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Short communication

Could interspecies differences in the protein binding of flavone acetic acid contribute to the failure to predict lack of efficacy in patients?

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Summary. We investigated the differences in plasma protein binding of flavone acetic acid (FAA) in mice and men in an attempt to explain the inter-species differences in response. In vitro data indicate both qualitative and quantitative differences in FAA protein binding: approximately 80% is bound in humans, with two different types of binding site identified; in mice, 70% is bound and only one binding site could be described. Protein binding is dose-dependent in both species. Plasma samples from 20 patients receiving FAA showed that most achieved levels that would be active in mice. We conclude that these differences in protein binding are insufficient to explain totally the observed differences in response.

Introduction

Flavone acetic acid (FAA) exhibits a wide spectrum of anti-tumour activity in murine solid tumours resistant to conventional cytotoxic agents [3, 5]. Pharmacokinetic studies have been carried out in mice during preclinical development: Zaharko et al. [8] identified a therapeutic window of total drug concentrations of 100–600 μg/ml. Furthermore, the duration of exposure above the critical threshold seemed to be an important determinant of toxicity and response; this was confirmed in a series of in vitro experiments [2]. We conducted a phase I study of this agent [4]; despite achieving plasma concentrations within this therapeutic window, we did not see any clinical responses. Although disappointing, this is not uncommon in phase I studies (e.g., advanced-stage disease, multiple previous therapies, and tumours considered to be unresponsive to currently available agents). However, there have as yet been no reports of activity in phase II trials.

It is generally accepted that only the free or unbound fraction of a drug is available for pharmacological responses at the cellular level. Standard drug-assay methods measure total drug concentrations, and the therapeutic window described above is stated in such terms.

Inter-species differences in plasma protein binding have been described; therefore, we investigated the plasma protein binding of FAA in mice and men, as this could contribute to the difference in activity noted between patients and preclinical tumour models. We also attempted to predict free levels of FAA from the total levels measured in patients.

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Materials and methods

High-pressure liquid chromatography (HPLC) was used for the measurement of both total and free drug concentrations. *p*-Dimethyl amino benzaldehyde (BDH Chemicals, England) was used as an internal standard. The mobile phase was composed of 12.5% methanol, 12.5% isopropyl alcohol, 12.5% acetonitrile and 62.5% 0.005 *M* phosphoric acid. Double-distilled deionised water from a Quartz glass still was used throughout, and purity standards for FAA were supplied by Lipha Lyonnaisse Industrielle Pharmaceutique (Lyon, France).

The extraction of FAA involved the addition of 0.1 μg internal standard to 1 ml plasma. After the vortexing step, 200 μl 5% trichloroacetic acid was added, followed by 10 ml chloroform. Each sample was then vortexed at room temperature for 1 h; the precipitate and aqueous phase were then sedimented by centrifugation for 15 min at 2,000 rpm. The drug-containing organic layer was then removed and evaporated at ambient temperature using a Buchler vortex evaporator, and the residue was re-dissolved in 200 μl methanol.

The HPLC system (model Altex 100A; Altex Scientific, Berkeley, Calif) contained a single constant-flow pump, which delivered a flow rate of 1.5 ml/min. The stainless-steel column (inside diameter, 250 × 5 mm) was packed with (5-μm) C18 Bondapack. Samples were injected onto the column via a manual injection port containing a 20-μl loop (Altex 210). The eluant was freshly prepared and degassed. A variable-wavelength UV detector (LC-UV Pye; Unicam, England) was set at 303 nm and 0.02 AUFS to detect the eluting compounds; the output signal was recorded by a Tekman potentiometric pen recorder. All separations were carried out at ambient temperature using isocratic elution. The assay was sensitive to a drug concentration of 1 μg/ml, the lowest reliable peak height being taken as 3 times the height of baseline noise. The accuracy of this assay is 94%; inter- and intra-assay coefficients of variation are both < 10%.

In vitro binding. Pooled plasma from cancer patients or mice was incubated for 1 h at 37° C with various concentrations of FAA (range, 50–1,000 μg/ml). The samples were adjusted to pH 7.4 and divided into two: one half was analysed by HPLC for total FAA concentration; the other was filtered through an Amicon YMT membrane at 4,000 rpm (1,930 *g*) for 20 min at 4° C, and the resulting ultrafiltrate was injected directly onto the HPLC column.

The molecular weight cut-off of this membrane is 30,000 daltons and its serum protein retention is 99.9%. No FAA could be detected in washings of the membranes after ultrafiltration, suggesting that FAA membrane binding did not occur.

In vivo binding. Blood samples were obtained from 20 patients at various times during and after FAA administration; they were analysed for both total and free FAA concentration by the methods described above. Attempts were made by simple graphical means (comparing the percentage of bound vs total drug concentration plots) as well as the more complex use of binding isotherms to predict free FAA levels in patients from their observed total plasma concentrations.

