To our surprise, the oxidized form of lipoate was equally effective in scavenging HOCl as dihydrolipoate (Fig. 4). In contrast to oxidized glutathione, oxidized lipoate appears to compete with the methionine residue of α_1-AP in the reaction with HOCl.

Discussion

Data from literature and also from this study indicate that several thiol-containing compounds, like cysteamine [7], *N*-acetylcysteine [8, this study] and GSH [this study] are effective scavengers of HOCl. Therefore, it did not come as a surprise that reduce lipoate, a molecule that contains two thiol groups, is also a potent scavenger of HOCl. Moreover, it was found that GSMe was a good scavenger. That a compound with a methylated thiol group can act as a scavenger of HOCl is also not surprising, because it is known that a methionine residue (which also contains a methylated thiol group) in α_1-AP is attacked by HOCl on its sulphur atom [3].

In addition, we found that GSSG is only a poor scavenger of HOCl. GSSG reacts with HOCl, but the reaction rate is too slow to provide an efficient protection of the methionine residue of α_1-AP. Unexpectedly, we found that oxidized lipoate is a potent scavenger of HOCl. In the past, the antioxidant activity of lipoate has been ascribed to its reduced form. Dihydrolipoate is able to elevate the concentration of GSH by reducing GSSG [4]. In addition, it has been stated that dihydrolipoate may protect against lipid peroxidation in an interaction with vitamin E [5]. The oxidized form of lipoate has no protective effect on the process of lipid peroxidation [4, 5]. The findings of this study indicate that, with respect to the scavenging of HOCl, oxidized lipoate, as well as reduced lipoate, acts as a potent antioxidant. This is of special importance because lipoate is used therapeutically in its oxidized form.

The most striking result in this study was the difference between GSSG, not a potent scavenger of HOCl, and oxidized lipoate, a very good scavenger of HOCl. The explanation for this difference may be found in the nature of the disulfide bridge in both molecules. Dihydrolipoate contains an intramolecular S—S bridge. Because this bridge is fixed in a 5-membered ring, some ring strain exists. It has been reported that the angle between both sulphur molecules is energetically not optimal [9]. In GSSG no ring strain exists because it is a flexible molecule. The difference of the S—S bridges probably explains the difference between GSSG and oxidized lipoate in their ability to scavenge HOCl. By scavenging HOCl, oxidized lipoate is probably converted into a sulphoxide. The sulphoxide of dihydrolipoate is also formed in the reaction of dihydrolipoate with hydrogen peroxide [10].

In summary, it was found that reduced GSH, *N*-acetylcysteine and GSMe are potent scavengers of HOCl, while GSSG is not. Surprisingly, not only reduced but also oxidized lipoate is an effective scavenger. The difference in scavenging effect between oxidized GSH and oxidized lipoate is probably caused by the difference in reactivity of the disulfide bridge in both molecules. The present results indicate that lipoate might be effective in the treatment of emphysema caused by the destruction of α_1-antiproteinase.

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Interspecies differences in *in vitro* etoposide plasma protein binding

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Preclinical studies in laboratory animals are used to define initial pharmacologic and toxicologic endpoints of anticancer drugs. Phase I protocols of anticancer drugs initiate doses in humans equal to or less than one-tenth the mouse lethal dose (LD_{10}), and titrate the dose upwards until dose-limiting toxicity is observed. This procedure is based on previous observations that a quantitative relationship exists between toxic doses of anticancer drugs in animals and humans [1]. Despite these similarities, often several dose escalations are required to reach the maximum

tolerated dose (MTD) in humans resulting in considerable delay and expense in the drug development process [2].

Significant variability in the pharmacokinetic parameters of anticancer drugs has been noted between cancer patients and laboratory animals [3]. These differences may contribute to the differences in the MTD of some anticancer agents between humans and animals. Because of these pharmacokinetic differences, investigators have proposed basing dose escalations on preclinical plasma pharmacokinetic data of anticancer agents rather than dose alone [2, 4]. This assumes that toxicity occurs at similar concentrations of an anticancer drug, regardless of animal species. Results from recent studies have shown that the area under the plasma concentration versus time curve (AUC) for anticancer drugs is a more useful parameter than dose alone for escalating doses of anticancer agents in Phase I protocols [2].

Thus far, these studies have all measured total drug concentration which consists of both drug bound to plasma protein and unbound drug. Pharmacologic theory states that the unbound drug is the active species [5]. For drugs extensively bound to plasma proteins, unbound drug concentrations correlate best with indices of pharmacologic effect [6, 7]. Thus, if significant interspecies variability exists in the plasma protein binding of anticancer drugs, comparisons between unbound drug concentrations and toxicity in humans and animals may be more appropriate. Interspecies differences in the serum or plasma protein binding of several drugs have been reported [8–10], including anticancer drugs [11, 12].

