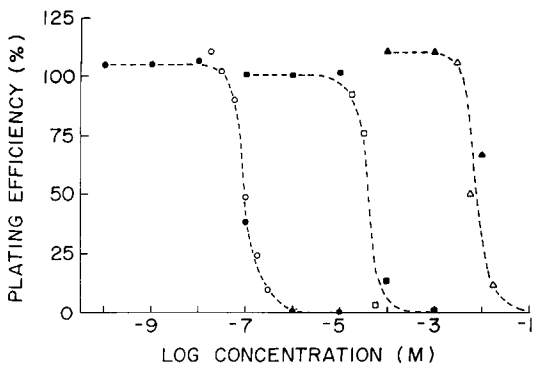

Fig. 1. Comparison of the effect of treating CHO cells with nitroimidazoles and a nitrosoimidazole. Adherent CHO cells were treated for 10 days with various concentrations of metronidazole (▲, △), 5-NO_2 (■, □), or 5-NO (●, ○) under aerobic conditions, and colony formation was monitored. Open and closed symbols indicate the results of separate experiments.

50 μl of DMSO. Each datum point was the average of determinations of five plates (ten plates for controls).

Bacterial mutagenicity was carried out according to the method of Ames *et al.* [13], with the omission of the S-9 liver microsomal fraction; test compounds were the last addition made in the preparation of top agar, since the nitrosoimidazoles decompose readily [4].

RESULTS AND DISCUSSION

Colony-forming ability was decreased by 50% when CHO cells were exposed to metronidazole at a concentration of 8 mM (Fig. 1), a value similar to that (10 mM) observed under analogous conditions by Mohindra and Rauth [8]. With 5-NO_2, on the other hand, a 50% inhibition of colony-forming ability occurred at a concentration of 6×10^{-2} mM, indicating that 5-NO_2 is approximately 100 times more potent than metronidazole for these cells, as is the case for their relative antibacterial activity [4]. In other experiments (Table 1), we found, as expected, that 4-NO_2 had no effect on plating efficiency at concentrations up to 1 mM (approximately its limit of solubility in the medium). On the other hand, 5-NO decreased colony formation by 50% at a concentration of 10^{-4} mM. The other nitrosoimidazoles were also potent; 4-NO and 4/5-NO decreased colony formation by 50% at concentrations of 8×10^{-4} mM and 3×10^{-3} mM respectively.

Table 1. Concentrations of various nitrosoimidazoles and nitroimidazoles causing 50% reduction in colony formation from plated CHO cells*

Compound	Concentration for 50% decrease in plating efficiency (μM)
Metronidazole	8000
5-NO_2	60
4-NO_2	>1000
5-NO	0.1
4/5-NO	3
4-NO	0.8

* Cells were treated for 10 days with the compounds indicated, and colony formation was monitored as described in Fig. 1 and Experimental Procedure.

Under the normal aerobic growth conditions of the experiments described in Fig. 1 and Table 1, the nitroimidazoles are merely inhibitory, not cytotoxic [8, 9]. Nevertheless, higher concentrations of such drugs as metronidazole are slightly cytotoxic for CHO cells under anaerobic conditions [8]. It is unclear, however, whether such cytotoxicity is a manifestation merely of the limited growth and survival of CHO cells under anaerobic conditions [8] or relates to the bactericidal activity of the nitroimidazoles, which occurs at lower concentrations of nitroimidazoles and only under anaerobic conditions [4].

Unlike the nitroimidazoles, the enhanced bactericidal activity of nitrosoimidazoles is expressed essentially equally under both aerobic and anaerobic conditions, apparently because the nitrosoimidazoles are more proximate to a common reactive species [4]. Tissue culture susceptibility to a nitrosoimidazole may therefore demonstrate the effect of a nitroimidazole's active species under conditions that are normal for mammalian cells. The nitrosoimidazoles, however, are labile compounds. The half-life of 5-NO, as estimated from the disappearance of the characteristic absorption at 366 nm, is 42 ± 3 min in the growth medium of CHO cells at 37°; such decomposition is accompanied by a complete loss of biological activity [4]. In contrast, 5-NO_2 remains in the growth medium throughout the 10 days of incubation. Figure 1, therefore, portrays the short-term toxicity of 5-NO, but the long-term inhibition of 5-NO_2.