Analysis of binding data

Using the method of Scatchard [6], plots of bound/free vs bound FAA were constructed for the in vitro data; the resulting curves can be described by a single straight line or a combination of straight lines corresponding to the number of types of separate binding sites. Data were fitted to these models by computer, using the least-squares method. The choice between competing models was made using the general linear (F) test and the Aikake information criterion (AIC). The SD in the difference in AIC (Δ AIC) is given by the equation

$$SD = \sqrt{\text{difference in number of parameters}/2}$$

and since the difference in the number of parameters is 2 (between a straight line and a two-binding-site Scatchard plot), the SD is 1. It follows that if ΔAIC is >3, it lies outwith 3 SDs i.e., the difference is statistically significant [1].

The data were also fitted to a descriptive binding isotherm developed by Zaharko and McCormack [7]. Briefly,

D = free drug (μg/ml)
S = free binding sites (μg/ml equivalents)
K = equilibrium constant
B = bound drug (μg/ml)
R = total binding sites (μg/ml equivalents)
T = total drug (μg/ml)
n = order of reaction

$D + S \leftrightharpoons B^n$; at equilibrium, $K_1/K_2 = K$, and since $K_1 \cdot D \cdot S = K_2 \cdot B^n$,

$$D = B^n/K \cdot S \qquad (1)$$

From Eq. (1), since $S = R - B$ and $B = T - D$ and a fit to the in vitro data indicated that $n = 2$, it follows that

$$(1 - K)\,D^2 + (KT - KR - 2T)\,D + T^2 = 0,$$

and by binomial expansion using

$$x = \frac{-b +/- \sqrt{bz - 4AC}}{2a},$$

results in the expression

$$D = \frac{K(R-T) + 2T - \sqrt{(KT - KR - 2T)^2 - 4(1-K)T^2}}{2(1-K)}, \qquad (2)$$

such that free drug can be determined from K, R and T.

Results

Plots of the percentage of in vitro binding vs total drug concentration are shown for humans in Fig. 1 and for mice in Fig. 2. In the proposed therapeutic range of total FAA concentrations, approx. 80% is protein-bound in humans,

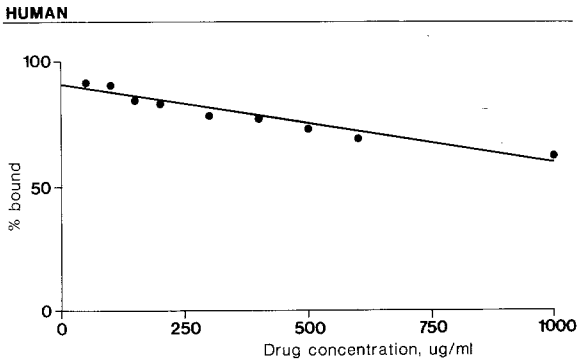

Fig. 1. Percentage of in vitro binding vs total drug concentration for humans. $r = 0.97$

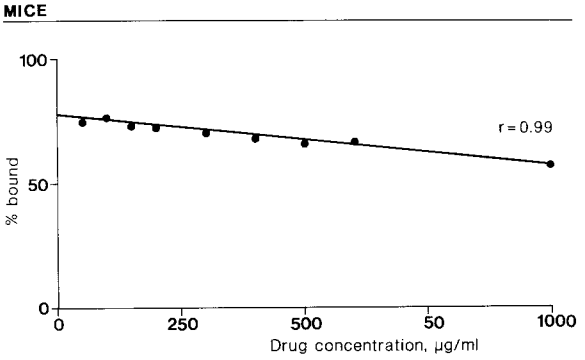

Fig. 2. Percentage of in vitro binding vs total drug concentration for mice

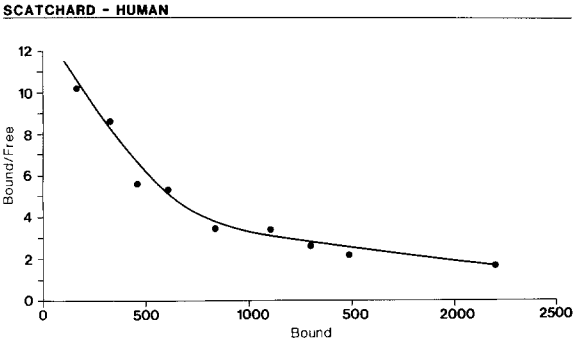

Fig. 3. Scatchard plot of the in vitro data for humans

whereas in mice ca. 70% is bound over the same concentration range. In both species, increasing drug concentration results in reduced binding, indicating that protein binding is saturable in both man and mouse. At higher concentrations the differences in binding are smaller; that is, the lines tend to converge. However, at any given concentration, protein binding in the mouse is less than that in man.

