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Flavone acetic acid and plasma protein binding*

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Summary. Both the capacity of healthy human, cancer patient, and mouse plasma proteins to bind flavone acetic acid (FAA) and the qualitative differences in the plasma protein-binding site were studied. The binding capacity of plasma proteins for FAA was saturated within the therapeutic range in both species. The binding of FAA to plasma protein was significantly greater in both healthy human and cancer patient plasma than in mouse plasma. Plasma from patients with cancer bound on the average less FAA than did healthy patient plasma. The concentration of albumin in the plasma varied between healthy humans, cancer patients, and mice, being 5.3 ± 0.7, 4.7 ± 0.8, and 3.9 ± 0.3 g/100 ml, respectively. The protein binding of FAA was found to be dependent on the plasma albumin concentration, but albumin concentration alone was not adequate for the accurate prediction of the percentage of FAA protein bound. Scatchard plots indicated that healthy human plasma had a greater number of high-affinity binding sites than did mouse plasma. FAA binds at the indolebenzodiazepine binding area on albumin and can be displaced from this site by salicylic acid and clofibric acid, but only at supratherapeutic concentrations. Our results indicate that alterations in plasma albumin could contribute to a variable effect with FAA. Therefore, the influence of serum albumin concentration and the nonlinearity of FAA protein binding should be considered in assessment of the appropriateness of a dose schedule for FAA.

Introduction

Flavone acetic acid (FAA) represents a new class of chemotherapeutic agents with a broad spectrum of activity against murine solid tumors [8, 22] and little, if any, activity against murine leukemias [20, 28]. FAA has demonstrated activity against human cell lines in vitro, albeit at high concentrations, and against human tumor xenografts in vivo [3]. Because of its profound effect in vivo against solid tumors, FAA entered phase I clinical trials in 1985. Although the trials are still in progress, excitement about this compound has substantially diminished because of both the large amount required for a maximum tolerated dose (MTD) and the lack of activity noted in humans to date [30].

Its mechanism of action is unknown, although it has been shown to have a direct cytotoxic effect in vitro [8] and to have an inhibitory effect on ATP levels that is coupled with a DNA fragmentation effect in vivo [2, 12]. A dramatic reduction in blood flow has been suggested to mediate the inhibition of ATP levels [12]. In vitro, FAA incubated with tumor cells for 2 h resulted in an inhibition of DNA and RNA synthesis, but no DNA fragmentation was detected [3]. Cummings et al. [9] have suggested that DNA fragmentation in vivo may be a secondary event rather than the primary cause of cell death. Ching and Baguley [5] and Finlay et al. [15] have reported immune-stimulatory effects of FAA and suggested this as a third possible mechanism of action. Thus, although FAA displays a number of biological effects typified by flavonoids in general [6, 18], the biochemical or molecular lesion leading to both cell and tumor cytotoxicity remains unclear.

Although it is one of the most active antitumor agents identified to date for murine solid tumors, FAA does suffer from several limitations [8, 31]. The compound exhibits both a steep dose-response curve and threshold-dose behavior. Duration of exposure above a critical minimal level may be required for a response [31]. Therefore, an understanding of the parameters controlling the disposition and pharmacokinetics of FAA are essential to obtaining an efficacious administration of this agent. Since the affinity a compound has for plasma proteins and the extent to which it is bound can modulate the distribution, excretion, and, therefore, efficacy of the compound, the present study was undertaken to quantitate the extent of plasma protein bind-

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Table 1. Binding percentages of flavone acetic acid[a]

Plasma type	Initial FAA concentration (μg/ml)				
	10	50	200	500	700
Healthy human	100 ±0 (8)	99.2±0.3 (8)	96.9±0.6 (9)	86.9±1.2 (7)	68.3±3.7 (7)
Cancer patient	100 ±0 (3)	98 ±0.5 (5)	93.8±2.2 (9)	72.9±3.1 (10)	60.6±4.4 (9)
Mouse	90.9±1.3 (4)	89.8±1.1 (4)	72.3±4.0 (6)	54.7±1.6 (3)	42.8±2.1 (3)

[a] Entries represent the mean, SEM, and number of determinations (in parentheses) for each FAA binding percentage. A preliminary analysis of variance model showed a nonsignificant interaction effect ($P = 0.054$) of plasma type and FAA concentration. That permitted straightforward interpretation of the simultaneous main effects of the two classification variables found in the ANOVA using the Tukey multiple-comparisons procedure. Healthy human plasma, cancer patient plasma, and mouse plasma had albumin concentrations of 5.3±0.7, 4.7±0.8, and 3.9±0.3 g/100 ml, respectively

ing of FAA and to identify and characterize its plasma binding site.