To compare the short-term toxicity of 5-NO_2 with that of 5-NO, CHO cells were treated for 2 hr with 1 mM 5-NO_2 (essentially its limit of solubility), as in the experiments of Fig. 1. The medium was then removed, the adherent cells were washed three times with fresh medium, and the plates were then incubated for 10 days in 5 ml of medium that contained only 1% DMSO. Such treatment with 5-NO_2, which simulates the length of exposure that the cells receive with 5-NO, was found to have no effect on colony formation, suggesting that 5-NO may more realistically be considered to be at least 10,000 times more toxic than 5-NO_2 for CHO cells.

To investigate the relative mutagenicity of the nitroimidazoles and their related nitrosoimidazoles, tests were carried out with the Ames *Salmonella typhimurium* histidine auxotroph strain TA100 [13], for which many nitroimidazoles, including metronidazole, are known to be mutagenic [14, 15]. Such studies (Table 2) indicate that 5-NO_2 is more mutagenic than metronidazole, but that 4-NO_2 exhibits little activity, and the tautomer 4/5-NO_2 shows intermediate activity. The relative bacterial mutagenicities of 5-NO_2 and 4-NO_2 correspond to their relative bactericidal potencies [4] as well as with their relative inhibition of CHO cell growth (Table 1), and is in accord with previous associations made between one-electron reduction potential and mutagenicity [16], cytotoxicity [17], and other properties [18]. Despite the complete decomposition of the nitro-

Table 2. Revertant response of *S. typhimurium* TA100 to various nitroimidazoles and nitrosoimidazoles

Compound	Concn* (μM)	Revertants/plate Expt. A	Revertants/plate Expt. B	Average relative activity†
None (5% DMSO)	—	147, 141, 101	117, 130, 111	
Metronidazole	300	800, 772	702	6.0
4-NO_2	100	212, 311		2.0
4/5-NO_2	10	145, 150		1.1
	100	400, 358		2.9
	1000	⩾1000		>10
5-NO_2	2.5	442, 345		3.0
	5	693, 772		5.6
	10	⩾1000		>10
	100	‡		‡
4-NO	0.01		110, 99, 122	0.9
	0.02	138, 181		1.2
	0.1		188, 207, 177	1.6
	1		1023, 921, 1135	8.6
4/5-NO	0.01		66, 96, 104	0.7
	0.1		132, 133, 131	1.1
	1		350, 312, 297	2.7
	10		‡	‡
5-NO	0.0025	148, 244		1.5
	0.005	190, 307		1.9
	0.01	484, 507	464, 500	3.9
	0.02	632, 900		5.9
	0.1	‡		‡

* The compound (in DMSO solution) was added to achieve the concentration indicated in the top agar and a final DMSO concentration of 5%.

† Expressed as the ratio of the number of revertants/plate in the presence of the test compound to that of the average revertants/plate for the corresponding control.

‡ Concentration was bactericidal.

soimidazoles within minutes in bacterial cultures [4], we found that 5-NO is at least two orders of magnitude more mutagenic than 5-NO_2. That 4/5-NO and 4-NO were also quite mutagenic, whereas 4/5-NO_2 and 4-NO_2 were much less active, lends further support to the view that the ease of nitro group reduction is the main determinant of nitroimidazole activity.

The greater biological activity of a nitrosoimidazole compared to that of the nitroimidazole from which it is formally derived can now be generalized to include cytotoxicity and bacterial mutagenesis, as well as bactericidal activity. Not only were the three nitrosoimidazoles more potent than their corresponding nitroimidazoles, but they displayed their biological activities under aerobic conditions. Such findings indicate that the nitrosoimidazole may either be a biologically active species derived from the nitroimidazole or that the nitrosoimidazole is readily converted to such an active species. Since the nitrosoimidazoles appear just as toxic for mammalian cells as they are for nitroimidazole-susceptible bacteria, the data of this paper support previous studies indicating that mammalian cells are simply not able to reduce 5-nitroimidazoles [12] sufficiently to produce toxicologically significant amounts of the activated intermediate(s) in the presence of normal amounts of oxygen.

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