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Alkylation of human albumin by the antimalarial artemisinin

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Abstract—The interaction between artemisinin and human serum was studied *in vitro* using [^{3}H]-dihydroartemisinin and [^{14}C]artemisinin. Approximately 20% of added drug was covalently bound to albumin in 24 hr. The results of electrospray ionization mass spectra showed that albumin had an M_r value of 66,745 ± 35 and the drug-bound albumin had an M_r of 67,223 ± 34. The binding was blocked 15 and 58% by iodoacetamide (IA) and *N*-ethylmaleimide, respectively, and 80% by the combination of IA and succinic anhydride. Hemin and Fe^{2+} increased the binding by 40 and 10%, respectively, whereas deferoxamine inhibited the binding by 10%. Therefore, we conclude that the binding between artemisinin and albumin probably involves thiol and amino groups via both iron-dependent and -independent reactions.

Artemisinin (qinghaosu) and its derivatives, such as dihydroartemisinin, artesunate, and artemether, are among the most promising new antimalarials under development. Although administered to over 2 million people in China and Southeast Asia, no clinical resistance or serious adverse effects have been seen [1]. Several artemisinin derivatives are currently undergoing Phase I and Phase II studies [1].

Iron catalyzes the activation of artemisinin to produce free radicals [2] and reactive aldehydes [3], and these reactions appear to be a critical step in the mechanism of the action of the drug [4–6]. To determine whether similar reactions can occur with host proteins, we have now studied the reactions of artemisinin with human serum albumin. The occurrence of reactions between the drug and host proteins would be important for two reasons. First, it may explain the dose-related adverse effects seen in animal studies [7,8]. Second, it may explain why the drug appears to disappear from blood samples upon storage [9–11].

Materials and Methods

[^{3}H]Dihydroartemisinin (15 Ci/mmol) was the gift of Dr. Achille Benakis, University of Geneva, Switzerland. [^{14}C]Artemisinin (45.4 mCi/mmol) was a gift from the Division of Experimental Therapeutics, Walter Reed Army Institute of Research, Washington, DC. Human serum was purchased from the Interstate Blood Bank, Memphis, TN. All other reagents were purchased from the Sigma Chemical Co. (St. Louis, MO).

Protection of thiol and amino groups. A mixture of human serum and Tris–HCl buffer (250 mM, pH 8.0) (1:4 v/v) was incubated for 20 min at room temperature in the presence of 30 mM iodoacetamide (IA*), 10 mM *N*-ethylmaleimide (NEM), or no addition. Two hundred microliters of each solution were then diluted with 300 μL of elution buffer (250 mM Tris–HCl/1 mM EDTA, pH 8.0), and the protein was separated from free IA and NEM by gel filtration on an NAP-5 column (Pharmacia, Uppsala, Sweden). The thiol content of the eluted serum proteins was assayed by measuring the difference in absorption at 412 nm before and 15 min after adding 150 μM 5,5′-dithionitrobenzoic acid (DTNB) [12, 13]. An aliquot of the IA-treated serum after gel filtration was then incubated with succinic anhydride (SA; 30 mg/mL), and the protein was freed of SA as above using an NAP-5 column. As a control for the IA/SA-treated serum, untreated serum was subjected to two gel filtration steps.

Binding of drug to albumin. To each serum sample, (diluted with Tris–HCl as above) 5.5 μCi/mL of [^{3}H]-dihydroartemisinin was added and incubated at 37°. At times 0, 2, 6 and 24 hr, 200 μL of each mixture were removed and stored in −20°. Three hundred microliters of elution buffer were then added to each thawed fraction, and the protein and unbound drug were applied to an NAP-5 column. The proteins were eluted in a single 0.9-mL fraction containing 99% of the total protein as determined by the method of Bradford [14] and counted using Scintivers BD (Fisher Scientific, Fair Lawn, NJ) on a Beckman LS 7000 Scintillation Counter.