Materials and methods

Materials. Bovine serum albumin (A-4378), salicylic acid, clofibric acid, phenylbutazone, and indomethicin were obtained from Sigma Chemical Co. (St. Louis, Mo.). Flufenamic acid and ibuprofen were purchased from Aldrich Chemical Co. (Milwaukee, Wis.). FAA was obtained from the Drug Evaluation Branch, National Cancer Institute. FAA was dissolved in 7.5% sodium bicarbonate at a concentration of 20 mg/ml, with further dilutions being made in phosphate-buffered saline (PBS).

Plasma. Human plasma was obtained from healthy human volunteers and from cancer patients who had not previously received chemotherapy. Mouse plasma was obtained from CDF_1 mice by bleeding from the retro-orbital venous plexus, followed by centrifugation for 7 min at 3,000 rpm.

HPLC analysis. A 200-μl sample of plasma, albumin, or ultrafiltrate was mixed with 0.9 ml methanol and 0.1 ml *p*-dimethylaminobenzaldehyde (200 μg/ml) as an internal standard. The mixture was centrifuged at 13,000 *g* for 5 min. A 50-μl aliquot of the supernatant was taken for injection onto the HPLC. The HPLC system used a 30-cm uBondapak C-18 3.9-mm column (Waters Associates) with an elution gradient of 30%–60% organic solvent (methanol, acetonitrile, isopropyl; 1 : 1 : 1, by vol.) in 5 m*M* phosphoric acid (pH 3.5) operating at a flow rate of 1.5 ml/min (Waters pump M-45). Eluting compounds were detected by their UV absorbance at 300 nm on a Spectroflow 773 Kratos detector integrated with a Waters Wisp System. The assay was linear from 0.5 to 1,000 μg/ml.

Plasma protein binding. Plasma protein-binding studies used the Amicon Centrifree micropartition system (Amicon Corp., Danvers, Mass.). The YMT filter membrane used in this system retains >99.9% of serum protein. Plasma was spiked with the indicated concentration of FAA and then incubated for 15 min at 37° C. A 200-μl aliquot of the plasma was added to 0.9 ml methanol and the total concentration of FAA was determined by HPLC assay. A 300-μl aliquot of the same plasma sample was placed in the upper compartment of the micropartition system and was centrifuged for 15 min at 2,000 *g* (IEC Sentra 7 centrifuge, fixed-angle rotor) at 25° C. The quantity of drug present in the ultrafiltrate was determined by HPLC assay, with the ratio of ultrafiltrate/unfiltered drug representing the percentage of free drug. Control studies using FAA in saline were conducted to determine whether FAA would bind to the filter membrane of degrade during centrifugation.

Competitive binding inhibitors. Plasma was spiked with FAA at a concentration of 100 μg/ml. Following a 10-min incubation at 37° C, the second agent (100 or 1,000 μg/ml) was added to the plasma, which was then incubated for an additional 10 min. The total and free concentrations of FAA were determined as described above.

Binding site determination. Specific drug-binding sites on plasma albumin were determined by the method of Sudlow et al. [26]. The fluorescence of solutions containing 1×10^{-5} *M* bovine serum albumin and 5×10^{-6} *M* probe was measured at 475 nm, before and after the addition of FAA, using an excitation wavelength of 370 nm. Fluorescence (F) as a percentage of initial fluorescence is given by $(F_{\text{before FAA}}/F_{\text{after FAA}}) \times 100$.

Plasma albumin. Plasma albumin concentration was determined using bromocresol green [11].

Statistical methods. The dependent variable analyzed was the percentage of FAA bound to albumin. The simultaneous statistical effects of subject group (healthy human, cancer patient, and mouse) and blood FAA concentration on the mean percentage of FAA bound to albumin were explored. This was performed with two-way, unbalanced, fixed-effects analysis of variance (ANOVA) methods using a general linear-models approach [16]. Tests for normality of the dependent variable were performed before the ANOVA was begun. A rank transformation of the percentage of FAA binding was necessary for sufficient normalization of the data to satisfy that ANOVA assumption reasonably. Analyses were carried out on the rank-transformed data, although for presentation purposes, arithmetic means are reported as summary statistics [7].

