Brief Articles

Reductive Heme-Dependent Activation of the *N*-Oxide Prodrug AQ4N by Nitric Oxide Synthase

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Anaerobic reduction of anticancer prodrugs is a promising route to achieve targeting and selectivity in anticancer drug design. Most reductive prodrug activations involve simple electron transfer from a flavoprotein and are not amenable to specific targeting. Here, we report that the *N*-oxide AQ4N is reduced by a nitric oxide synthase. This reduction involves interaction with the heme iron atom in the active site and is thus subject to specific protein constraints.

Introduction

One of the crucial problems in cancer chemotherapy is specific targeting of the therapeutic agent to tumor rather than normal cells, as anticancer agents generally interfere equally well with the intended target in both types of cells. One of the features of cancer cells in solid tumors that has been exploited for this purpose is the relatively low oxygen tension inside the tumor due to poor vascularization of the tissue.[1] This hypoxia enables the reductive activation of anticancer prodrugs that cannot be similarly activated in normoxic tissues due to competing oxidative pathways. Among the drugs and potential anticancer agents that are activated in this manner are tirapazamine,[2] mitomycin,[3] **1** (NLCQ-1[a]),[4] **2** (KS119),[5] and **3** (AQ4N).[6]

The reductive activation of most prodrugs in hypoxic tissues is generally mediated by electron transfer from the flavoprotein NADPH-cytochrome P450 reductase.[4,7] However, it has more recently been shown that the flavoprotein domain of nitric oxide synthases (NOS) lacking the heme domain can also provide electrons for activation of some of these agents, including tirapazamine and doxorubicin.[8] The NOS are of interest in this context because their concentration is often elevated in tumor tissues.[9] They may therefore make a significant contribution to the activation of prodrugs in hypoxic tumor cells and could in principle be exploited as specific targets for such activation. This would be desirable, as it would impose an additional constraint favoring tumor-specific activation of the agent. Unfortunately, electron transfer from both the NADPH-cytochrome P450 reductase and the NOS flavoprotein domain is relatively nonspecific so that targeting of prodrugs for specific activation by flavoprotein electron donors may not be feasible.

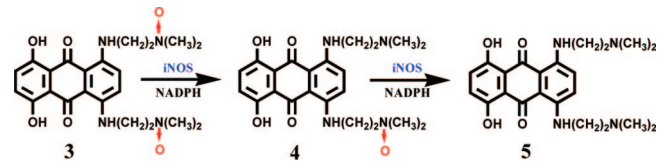

Figure 1

3 is a bis-*N*-oxide anticancer prodrug that, when reduced to the bis-amine, is a potent inhibitor of topoisomerase II.[10] The prodrug itself has virtually no activity against this target enzyme and, in the absence of reduction, is relatively nontoxic. **3** differs from the other agents because it is not activated by simple electron transfer from P450 reductase. However, it is activated by cytochrome P450 enzymes, with the highest activity reported for human CYP3A4.[11] It is likely that this involves direct interaction of **3** with the active site iron atom, although the only actual evidence for this is that carbon monoxide inhibits the bioactivation reaction.[12] In view of the elevation of the NOS enzymes in tumor cells, we have investigated whether the nitric oxide synthases can activate **3**, have examined the nature of the reaction, and have compared the activity of human hepatic inducible nitric oxide synthase (iNOS) with that of CYP3A4. The results open the exciting possibility that anaerobically activated prodrugs can be selectively targeted for activation by NOS enzymes.

Discussion

Incubation of iNOS and NADPH with **3** under anaerobic conditions (glovebox) led to sequential reduction of **3** to the mono-*N*-oxide **4** (AQ4M) and then to the active drug **5** (AQ4) (Figure 1). The products were identified by HPLC comparison with authentic standards. A time course of the reaction showed that **3** was first converted to **4**, which accumulated in the medium and was subsequently converted to **5** (Figure 2). No product formation was observed in the absence of NADPH or NOS, under aerobic conditions, or if the NOS enzyme was replaced by purified NADPH-cytochrome P450 reductase. Beyond their sequence homology, CYP reductase and the iNOS reductase domain are comparable in terms of activities with regard to reduction of small molecule electron acceptors such as ferricyanide and of protein electron acceptors such as cytochrome *c*. Thus, these results clearly show that the reaction requires

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[a] Abbreviations: AQ4N, 1,4-bis-{[2-(dimethylamino-*N*-oxide)ethyl]amino}-5,8-dihydroxyanthracene-9,10-dione; AQ4M, 1-{[2-(dimethylamino-*N*-oxide)ethyl]amino}-4-{[2-(dimethylamino)ethyl]amino}-5,8-dihydroxyanthracene-9,10-dione; AQ4, 1,4-bis{[2-(dimethylamino)ethyl]amino}-5,8-dihydroxyanthracene-9,10-dione; CYP, cytochrome P450; HPLC, high-performance liquid chromatography; KS119, 1,2-bis(methylsulfonyl)-1-(2-chloroethyl)-2-[[1-(4-nitrophenyl)ethoxy]carbonyl]hydrazine; iNOS, inducible isoform of nitric oxide synthase (NOS-2); nNOS, neuronal isoform of NOS (NOS-1); NADPH, nicotinamide adenine dinucleotide phosphate (reduced form); NLCQ-1, 4-[3-(2-nitro-1-imidazolyl)propylamino]-7-chloroquinoline hydrochloride.

