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The correlation of plasma proteins binding capacity and flavopiridol cellular and clinical trial studies

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Abstract. Previous clinical research has suggested high-affinity binding of flavopiridol (FLAP) to human blood serum proteins, specifically either human serum albumin (HSA) or human alpha-1-acid glycoprotein (hAGP), when compared to fetal bovine serum albumin (BSA) or bovine alpha-1-acid glycoprotein (bAGP) used in pre-clinical assays. This high-affinity binding was suggested as the reason for its poor human clinical trial performance as a treatment for chronic lymphocytic leukaemia (CLL). Using three biophysical techniques, specifically circular dichroism (CD), isothermal calorimetry (ITC) and fluorescence spectroscopy, I show that FLAP does not have an overly high-affinity for either fetal BSA, HSA, bAGP or hAGP. I therefore suggest an alternate hypothesis that models the albumin and alpha-1-acid glycoprotein (AGP) binding sites at the different protein concentrations used in the fetal bovine pre-clinical assay and human physiological conditions. I use analytical ultracentrifugation (AUC) experiments to determine the validity of the theoretical models. The models can also be altered to account for the elevated AGP levels and reduced albumin levels seen in human cancer patients. Major differences in the concentrations of free available FLAP are observed between the fetal bovine pre-clinical model and human physiological conditions. A number of recommendations can therefore be made on how future pre-clinical assay studies should be conducted.

Keywords: Cancer, drug binding, serum albumin, alpha-1-acid glycoprotein, flavopiridol

Abbreviations

AGP	alpha-1-acid glycoprotein
AUC	analytical ultracentrifugation
bAGP	bovine AGP
BSA	bovine serum albumin
BSA_{Aff}	fatty acid free BSA
BSA_{FA}	fatty acid bound BSA
CD	circular dichroism
CDK's	cyclin dependent kinases
CLL	chronic lymphocytic leukaemia
DMSO	dimethylsulfoxide
FBS	fetal bovine serum

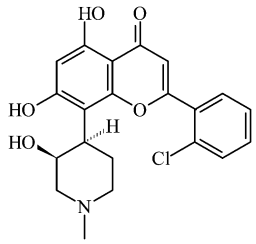

Fig. 1. The molecular structure of flavopiridol (FLAP).

FLAP	flavopiridol
hAGP	human AGP
HAS	human serum albumin
HSA_{Aff}	fatty acid free HSA
ITC	isothermal calorimetry

1. Introduction

Flavopiridol (FLAP, see Fig. 1) is a potent, cyclin-dependent kinase (CDK) inhibitor with high anti-cancer activity for chronic lymphocytic leukaemia (CLL) in pre-clinical fetal bovine serum cell assays [4]. However, in human clinical trials, FLAP demonstrated a poor therapeutic effect. This has been suggested to be due to high-binding affinity to either of the two main human blood serum proteins [6], namely serum albumin and alpha-1-acid glycoprotein (AGP). Serum albumin and AGP are known to bind a range of drugs, reducing the concentration and distribution of the free drug species, and, hence, their efficacy [8].

The binding affinities of flavopiridol for BSA, HSA, bAGP and hAGP have been experimentally determined using circular dichroism (CD), isothermal calorimetry (ITC) and fluorescence spectroscopy. A theoretical model was then constructed using the experimentally determined binding affinities to determine the concentration of free, unbound FLAP in fetal bovine cell assays and in healthy or disease state (i.e. cancerous) human patients. The amount of free drug is critical because it dictates how much of the drug is available in the blood stream to reach the drug's cellular target (in this case, cyclin-dependent kinases (CDK's)). There have been a number of examples of differential species dependent drug binding [1,19], but not as much attention has been paid to different protein concentrations in different experimental conditions. This can be of increased importance in disease states, such as cancer, when levels of AGP can increase by 2–10 fold and levels of serum albumin can decrease by 20–30% [2]. The level of changes observed depends on a number of factors, including cancer type, severity and the use of surgery [2,10,12]. The veracity of the theoretical models produced are then experimentally tested using an analytical ultracentrifugation (AUC) experiment.