The statistical interaction effect of subject group and blood albumin concentration was tested by the inclusion of a cross-product term of those two variables in preliminary ANOVA models. A significant interaction was to be followed up with one-way ANOVA models comparing each study variable (subject group, concentration), stratified by the other. A nonsignificant interaction resulted in our dropping this effect from the model and refitting the ANOVA with the two main effects only. In investigations of significant main effects found in the ANOVA, the Tukey multiple-comparisons procedure was used to maintain the experimentwise type-I error rate at 0.05 [16].

Results

FAA (10–1,000 μg/ml) was added to either mouse, healthy human, or cancer patient plasma and the percentage of FAA bound to plasma protein was determined. As the concentration of FAA increased in the plasma, the percentage of FAA bound to plasma protein was found to decrease (Table 1). The effect of FAA concentration (controlling for plasma type) on plasma protein binding was

Table 2. Binding percentages of flavone acetic acid[a]

Sample	Initial FAA concentration (μg/ml)				
	10	50	200	500	700
Bovine albumin 5%	97.2±1.5 (4)	95.8±0.4 (4)	94.4±0.4 (4)	83.2±0.9 (4)	70.9±0.9 (3)
Bovine albumin 4%	94.6±2.9 (3)	95.2±1.1 (3)	91.0±0.5 (3)	73.7±2.1 (4)	61.9±0.1 (3)
Bovine albumin 3%	95.0±0.8 (3)	94.1±1.1 (3)	84.9±4.1 (5)	64.6±2.7 (4)	55.2±0.6 (3)

[a] Entries represent the mean, SEM, and number of determinations (in parentheses) for each FAA binding percentage

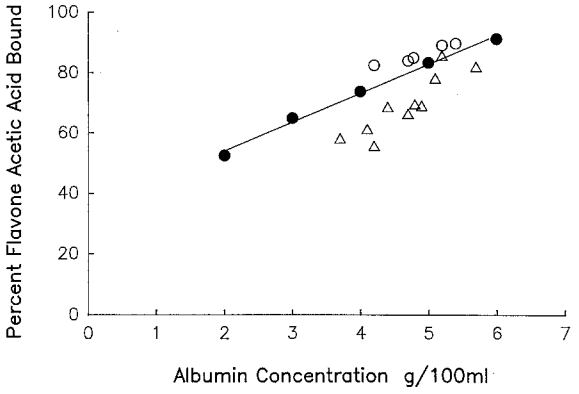

Fig. 1. Percentage of flavone acetic acid (FAA) bound to either albumin (●), healthy human plasma (○), or cancer patient plasma (△). The initial FAA concentration was 500 μg/ml. Each data point represents the mean of at least three determinations

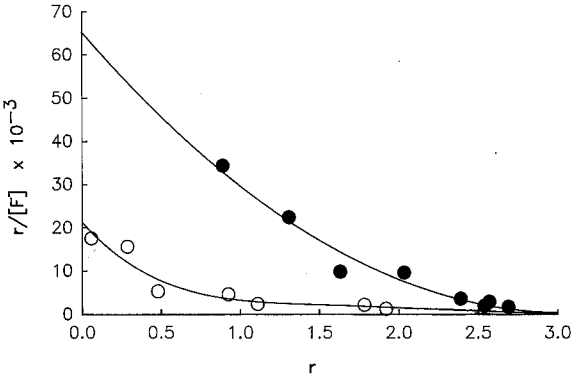

Fig. 2. Scatchard plot of binding of flavone acetic acid (FAA) to human (●) and mouse (○) plasma. [*F*] denotes the molar free concentration of FAA, and *r* denotes the ratio of moles of FAA bound/moles of albumin present in the plasma. The healthy human plasma and mouse plasma had albumin concentrations of 5.3±0.7 and 3.9±0.3 g/100 ml, respectively. Each data point represents the mean of at least four determinations

highly statistically significant ($P < 0.0001$). The mean FAA binding percentage showed very statistically significant decreases at each successively higher FAA concentration, beginning with 50 vs 200 μg/ml ($P < 0.005$). The effect of plasma type (controlling for FAA concentration) was highly statistically significant ($P < 0.0001$). The extent of protein binding for FAA was greater in human plasma than in mouse plasma at equivalent FAA concentrations for both healthy and cancer patient plasma ($P < 0.05$). Plasma from patients with cancer bound on the average less FAA than did healthy patient plasma ($P < 0.05$; controlling for FAA concentration).