Gel electrophoresis and autoradiography. Sodium dodecyl sulfate–polyacrylamide gel (10%, w/v) electrophoresis (SDS–PAGE) [15] was performed after the material to be analyzed was incubated for 1 hr at room temperature in the presence of 5% (v/v) mercaptoethanol, 8 M urea and no addition (control). After the gels had been stained and destained, they were impregnated with EN^{3}HANCE autoradiography enhancer solution (E.I. du Pont de Nemours & Co., NEN Products, Boston, MA) for 1 hr under gentle agitation. Following impregnation, the fluorescent material inside the gel was precipitated by an excess of cold water for 0.5 hr. The gels were dried on a Bio-Rad gel drier, model 583 (Bio-Rad Laboratories, Melville, NY). Finally, the gels were exposed on Kodak X-Omat AR films (Eastman Kodak Co., Rochester, NY) at −80° for 1 week.

Electrospray ionization–mass spectra (ESI–MS). Human serum albumin (0.5 mg) was incubated with and without 1 mM artemisinin at 37° for 48 hr in Tris–HCl buffer (150 mM, pH 8.6) containing 10% (v/v) ethanol. The mixtures were then transferred to Centricon-30 microconcentrators (Amicon Division, W. R. Grace & Co., Beverly, MA), and centrifuged and resuspended four times, during which time more than 99% of the free artemisinin was removed. The final concentrate was collected and diluted to a protein concentration of 1 mg/mL. ESI–MS were obtained using a Vestec electrospray source and a model 201 single quadrupole mass spectrometer (Vestec Corp., Houston, TX) fitted with a 2000 *m/z* range [16, 17]. Samples were delivered to the source in a 10-μL injection loop at 5 μL/min in 4% acetic acid/50% acetonitrile.

Iron-dependence study. To determine the effects of various reagents on the reaction, human serum was mixed with 4 vol. of Tris buffer and incubated with 5.5 μCi/mL of [^{3}H]dihydroartemisinin for 24 hr at 37° in the presence of $FeSO_4$ (100 μM), deferoxamine (DFO, 1 mM), hemin (100 μM, diluted from a fresh stock solution prepared as in Ref. 2), and reduced glutathione (100 μM). The incubation mixture was then subjected to gel filtration as described above. Columns were run in triplicate.

Controls. To show that albumin labeling was not due to

* Abbreviations: IA, iodoacetamide; NEM, *N*-ethylmaleimide; SA, succinic anhydride; DFO, deferoxamine; DTNB, 5,5′-dithionitrobenzoic acid; and ESI–MS, electrospray ionization–mass spectra.

