Oxygen Transfer from the Nitro Group of a Nitroaromatic Radiosensitizer to a DNA Sugar Damage Product†

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ABSTRACT: Mechanisms based on one-electron oxidation appear incomplete in explaining cellular radiosensitization by nitroaromatic compounds such as misonidazole. Evidence is presented for a novel mechanism that may be involved in enhancing DNA strand breakage due to a variety of agents, including ionizing radiation, that generate carbon-centered radicals on DNA deoxyribose. Under anaerobic conditions the carbon-centered radical generated selectively at C-5′ of deoxyribose of thymidylate residues in DNA by the antitumor antibiotic neocarzinostatin reacts with misonidazole to produce a DNA damage product in the form of 3′-(formyl phosphate)-ended DNA. In an ^{18}O-transfer experiment we find that the carbonyl oxygen of the activated formyl moiety (trapped as formyl-Tris) is derived from the nitro group oxygen of misonidazole. This result strongly supports a mechanism in which a nitroxide radical adduct, formed by the addition of misonidazole to the radical at C-5′ of deoxyribose, cleaves between the N and O so as to form an oxy radical precursor of the formyl moiety and a two-electron reduction species of misonidazole.

Much attention has been devoted to the development of nitroaromatic ("electron-affinic") compounds as sensitizers of radioresistant hypoxic tumor cells to the therapeutic effects of ionizing radiation. Despite considerable study, the underlying molecular mechanisms of the radiosensitization process remain poorly understood (Wardman, 1987). This undertaking is made especially difficult by the fact that ionizing radiation generates a wide variety of carbon-centered radical species on the various carbon atoms of both the bases and the deoxyribose components of DNA, the main target for its cell killing action (von Sonntag, 1987).

It has been generally believed that nitroaromatic compounds act as radiosensitizers primarily by causing one-electron oxidation of DNA base and/or sugar radicals (Wardman, 1987). The molecular nature of this reaction has been studied in greatest detail in model systems involving the oxidation of pyrimidine base radicals generated by ionizing radiation. A nitroxide radical adduct intermediate is formed between the sensitizer and the radical center on the base that then cleaves spontaneously between the oxygen of the nitro group and the pyrimidine carbon, by means of an OH^--catalyzed heterolysis, so as to produce an oxidized base, such as thymine glycol, and the reduced nitro radical anion of the sensitizer (Steenken & Jagannadham, 1985). Although the yield of thymine glycol increases in the presence of nitroaromatic sensitizers in irradiated solutions (Nishimoto et al., 1983), doubt has been cast that this is an important mechanism of cellular sensitization (von Sonntag, 1987; Remsen, 1985). Further, while nitroaromatic compounds have been found to sensitize phosphate elimination from 5′-nucleotides (Raleigh et al., 1973a,b), neither the sugar damage products nor the intermediates in the reaction have been identified.

An opportunity to explore the mechanism of action of radiosensitizers under more readily analyzable conditions has evolved from our studies, showing that the radiomimetic antitumor antibiotic neocarzinostatin (NCS), as a targeted diradical, selectively generates carbon-centered radicals at C-5′ (and to a lesser extent at C-1′ in certain sequences) of deoxyribose of mainly thymidylate residues in DNA (Kappen & Goldberg, 1985; Goldberg, 1987; Myers, 1987; Chin et al., 1988) and that nitroaromatic radiation sensitizers can substiture for oxygen in the ensuing DNA strand breakage (Kappen & Goldberg, 1984). This system has enabled us to identify the main sugar damage product and to show that the nitroaromatic compound misonidazole reacts directly with the nascent DNA damage (Chin et al., 1987). We now show that the carbonyl oxygen of the DNA sugar damage product (formate) derived from C-5′ comes from the nitro group of misonidazole by a novel mechanism.

Materials and Methods

Synthesis of ^{16}O- and ^{18}O-labeled misonidazole was as reported (Yang & Goldberg, 1989). FAB/MS analysis of synthetic ^{18}O-labeled misonidazole gave absolute peak intensities for the $^{18}O,^{18}O$ peak at m/z 206, 52.3%, the $^{18}O,^{16}O$ peak at m/z 204, 35.4%, and the $^{16}O,^{16}O$ peak at m/z 202, 12.3%. Formyl-Tris was prepared by reacting Tris [tris(hydroxymethyl)aminomethane] with acetic formic anhydride. Its structure was confirmed by ^1H NMR and high-resolution mass spectrometry. It was found that the carbonyl oxygen of formyl-Tris does not exchange with H_2O. Preparation of the NCS chromophore from the native drug was as previously described (Kappen & Goldberg, 1985).