Although albumin is the most abundant protein in plasma and one of the most common sites for drug binding, its concentration varies between the human and mouse samples studied (see footnote to Table 1). Therefore, the binding of FAA to 5, 4, and 3 g/100 ml bovine serum albumin was determined at various concentrations of FAA (Table 2). As shown in Fig. 1, binding of FAA to albumin was saturable, paralleling the results found with human and mouse plasma, with the degree of binding being directly proportional to the albumin concentration ($r = 0.997$). The percentage of FAA protein bound in healthy human plasma correlated with the plasma albumin concentration ($r = 0.965$) (Fig. 1). The binding capacity for human plasma was consistently slightly higher than that for bovine serum albumin, which possibly reflects slight differences in human and bovine albumin characteristics. The plasma albumin concentration in cancer patients varied from 3.6 to 5.7 g/100 ml. The amount of FAA protein bound correlated with the plasma albumin concentration ($r = 0.893$), although the correlation was lower than that found for healthy human plasma ($r = 0.965$). In contrast to that of healthy human plasma, the binding capacity of cancer patient plasma was consistently lower than that of bovine serum albumin. Furthermore, the percentage of FAA bound to protein in cancer patient plasma was less accurately predicted from the bovine albumin regression line (MSE = 164.6; mean square error) than from the value for healthy human plasma (MSE = 39.7).

The affinity and number of binding sites FAA has on human and mouse albumin was determined from the plasma data using a Scatchard plot (Fig. 2). Scatchard plots of the binding of FAA to mouse and human plasma suggest the presence of both a high- and a low-affinity binding site. The binding affinity and number of binding sites was calculated using the curve-fitting method developed by Feldman [14]. Among the high- and low-affinity sites, human plasma has 1.6 binding sites with an affinity of 40,014 M^{-1} and 1.5 binding sites with an affinity of 1,956 M^{-1}, respec-

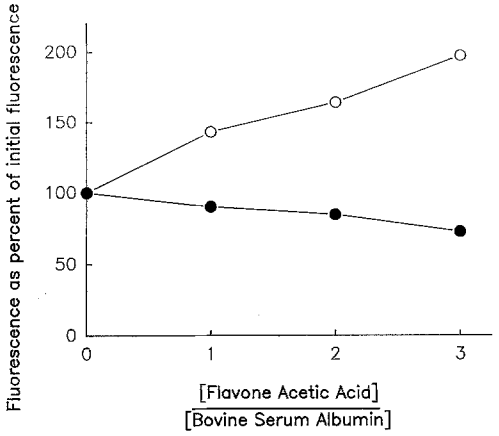

Fig. 3. Flavone acetic acid (FAA)-induced changes in the fluorescence of probes dansylamide (○) and dansylsarcosine (●) bound to bovine serum albumin. The fluorescence of solutions containing 1×10^{-5} *M* bovine serum albumin and 5×10^{-6} *M* probe was measured at 475 nm, before and after the addition of FAA, using an excitation wavelength of 370 nm. Each data point represents the mean of three determinations

tively. Mouse plasma has 0.5 binding sites with an affinity of 40,000 M^{-1} and 3 binding sites with an affinity of 800 M^{-1}, respectively.

Two distinct binding sites for anionic drugs on human plasma albumin have been characterized using fluorescent probes [26]. FAA decreased the fluorescence of dansylsarcosine (type II probe) by 28% when present at a 3:1 drug; albumin ratio, whereas it caused a 97% increase in the fluorescence of dansylamide (type I probe) (Fig. 3). The ability of the type-II binding-site agents salicylic acid, clofibric acid, indomethacin, flufenamic acid, and ibuprofen and the type I agent phenylbutazone to displace FAA was determined (Table 3). None of these agents could displace FAA when they were added at an equivalent concentration of 100 μg/ml. However, salicylic acid and clofibric acid displaced 16% of the bound FAA when present at a concentration 10-fold that of FAA. Flufenamic acid and ibuprofen effected a small (about 5%) displacement at this concentration. At therapeutic concentrations, salicylic acid (330 μg/ml) displaced only 6% of the FAA and clofibric acid (200 μg/ml) did not cause significant displacement.