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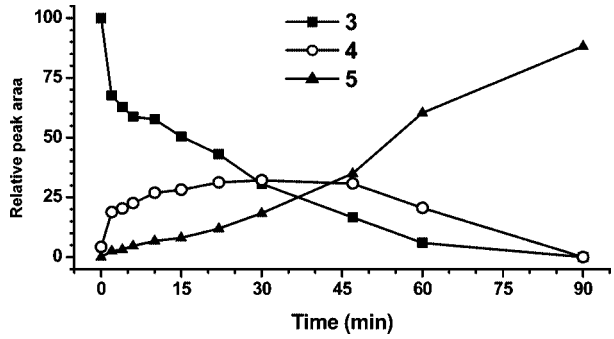

Figure 2. Time course of **3** reduction by iNOS.

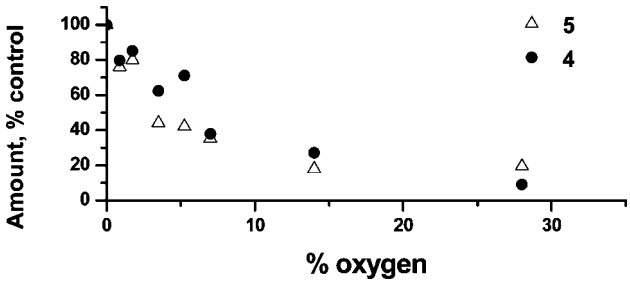

Figure 3. Dependence of **3** and **4** reduction on oxygen concentration. Different ratios of anaerobic and air-saturated solutions were mixed.[13]

the NOS heme domain and is not simply the result of electron transfer from the flavoprotein domain.

In order to evaluate the conditions required for **3** reduction, we have determined the K_m value for the iNOS reaction. The K_m for the conversion of **3** to **4** is 130 ± 30 μM, but the K_m for the conversion of **4** to **5** could not be determined. A plot of product formation versus **4** (substrate) concentration did not reach a plateau at nominal concentrations below 500 μM. Although **3** has two *N*-oxide moieties, it bears no net formal charge and is much less polar than **4**, which has a trialkylamine substituent that is protonated at pH 7. It is possible that only the unprotonated **3** is bound in the NOS active site and thus that only a small fraction of the added **4** is available to the enzyme. Nevertheless, as the time-course studies demonstrate, the NOS enzymes can fully reduce **3** to the anticancer-active agent **5**.

We have also determined the oxygen sensitivity of the reductive reaction by measuring the rate of **4** or **5** formation as a function of increasing oxygen concentration (Figure 3). As shown in the figure, the highest product formation is observed under fully anaerobic conditions, but half-maximal product formation occurs at 4−5% oxygen.

A comparison of the activities of iNOS and CYP3A4 is informative, as it shows that iNOS, with a rate of ~5 min^{-1}, has a 10-fold higher activity than CYP3A4, for which the activity is ~0.5 min^{-1} in assays with the reconstituted purified enzyme and CYP3A4 insect cell microsomes (BD Biosciences). Comparison with other NOS isoforms demonstrates a correlation between native NOS activity and reduction of **3**. The neuronal isoform (nNOS) exhibited an observed activity of ~2 min^{-1}, and the level of endothelial NOS (eNOS) activity also closely mirrored the lower native activity observed for eNOS nitric oxide production from arginine compared to those of iNOS and nNOS; that is, turnover of **3** by eNOS was approximately 10-fold less than by iNOS, 0.5 min^{-1}.

It is of note that heme and NADPH alone can reduce **3** under anaerobic conditions, including under quench conditions. However, since the background reaction of iNOS under quench conditions was negligible, the observed products were formed by iNOS rather than free heme.

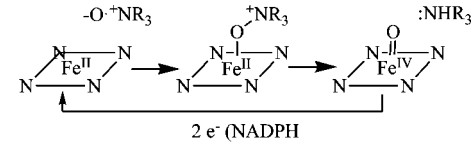

Figure 4. Possible mechanism of *N*-oxide reduction by P450 and NOS involving direct *N*-oxide coordination to ferrous iron.

Arginine at 1 mM (apparent K_m for iNOS of 2.8 μM[14]) did not inhibit anaerobic reduction of **3**. Although the K_m for **3** is fairly high at 130 μM, reduction of an *N*-oxide via heme coordination under anaerobic conditions may be noncompetitive with arginine binding. In contrast, evidence in support of the reaction of **3** with the heme iron is provided by the 50% inhibition of **3** reduction to **5** by 50 μM econazole or clotrimazole. Both of these azole agents are known to coordinate to the NOS heme iron atom.

If reduction of **3** involves direct oxygen transfer from the *N*-oxide to the heme of iNOS, as indicated by our results, the *N*-oxide would be expected to coordinate to the heme iron atom at some point in the reductive mechanism. However, we have not detected direct heme coordination by UV−vis spectroscopy in a titration of ferric iNOS with up to 450 μM **3** or **4**. It is therefore likely that coordination only occurs with the ferrous iron, to which the oxygen atom is then immediately transferred (Figure 4). The resulting hypervalent iron would then be reduced by further electron transfer from the NOS reductase domain.

Conclusion

Targeting prodrugs such as **3** for reductive activation in relatively anaerobic tissues by NOS enzymes may provide a new dimension that is relevant to, for example, agents directed at the treatment of solid tumors.

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Supporting Information Available: Experimental methods and azole inhibition of **3** reduction. This material is available free of charge via the Internet at http://pubs.acs.org.

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