Anaerobic DNA damage reactions (Kappen & Goldberg, 1985) (room temperature, 1 h; final pH 8.0) contained 10 mM sodium citrate (pH 4.0), 1 mM diethylenetriaminepentaacetic acid, 47.5 mM Tris-HCl (pH 9), 1 mM glutathione, 500 μM sonicated calf thymus DNA, 50 μM NCS chromophore, and 10 mM misonidazole having the ^{16}O or ^{18}O label in its nitro group. After removal of DNA by passage through a Sep-Pak C_{18} cartridge (Waters Associates), separation of the product (retention time 5–6 min) was achieved by HPLC on a Waters C_{18} column (3.9 mm × 30 cm), eluted with ammonium acetate (pH 5.4). The product was freed of salts by ion-exchange column chromatography on DEAE-Sephadex A25 using am-

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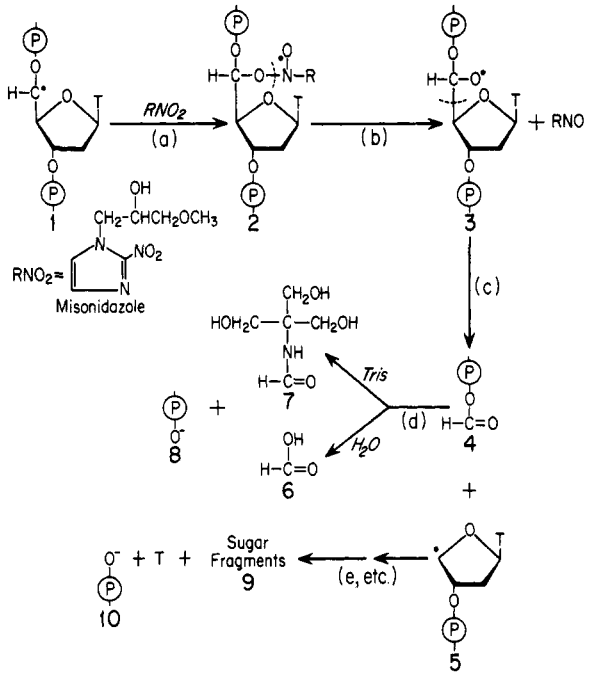
Scheme I: Proposed Mechanism of Involvement of Misonidazole in the DNA Sugar Damage Reaction



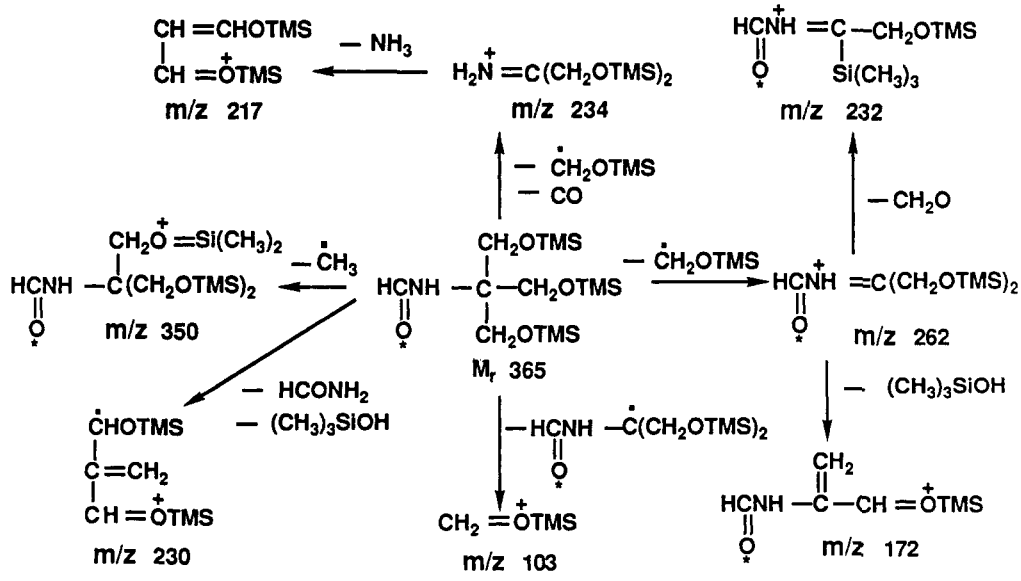
monium acetate (pH 8.0) followed by CM-Sephadex C25 in ammonium acetate (pH 6.5). A trace amount of ^{3}H-labeled formyl-Tris (isolated from a similar drug reaction containing [5′-^{3}H]thymidine-labeled DNA and misonidazole) added to the reaction served as a marker in the purification steps. Product recoveries from both reactions were identical (74%). TMS derivatives prepared by heating the product (80 °C, 45 min) in a 1:1 mixture of *N,O*-bis(trimethylsilyl)trifluoroacetamide/1% trimethylchlorosilane and CH_3CN were introduced through an Alltech 5% SE-30 column on a gradient of 80–180° C (16 °C/min) with a Hewlett-Packard 5992A GC/MS spectrometer run at 70 eV.