Discussion

Our results indicate that if free FAA is the molecule of therapeutic importance, alterations in plasma albumin could contribute to a variable therapeutic effect with FAA in cancer patients. Evidence is presented that suggests qualitative and quantitative differences in interspecies protein-binding characteristics and the dependence of binding on albumin concentration.

In the present study, the binding of FAA to plasma protein was significantly greater in both healthy human and cancer patient plasma than in mouse plasma at equivalent drug concentrations. Other investigators have also noted an interspecies variation in plasma protein binding for FAA. Bibby et al. [1] noted a lesser extent of binding

Table 3. Binding percentages of flavone acetic acid to human plasma protein in the presence of a second protein-bound agent[a]

Second agent	Concentration of second agent (μg/ml)	
	1,000	100
Salicylic acid	78.7±0.6	92.6±2.2
Clofibric acid	79.1±3.9	93.6±2.2
Phenylbutazone	91.1±1.8	93.7±2.0
Indomethacin	87.3±2.0	93.4±2.2
Flufenamic acid	85.1±0.2	91.1±0.7
Ibuprofen	83.6±2.7	91.4±0.2

[a] The concentration of FAA was 100 μg/ml. The percentage of FAA bound to human plasma protein in the absence of a second agent was 93.4%±3.8%. The values represent the mean of three determinations (± SEM)

for mouse serum than for human serum (47% vs 81% bound at 250 μg/ml), although they did not observe the concentration-dependent binding seen in the present study. Recently, Cassidy et al. [4] reported concentration-dependent binding of FAA and interspecies differences in the protein binding of FAA in plasma obtained from mice and human cancer patients. As shown in the present study by linear regression of the percentage of FAA bound to protein vs the bovine albumin concentration, the protein binding of FAA is dependent on albumin concentration. The variability in the percentage of FAA protein bound in plasma from cancer patients at a single FAA concentration is related to the level of plasma albumin in the individual patients. Although it accounted for most of the binding capacity, albumin concentration alone was not adequate for accurate prediction of the percentage of FAA bound. Cassidy et al. [4] found that the in vivo binding capacity of patients receiving intravenous FAA could not be predicted from in vitro data. Albumin metabolism [25, 29] and electrophoretic behavior [21, 23] are modified by the presence of neoplastic disease; thus, alterations in albumin's binding characteristics may have caused the deviation from predicted values. Staubus et al. [24] have suggested that a lack of correlation between in vitro and in vivo data may be related to competition for binding sites by conjugated metabolites.

Healthy human plasma appears to have a greater number of high-affinity binding sites for FAA and a greater concentration of albumin than does mouse plasma. Mouse plasma has a greater number of low-affinity binding sites, but these sites are thought to be nonspecific [19]. The percentage of FAA bound to mouse plasma protein at 500 μg/ml FAA is 54.7%. The predicted value from the bovine albumin regression curve at 3.9 g albumin/100 ml is 72%, which is 23% more than was actually bound to mouse plasma. The degree of plasma protein binding in the mouse was not accurately predicted from the bovine serum albumin regression line, most likely because of these qualitative and quantitative differences in mouse albumin. Interspecies differences in the efficacy and pharmacokinetics of several anionic compounds have been related to interspecies differences in binding of the compounds to albumin [27].

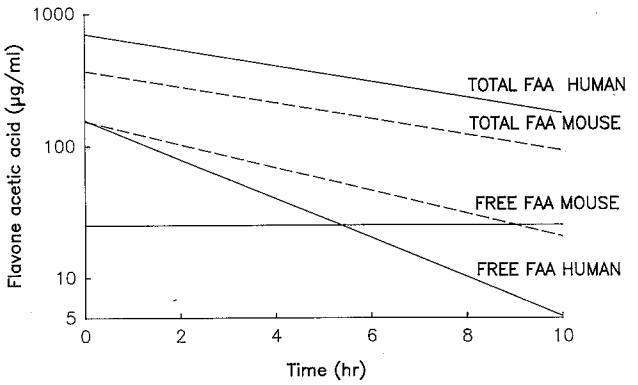

Fig. 4. Theoretical mouse and human plasma elimination curves for flavone acetic acid (FAA). (Human and mouse plasma concentrations were calculated from pharmacokinetic parameters reported in [17] and [31]). To achieve these plasma concentrations, a dose of about 4.8 g/m^2 would need to be infused over 1 h in humans and that of 200 mg/kg, by i.v. bolus in mice. The free FAA plasma concentration in human and mouse was predicted from our in vitro data. The solid horizontal line corresponds to 25 μg/ml and represents the projected therapeutic threshold concentration of free FAA