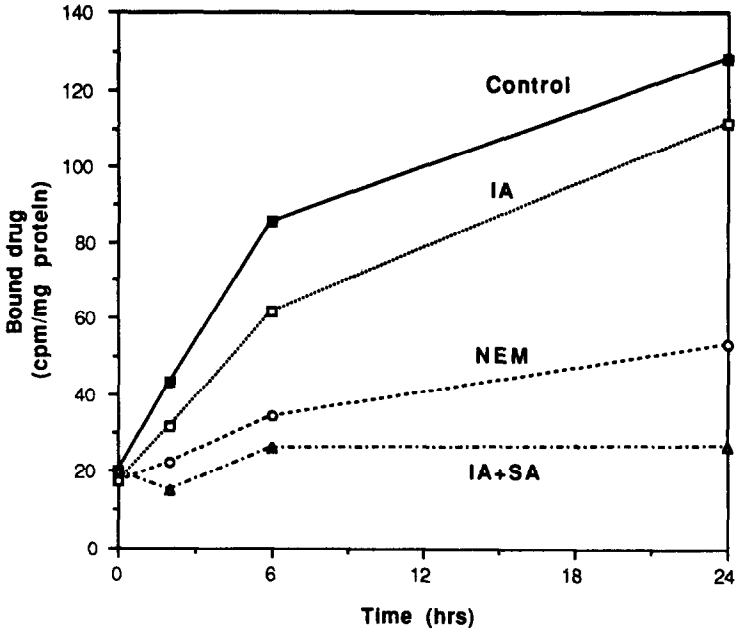

Fig. 1. Effects of IA-, NEM- and IA + SA-pretreatment on [^{3}H]dihydroartemisinin binding to serum. Key: control (—■—); IA pretreated (···□···); NEM pretreated (---○---); and IA and SA pretreated (-·-·△-·-·).

tritium exchange, human serum (mixed with 4 vol. of Tris buffer) was incubated in the presence of 0.25 μCi/mL of [^{14}C]artemisinin for 24 hr at 37°. As control for buffer, human serum was incubated with 5.5 μCi/mL of [^{3}H]-dihydroartemisinin as described above, but mixed with 4 vol. of Dulbecco's phosphate-buffered saline, pH 7.4.

Results

[^{3}H]Dihydroartemisinin bound to serum in a time-dependent manner (Fig. 1). After 24 hr, 17–20% of the added drug eluted in the high molecular weight fraction from the NAP-5 columns. To determine whether the binding required the presence of free thiols, serum was pretreated with two thiol blocking reagents, IA and NEM. Fifteen and 58% less drug bound to IA- or NEM-pretreated serum, respectively. Both treatments blocked >90% of the measurable thiols. Unlike IA, NEM is also capable of reacting with free amino groups [18], and the fact that NEM was more protective than IA suggests that nucleophilic amino groups are also involved. To test this, serum was

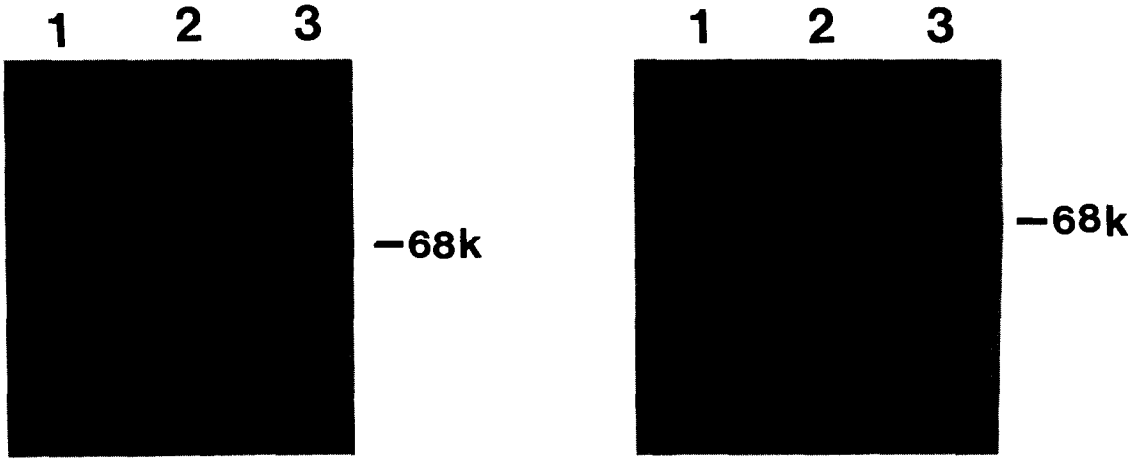

Fig. 2. SDS–PAGE autoradiograms of serum incubated with [^{3}H]dihydroartemisinin in the absence (left panel) or presence (right panel) of hemin. Incubation conditions are described in Materials and Methods. Samples were incubated for 1 hr at room temperature with no addition (lane 1), 5% mercaptoethanol (lane 2), and 8 M urea (lane 3) before electrophoresis.