Results and Discussion

The strand break formed under anaerobic conditions by NCS in the presence of misonidazole actually consists of a gap, bounded by phosphates at either end, from which an unmodified base and deoxyribose fragments have been released (Kappen & Goldberg, 1984). Formate, derived from C-5′ of thymidylate, has been identified as a main product of the reaction, and the intermediacy of a labile, reactive 3′-(formyl phosphate)-ended DNA has been proposed in its formation (Chin et al., 1987). The finding that misonidazole reduction in the anaerobic reaction was DNA dependent clearly implicated a nascent form of DNA damage, presumably the carbon-centered radical at C-5′, in the reduction process and suggested that a nitroxide radical adduct at C-5′ was an intermediate in the reaction. In the reaction scheme (Scheme I) proposed to account for these and other findings, thiol-activated NCS abstracts a hydrogen atom from C-5′ to generate a carbon-centered radical at C-5′ (**1**) which forms a labile nitroxide radical adduct (**2**) with misonidazole. The adduct cleaves between the N and O to generate an oxy radical (**3**) on the DNA which undergoes β fragmentation, resulting in cleavage between C-5′ and C-4′ to form 3′-(formyl phosphate)-ended DNA (**4**), and the nitroso reduction product of misonidazole. Whereas heterolytic cleavage between the O and C is a one-electron process, that between the N and O, for which there is chemical precedence (Perkins & Roberts, 1974), is a two-electron process. The reactive form of formate can be trapped by nucleophiles such as Tris or hydroxylamine to form formyl-Tris (**7**) or formylhydroxamic acid, respectively, for purposes of identification and quantitation. The scheme predicts that the carbonyl oxygen of the formate is derived from the nitro group of misonidazole. In order to test this possibility, the NCS-induced DNA damage reaction was performed with the misonidazole labeled with ^{18}O in its nitro group.

Two parallel anaerobic reactions were carried out in the presence of misonidazole, one having the ^{16}O and the other the ^{18}O label in its nitro group. The DNA damage product formyl-Tris was purified, and its trimethylsilyl (TMS) derivative was subjected to gas chromatography/mass spectrometry (GC/MS). The fragmentation pattern obtained for the product from the [^{16}O]misonidazole-containing reaction (Figure 1B) is identical with that for authentic formyl-Tris (Figure 1A) in mass and relative abundance of the fragments.

Scheme II: Proposed Fragmentation of Formyl-Tris To Rationalize the Observed Mass Spectrum



[a] TMS represents $Si(CH_3)_3$. The peaks at *m/z* 73, 75, and 147 are most likely due to $^+Si(CH_3)_3$, $(CH_3)_2Si{=}O^+H$ and $(CH_3)_3SiO^+{=}Si(CH_3)_2$, respectively, as commonly observed in the mass spectrum of TMS derivatives (Diekman et al., 1968). An asterisk denotes the oxygen carrying the ^{18}O label.