An efficacious peak plasma concentration of FAA in mice is 368 μg/ml [31], which from our data yields a free FAA level of 155 μg/ml. To attain a free FAA level comparable with that in the mouse, it would be necessary to achieve a plasma level of 700 μg/ml in humans with healthy serum albumin. Peak plasma levels of 700 μg/ml have been reported for patients receiving FAA [4, 17]; however, very high doses (about 4.8 g/m^2 infused over 1 h) were necessary before this concentration could be reached [17]. Cassidy et al. [4] have concluded that since free drug concentrations in humans attained a range comparable with the therapeutic range of free FAA in mice, plasma protein binding does not completely explain the differences in response. Achievement of a therapeutic plasma level can be misleading since it does not take into account the saturation of protein binding within the therapeutic dose range.

Figure 4 illustrates theoretical total as well as free FAA plasma concentration curves in mice and humans, based on the above peak plasma levels and a half-life of 5 h. A half-life of 4.8 ± 2.3 h has been reported for FAA in humans [17], and that of 2–5 h has been obtained in mice [31]. The free level of FAA was calculated using our in vitro results. The threshold therapeutic level for FAA in mice has been calculated to be 100 μg/ml [31], which would result in a free FAA level of 25 μg/ml. It would take 9 h for the mouse to achieve plasma levels of <25 μg/ml, whereas it would take only 5 h for the human; the latter would be exposed to a therapeutic concentration for about one-half the time of exposure necessary for the mouse. These calculations are based on in vitro binding data and suggest a possible model for in vivo plasma concentration curves.

Humans metabolize FAA extensively to glucuronide conjugates, whereas this represents a minor elimination pathway in mice [10]. It has been suggested that in vivo, FAA metabolites compete for the FAA binding sites in humans and alter the percentage of protein bound [24]. Therefore, although interspecies differences in plasma protein binding may not completely explain the lack of response in humans, evaluation of optimal therapy should consider all kinetic characteristics.

One of the problems encountered in the phase I clinical trial was the large amount of FAA required to reach the MTD. A possible approach for achieving the desired free drug levels in humans at a lower total dose involves competition with other highly protein-bound compounds for the FAA binding site. Serum albumin has been shown to have a small number of distinct binding sites [13]. The two most important binding sites for anionic drugs include the warfarin-azapropazone binding area (site I) and the indole-benzodiazepine binding area (site II). Specific probes have been used to identify the specificity and relative strength of drugs binding to these sites. An agent that binds to the type I binding site displaces the probe dansylamide, resulting in a decrease in the fluorescence of this probe. A type-II binding-site agent displaces the probe dansylsarcosine. FAA displaced dansylsarcosine and increased the binding of dansylamide, suggesting that FAA binds at site II. An increase in the fluorescence of probes bound to site I by agents that bind at site II is thought to be mediated by drug-induced change in the conformation of the albumin molecule [26]. The type-II binding-site agents salicylic acid and clofibric acid could inhibit the binding of FAA at supratherapeutic concentrations, but no significant displacement occurred at therapeutic concentrations. The site I binding agent phenylbutazone caused no displacement of FAA, confirming that FAA does not bind at site I. The high dose requirements for FAA will not be overcome by displacing FAA from albumin with these competing agents. The ideal displacing agent would have to cause consistent displacement of FAA so as to avoid the introduction of additional interindividual variability in protein-binding characteristics.

Another possibility that we are pursuing to overcome the binding limitation is to obtain analogs of FAA with a lower albumin-binding capacity. We have incorporated the study of murine and human plasma binding as a factor to be considered in the development of new clinical candidates for FAA analogs.

In summary, the protein binding of FAA is dependent on both albumin concentration and plasma type and is saturated within the therapeutic range. The results of the present investigation as well as previously reported studies illustrate the necessity of determining the in vitro and in vivo binding characteristics of FAA and FAA analogues prior to assessing the efficacy of a dose schedule.

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