Scatchard plots of the in vitro data are shown in Fig. 3 for humans and in Fig. 4 for mice. The curvilinear plot for humans indicates the presence of more than one type of binding site, or binding-site interaction; computer fitting of the curve indicates that two separate types of binding site can be modeled: a major and minor site. Regression analysis gives values of

$N_1 = 2294 +/- 34$ $\quad K_1 = 0\ 0.0015 +/- 0.0004$ and
$N_2 = 331 +/- 6$ $\quad K_2 = 0.03 +/- 0.002$,

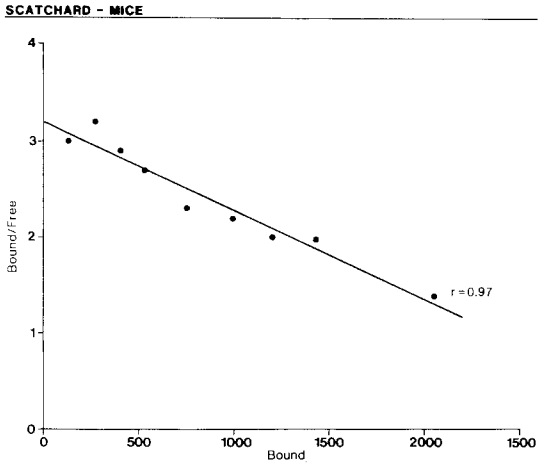

Fig. 4. Scatchard plot of the in vitro data for mice

where N is the total number of binding sites in μg/ml equivalents and K is the equilibrium constant.

On the other hand, Scatchard plots of mouse in vitro data more closely approximate a monophasic than a biphasic isotherm (ΔAIC = 9), indicating that only one type of binding site is necessary for modeling, where

$N = 3436 +/- 40 \qquad K = 0.0009 +/- 0.0001.$

From our own human in vitro data, we derived values of

K = 0.08
R = 4400
n = 2 (approx.)

by fitting the data to Eq. (1) above. Using these values, we predicted the free drug level for individual patients from the total plasma drug measured (Table 1). We also attempted to predict free drug levels using the simple graphs of the percentage of drug bound. Table 1 summarises these results but shows only peak plasma levels for clarity.

Discussion

This study demonstrates both qualitative and quantitative differences in the protein binding of FAA between mice and men. However, in both, the percentage of binding decreases with increasing total drug concentration, suggesting a degree of binding-site saturation in both species.

FAA has dose-dependent pharmacokinetics, with a non-linear relationship between dose and the AUC for the plasma concentration-time curve and the end-of-infusion peak drug concentrations. The mean total-body plasma clearance decreased as the dose was escalated, whereas the mean steady-state volume of distribution tended to decrease with increasing dose. The fact that FAA protein binding is also dose-dependent contributes to the dose-dependent pharmacokinetics of this drug.

From consideration of our in vitro data we could derive a therapeutic range of free FAA (24–210 μg/ml) in mice and hence the range of total concentrations required to achieve these free concentrations in humans, 175–675 μg/ml. All of our 20 patient profiles attained these levels. The duration of exposure (above the threshold) is also important for an anti-tumour effect with this agent, and it is self-evident that increasing the threshold for efficacy will reduce the time within the confines of the therapeutic window.

We could not accurately predict free FAA levels from total levels for individual patients by any of the methods tested. This could be explained by wide inter-individual variations in factors relevant to plasma protein binding e.g., albumin concentration, the presence of displacing substances such as other drugs, pH, age, sex, or changes in binding affinity that are known to occur in many pathophysiological conditions. However, it is noteworthy that on their entry in our phase I study of FAA, all patients had normal renal (creatinine, <120 μmol/l) and hepatic function (bilirubin, <20 μmol/l).

Assuming that the preclinical pharmacology and therapeutic window concept was correct, we feel that the mag-

Table 1. Peak plasma levels for individual patients from the total plasma drug measured

Patient	Total observed drug concentration (μg/ml)	Free observed drug concentration (μg/ml)	Bound drug %	Free drug predicted graphically (μg/ml)	Free drug predicted by isotherm (μg/ml)
1	300	57	81	57	109
2	444	125	72	102	192
3	672	156	77	201	340
4	215	15	93	34	66
5	320	78	76	125	120
6	513	209	59	244	235
7	604	48	92	169	294
8	1042	86	92	438	604
9	270	42	84	48	93
10	372	161	57	78	149
11	270	37	86	48	93
12	325	83	74	63	122
13	382	180	53	82	155
14	397	154	61	85	163
15	326	107	67	64	123
16	385	175	55	81	156
17	423	176	58	95	179
18	497	185	63	124	225
19	390	146	63	84	159
20	266	79	70	47	91

nitude of the differences in FAA protein binding are insufficient to explain completely the differences in response to this drug observed to date. Perhaps differences in metabolism may be quantitatively more important; studies are currently under way to investigate this possibility.

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