Table 1. Effects of iron and iron chelators on the reaction between serum and [^{3}H]dihydroartemisinin

Reaction	Drug bound (cpm/mg protein)	% Control	P value
Serum + [^{3}H]artemisinin	63,660 ± 1310	100.0	
+ DFO (1 mM)	57,200 ± 970	89.9	0.0023
+ $FeSO_4$ (100 μM)	70,530 ± 830	110.8	0.0015
+ Hemin (100 μM)	86,230 ± 340	135.5	0.0001
+ GSH (100 μM)	63,500 ± 990	99.8	0.8748

Values are means ± SD, N = 3.

pretreated with both IA and SA to block both thiols and free amines [18]. After this treatment, almost 80% of the binding was blocked.

The labeled drug bound almost exclusively to albumin (Fig. 2A). There were no other detectable bands on the autoradiogram. The fact that the radioactivity remained associated with protein during electrophoresis suggests that there was either covalent binding or very tight non-covalent binding. There was no diminution in the intensity of the albumin band when the sample was treated with mercaptoethanol or urea prior to electrophoresis, suggesting that the binding was indeed covalent.

As further confirmation that the bond was covalent, ESI–MS were taken. The control sample (no addition of artemisinin) has an M_r of 66,745 ± 35 and the artemisinin bound albumin has an M_r of 67,223 ± 34. The difference in molecular weights was 478, suggesting that more than one drug molecule (molecular weight = 282) reacted per albumin molecule under the conditions used.

The observed reaction between drug and albumin was not due to tritium exchange. As with the [^{3}H]dihydroartemisinin, 21% of the [^{14}C]artemisinin reacted with albumin and eluted from the gel filtration column (not shown). Furthermore, 20% of [^{3}H]dihydroartemisinin reacted with albumin when Dulbecco's phosphate-buffered saline was used instead of Tris buffer (not shown), indicating that the reaction was not buffer dependent.

The reaction between [^{3}H]dihydroartemisinin and serum was only somewhat iron dependent (Table 1). There was a small but significant increase in the bound drug due to the presence of Fe^{2+} (11%, P = 0.0015), and a slight but significant inhibitory effect (10%) by the iron chelator DFO (P = 0.0023). Hemin had a larger stimulatory effect (35%, P = 0.0001). This latter increase could be due to heme alkylation, since hemin alone aggregates and elutes in the high molecular weight fraction from gel exclusion columns [13]. However, the increased association between the drug and albumin in the presence of hemin was not due to the alkylation of hemin by drug, since the NAP-5 eluate contained only labeled albumin as determined by autoradiography of the SDS–PAGE gels (Fig. 2B). The reaction was unaffected by the addition of reduced glutathione, suggesting that the reaction cannot be competitively inhibited by the addition of a nucleophilic reagent.

Discussion

In the present study, we have shown that artemisinin reacted covalently with human serum albumin, alkylating both thiol and amino moieties via iron-dependent and -independent reactions. These findings have implications for the understanding of the mechanism of action of the drug and its pharmacokinetics.

Artemisinin clearly reacted with albumin via several routes. However, our data argue that the most important reaction was iron independent because the effects of exogenous iron and chelator were relatively small. Furthermore, the fact that IA, which blocks SH groups, had a much smaller inhibitory effect than IA + SA, or NEM, which block both SH and amino groups, suggests that the reaction with amino groups was more important than the reaction with thiols.

The reaction between artemisinin and protein described here is important because it may help in understanding the mode of action of the drug. Previously, artemisinin has been shown to alkylate intraparasitic heme [13]. The data presented here suggest that the drug may be capable of alkylating parasitic proteins as well.

The alkylation of protein by artemisinin has implications for our understanding of the drug's pharmacology. The reactions described here may mediate some of the reported adverse effects seen at high doses [7, 8], and explain why the drug is rapidly eliminated in the circulation [19, 20], or from stored blood samples [9–11]. Further studies on the reactions of artemisinin with host proteins are in progress.

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