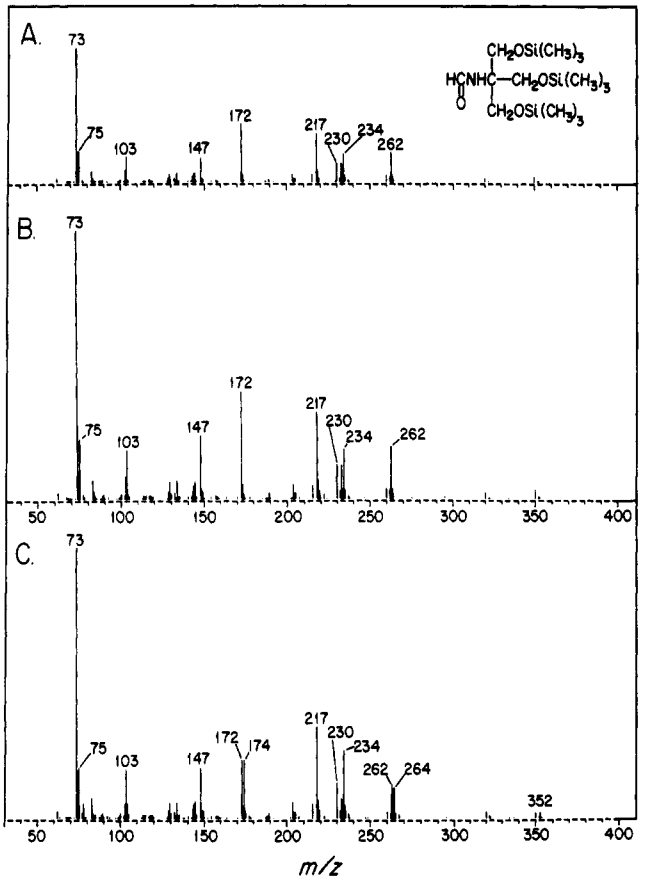


FIGURE 1: DNA damage product analysis by GC/MS. Purification of the DNA damage product was as described under Materials and Methods. The retention time of formyl-Tris was 10.7 min. (A) Authentic formyl-Tris; (B) product from [^{16}O]misonidazole-containing reaction; (C) product from [^{18}O]misonidazole-containing reaction.

The product from the [^{18}O]misonidazole-dependent reaction (Figure 1C) also gives a spectrum similar to those in panels A and B of Figure 1 but with three additional peaks, each representing a fragment with two mass units higher than those corresponding to m/z 350, 262, and 172. This is in accord with the fragmentation mode proposed in Scheme II, where these fragments retain the carbonyl oxygen of the formyl moiety. Further, the intensities of the peaks at m/z 350, 262, and 172 are reduced in Figure 1C by about 50% concomitant with the appearance, at nearly equal intensities, of the peaks at m/z 352, 264, and 174. In addition, the peak at m/z 232 also retains the carbonyl group, as evidenced by the decrease in its intensity with a corresponding increase in that of the peak at m/z 234. As expected, there are no ^{18}O counterpart peaks in Figure 1C for any of the other fragments that have lost the carbonyl oxygen. The ^{16}O to ^{18}O ratio of about 1:1 in the carbonyl-containing fragments is compatible with the ^{18}O content of misonidazole (70%). These results indicate transfer of ^{18}O from the nitro group of misonidazole to the site of DNA damage, supporting a mechanism in which the nitroxide radical adduct undergoes cleavage between the N and O positions.

In mammalian cells irradiated with X-rays nitroaromatic sensitizers increase the yield of strand breaks, and this correlates well with cell killing (Dugle et al., 1972). The ability of misonidazole to increase DNA strand breakage by a radiomimetic drug, which produces carbon-centered radicals at a specific location on the deoxyribose in DNA, suggests that a similar mechanism, involving oxygen transfer from misonidazole to any of the several sites of nascent DNA damage, may occur in the radiosensitization reaction. Although formate from deoxyribose has been identified as a DNA damage product due to ionizing radiation (von Sonntag, 1987), to our knowledge, there are no reports of the effect of radiosensitizers on its formation.

Although our studies do not provide unequivocable proof for the existence of a nitroxide radical adduct intermediate, the data are most easily explained by its participation in the reaction. In fact, an analogous intermediate has been proposed to form in the aerobic NCS-induced DNA strand breakage reaction where dioxygen, rather than acting as a one-electron acceptor, adds to the carbon-centered radical at C-5′ (Chin et al., 1984) to form a peroxy radical intermediate that eventuates in a strand break with a nucleoside 5′-aldehyde at the 5′ end (Kappen & Goldberg, 1985). It is intriguing to speculate as to why nitroxide radical adducts on pyrimidine bases and those on sugars in DNA appear to cleave in different ways. The interesting possibility exists that the DNA and/or the DNA-bound NCS participate in determining the preferred route of cleavage of the nitroxide radical adduct.

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