2. Material and methods

All chemicals and proteins were purchased from Sigma-Aldrich (UK). FLAP (CAS: 146426-40-6, (-)-2-(2 Chlorophenyl)-5,7-dihydroxy-8-[(3S,4R)-3-hydroxy-1-methyl-4-piperidinyl]-4H-1-benzopyran-4-one hydrochloride – purity $> 98\%$ confirmed by LC-MS and NMR) was solubilised in dimethylsulfoxide (DMSO) to a stock concentration of 14.167 mM. All proteins purchased with the highest analytical grade with a purity $\geqslant 99\%$ as determined by the supplier by agarose gel electrophoresis.

2.1. Circular dichroism titration experiments

The CD samples were prepared in ddH_2O. Human serum albumin fatty acid free (HSA_{Aff}) and *Bos taurus* bovine serum albumin fatty acid free (BSA_{Aff}) were made up to concentrations of 500 μM. Human (hAGP) and *Bos taurus* bovine (bAGP) alpha-1-acid glycoprotein with a concentration of 20 and 10 μM, respectively, were used. Sample concentrations were determined by weight and confirmed by A_{280} optical absorbance in accordance with the supplier product specification sheet. The pH of the system was checked at the start, during and at the end of each experiment; it remained constant at 7.4. All CD spectra were recorded on a nitrogen-flushed Chirascan Plus spectrophotometer (Applied Photophysics Ltd., UK). Spectra were recorded in units of millidegrees (mdeg) from a wavelength range of 250 to 550 nm using the following parameters: 2 nm bandwidth, 2 nm step, 1 second integration time, 25°C, 0.2 mm path length cell, and 9 repeats. The data obtained was processed and graphed using the CD automation program – an in-house designed piece of software. The binding affinity (K_D) of human and bovine AGP was determined at 284 and 310 nm respectively, using the single binding site, single wavelength, non-linear regression method set out by Siligardi et al. [17], which has been automated into the CDApps software program [9].

2.2. Isothermal calorimetry

Each ITC experiment was performed using a TA Instruments Nano Series machine using 1 mM FLAP solutions titrated in 50–5 μL aliquots into a 40 μM protein solution (solutions were matched for DMSO). Both solutions were degassed for 10 minutes immediately before the experiment. The HSA_{Aff} and BSA_{Aff} experiments were performed at 25°C, and the bovine alpha-1-acid glycoprotein experiment was performed at 37°C, utilising a 300 second injection delay and a 300 rpm stirring speed, which was done to maximise the exothermic signal obtained. The data was analysed by subtracting the baseline injection heats for the protein and drug controls, then modelling the data to a one binding site independent model using the NanoAnalyse software (TA Instruments).

2.3. Fluorescence

The fluorescence titration experiments were performed using the fluorescence attachment of a Chirascan-Plus spectrophotometer (Applied Photophysics Ltd., UK). A 2 μM solution of the protein was titrated with increasing concentrations of FLAP. Each protein that was examined had one tryptophan present in its binding site (BSA had an additional tryptophan near the surface of the protein). This allowed for tryptophan fluorescence of the protein in a 1 cm cell pathlength at 2 μM concentration, 1 nm bandwidth, 1 nm step, 1 second integration time and 4 repeats at 25°C with an excitation wavelength of 285 nm and an emission spectrum recorded over 300–460 nm. A 10 point average adjacent smoothing was applied to the raw data. The maximal emission wavelength was then used to produce a single wavelength binding curve. This curve was corrected for the reduction in fluorescence, due to the absorption of FLAP in the excitation and emission region (see Supplementary Material Part 1) for a full description of how this was achieved). The obtained binding curve was fitted to a single binding site ($n = 1$), non-linear regression curve to determine the binding affinity of the protein for FLAP using the equation below:

$$F - F_0 = \frac{F_{\max} \times [\text{FLAP}]}{K_D + [\text{FLAP}]} \tag{1}$$

Where $F - F_0 =$ the change in fluorescence due to the addition of FLAP (no units),
$F_{\max} =$ the maximum change due to the addition of FLAP, [FLAP] = the concentration of FLAP (in units of micromolar) and $K_D =$ the binding affinity of FLAP for the specified protein (in units of micromolar).