Etoposide, a semisynthetic derivative of podophyllotoxin, is currently used in combination with other cytotoxic agents in treating several malignancies including lung, testicular, and ovarian cancers [13]. Studies from our laboratory have indicated etoposide to be approximately 95% bound to human plasma proteins [14]. The objectives of this study were to evaluate the plasma protein binding of etoposide in several animal species, and compare the extent of binding in animals with that observed in human plasma.

Materials and Methods

Drugs. Analytical grade etoposide (purity >99.7%) was provided by the Bristol-Meyers Co., Oncology Division (Syracuse, NY). Tritiated etoposide (sp. act. 1 Ci/mmol) was obtained from Moravek Biochemicals (Brea, CA). The purity of the [^{3}H]etoposide was determined to be >96% by an HPLC method [15].

Animal and human plasma. Blood was collected from the following animal species: sheep, rabbit (New Zealand White), rat (Sprague–Dawley), canine (mongrel), and feline (domestic shorthair). Additionally, blood from three strains of mice (BDF_1, DBA/2, and Swiss–Webster) was collected in tubes containing heparin (10 I.U./mL). All animals were healthy and not receiving any drugs at the time of blood collection. Immediately after collection, the blood was centrifuged (10 min, 400 *g*). The plasma was transferred into tubes, and stored at −20° until protein binding experiments were performed. For determination of etoposide binding to human plasma, pooled, drug-free plasma was obtained. Albumin (BCG method), total protein (Biuret method) and total bilirubin concentrations (Grof method) were determined from an aliquot of each source of plasma by an automated procedure (American Monitor Perspective).

Protein binding methodology. Etoposide fraction unbound (f_u) in plasma was determined by an equilibrium dialysis technique. Plasma from animals and humans was spiked with etoposide to obtain a final concentration of 10 μg/mL (17 μM). Spiked plasma samples were adjusted to pH 7.4 with dilute phosphoric acid, and the volume of acid added was always less than 1% of the final sample volume. Sorensens' phosphate buffer (pH 7.4, 0.167 M) was spiked with a trace amount of [^{3}H]etoposide. The plasma samples (0.5 mL) were dialyzed against an equal volume of Sorensens' phosphate buffer for 6 hr at the corresponding normal body temperature of each animal species (i.e. human 37°, sheep 39.5°, rabbit 39.3°, cat 38.5°, dog 38.9°, rat 37.5°, and mice 37.2°) [16]. Preliminary studies indicated that equilibrium occurred in each plasma source after 6 hr of dialysis at the above-named temperatures. The plasma and buffer solutions were separated by a semipermeable membrane (Spectra-Por No. 2, molecular cutoff 12,000–14,000, Spectrum Medical, Los Angeles, CA). Six cells were dialyzed for each plasma source.

Determination of etoposide f_u and etoposide binding ratio. After the end of dialysis, an aliquot (300 μL) of plasma and of buffer were added to 5 mL of scintillation fluid (Biosafe II, Research Products International Corp., Mount Prospect, IL). Each sample was counted for 10 min, and etoposide f_u was calculated from the ratio of the disintegrations per minute in an aliquot of buffer to the disintegrations per minute in an aliquot of plasma. Correction for quench was by an external standardization method. Corrections for volume shift and radiochemical impurity were made by previously published methods [17, 18]. Etoposide binding ratio was calculated from the following equation:

$$\text{etoposide binding ratio} = f_{\text{bound}}/f_{\text{unbound}} \quad (1)$$

where f_{bound} equals the fraction of etoposide bound to plasma proteins, and f_{unbound} equals the fraction of etoposide unbound in plasma.

Statistical methodology. The mean and standard deviation of etoposide f_u were calculated for each species. Comparisons between the etoposide f_u in each animal species and that in humans were performed by the two-sample *t*-test with an *a priori* level of significance of $P < 0.05$. Bonferonni's correction ($P < 0.00625$) was used to correct for inequality of multiple tests. Analysis of variance with Scheffe's test was used to determine differences in etoposide f_u between strains. Correlation between etoposide fraction unbound or etoposide binding ratio and both albumin and total protein concentrations was assessed by simple linear regression analysis.

Results

Etoposide f_u in animal plasma demonstrated significant interspecies variability (Table 1). Etoposide f_u in sheep,

Table 1. Etoposide fraction unbound and plasma protein concentrations for each species

Species	Etoposide f_u*	Total protein (g/dL)	Albumin (g/dL)
Human	0.049 ± 0.001	6.5	3.69
Sheep	0.66 ± 0.01†	6.4	3.43
Rabbit	0.31 ± 0.01†	6.6	3.93
Feline	0.62 ± 0.01†	5.9	2.80
Canine	0.63 ± 0.01†	8.8	2.65
Rat	0.52 ± 0.01†	6.2	3.13
Mice			
Swiss–Webster	0.38 ± 0.01†‡	5.2	2.72
DBA/2	0.43 ± 0.01†‡	4.9	2.81
BDF_1	0.46 ± 0.01†‡	4.5	2.80

* Values are means ± SD, N = 6.

† Significantly different from human (two-sample *t*-test with Bonferonni's correction, $P < 0.00625$).

‡ Significant interstrain differences (Swiss–Webster 0.45 vs DBA/2 0.42 vs BDF, 0.37; Scheffe multiple comparison test, $P < 0.05$).

rabbit, feline, rat, and canine plasma were significantly greater than observed for human plasma ($P < 0.00625$), indicating decreased plasma protein binding in these species relative to humans. Likewise, the etoposide f_u in three strains of murine plasma was significantly greater than the binding in human plasma ($P < 0.00625$). Not only were differences observed between species, but also within species. Statistically significant differences were observed in the mean etoposide f_u for each of the three murine strains evaluated (Swiss–Webster 0.45 vs DBA/2 0.42 vs BDF_1 0.37; Scheffe multiple comparison test, $P < 0.05$).

The plasma concentration of total bilirubin was within normal limits for each species. The plasma concentrations of albumin and total protein for each species are presented in Table 1. Linear correlations between etoposide f_u for each animal species and either albumin or total protein concentration were assessed to evaluate the interspecies variability of etoposide f_u. The correlation between the etoposide binding ratio and albumin or total protein was not statistically significant and poorly predictive ($P > 0.05$).

Discussion

This study demonstrates that etoposide is extensively bound to human plasma proteins but to a lesser extent to plasma proteins from the other animal species evaluated. Although interspecies differences in albumin and total protein concentration can account for the interspecies variability of protein binding for some drugs (e.g. salicylate, penicillin, propranolol) [19], differences in etoposide plasma protein binding could not be attributed to differences in either albumin or total protein concentration. As has been observed for other drugs [8–10], the affinity of etoposide for its binding sites is the most likely explanation for the differences in binding observed in the animal plasma. Genetic differences in the structure and conformation of the albumin molecule have been shown to influence both the drug binding affinity and the number of binding sites on albumin [19].

We have reported previously that plasma albumin accounts for the majority of *in vitro* etoposide plasma binding in humans [20]. Bailey-Wood and colleagues [21] reported that the choice of serum protein (bovine or human) in a colony-forming assay of etoposide activity significantly influenced the inhibition of CFU-GM growth. An approximately 9-fold increase in total (i.e. bound and unbound) etoposide concentration was required in the presence of human serum albumin (HSA) to achieve 50% survival (D_{50}) of CFU-GM colonies as compared to colonies cultured in the presence of the same concentration of bovine serum albumin (BSA). The authors suggested that the difference in D_{50} could be attributed to a greater affinity for HSA than BSA, which would be consistent with the results of the present study.

Prelinical plasma pharmacokinetic data are useful in guiding dose escalations in Phase I trials of anticancer agents [2]. These preclinical studies are performed in a variety of animal species as well as in different strains of a species (e.g. mice). The use of data from preclinical studies in animals can decrease considerably the time and the expense of Phase I trials conducted in humans. When this approach has been applied to the scale-up of preclinical data for anticancer drugs, pharmacokinetic data based on total drug concentrations have been used (versus unbound drug concentrations).

Differences in both interspecies drug protein binding and clearance may hinder pharmacologically guided dose escalations based on total concentration alone, thus requiring the use of unbound pharmacokinetic data to efficiently escalate drug dose for Phase I studies. This is especially important for drugs which are highly protein bound or where interspecies differences in protein binding may be suspected based on chemical structure. As an example, comparable doses of the disubstituted amsacrine derivative, CI-921, in mice and humans result in substantial interspecies differences in total drug AUC values, but similar unbound AUC values [22]. Likewise, at similar dosages amsacrine is reported to have pronounced interspecies differences in total drug AUC values, but similar bound drug AUC values [22]. The authors suggest that interspecies differences in protein binding and metabolism of amsacrine and CI-921 may explain the differences observed in pharmacokinetic data.

In summary, etoposide plasma protein binding demonstrates marked interspecies and in the case of mice interstrain variability. Overall, etoposide f_u was statistically greater in sheep, rabbit, canine, feline, rat, and mice plasma than that determined in plasma from humans. This indicates that at any given plasma etoposide concentration, humans will be exposed to 7–16-fold less unbound drug, which is the pharmacologically active drug in plasma. Based on the pronounced interspecies differences we have observed in etoposide protein binding, the use of unbound pharmacokinetic data may be more appropriate for drugs which are highly protein bound. Results of this study may have important implications for the scale-up of preclinical studies, and the design of Phase I studies of anticancer drugs which are highly protein bound.

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