2.4. AUC

Sedimentation velocity experiments were conducted at 20°C at 129,024 g (RCF) using an An-60Ti rotor in a Beckman XL-I analytical ultracentrifuge. Absorption data was recorded at 280 nm. The resulting concentration distributions were processed exactly the same for each spectra and analysed by the SEDFIT program [7] to obtain the sedimentation coefficient distributions ($c(s) - S$) for 25 μM FLAP, 25 μM *Bos Taurus* fatty acid bound bovine serum albumin (BSA_{FA}) and a 1:1 molar ratio of 25 μM FLAP:BSA_{FA} in ddH_2O. BSA_{FA} was chosen due to its greater stability over BSA_{Aff} in AUC centrifuge experiments over time. FLAP does not compete at fatty acid binding sites, and it has a similar binding affinity for BSA_{Aff} [11]. The area of the free FLAP in the FLAP and FLAP:BSA_{FA} samples were then directly related to the free FLAP concentration. As the free FLAP concentration was known (25 μM), the FLAP:BSA_{FA} and the free FLAP concentrations could be determined (see Table 6).

2.5. Calculation

2.5.1. Building a theoretical model of the fetal bovine assay and human system

Two independent binding site system. For the derivation of a one binding site system please see Supplementary Material Part 2). The single binding site model can be expanded for a two binding site system. We can expand Supplementary Material Equation 16.3 to allow for two independent binding classes:

$$F_{\text{ub}} = \frac{1}{1 + \frac{N_{\text{TOT1}}}{K_{D1}+[D]} + \frac{N_{\text{TOT2}}}{K_{D2}+[D]}} \tag{2}$$

In the above equation, F_{ub} equals the fraction of unbound drug and N_{TOT1} and N_{TOT2} equal the number of binding sites per litre of binding class 1 and 2, respectively. K_{D1} and K_{D2} equal the dissociation constant of the drug for binding class 1 and 2, and $[D]$ equals the free drug concentration. We can use equation (2) to model two binding classes that bind to a drug (i.e. one molecule with two binding sites). We can model binding curves with two binding classes and compare them to the binding curves of those with one binding class.

Building a theoretical model. To build the fetal bovine assay and human theoretical models, the concentrations of all the binding components in the fetal bovine assay and the human system were determined below. Table 1 displays the derived concentrations of albumin, AGP and FLAP in a bovine cell assay and in humans.

Total protein human and bovine protein levels. The calculations below are an estimate of protein concentrations. The absolute concentration of any individual depends on a number of factors within a range.
Human. The concentration of total human serum protein is between 60–80 mg/mL of blood [12]. If we take a midpoint of this range, we get 70 mg/mL of blood. The human serum albumin concentration is estimated to be 600 μM, which is approximately 60% of the total protein amount. As a result, $0.6 \times 70 =$ 42 mg/mL, which is similar to the 600 μM, 600 μM = 40 mg/mL. The AGP concentration is given as

Table 1
Determining the total serum, albumin, AGP and FLAP concentrations in human physiological and bovine cell assay conditions

	Human physiological conditions	Bovine cell assay
Total protein	70 mg/mL (Range: 60–80 mg/ml)	37.5 mg/ml
[Albumin], μM	600	33.83
[AGP], μM	20.6	1.3
[FLAP], μM	388	0.18
[FLAP] in bloodstream	20.18	
μM of FLAP per μM of albumin	$20.18/600 = 0.0336$	$0.18/33.83 = 0.0053$

The concentrations of albumin and AGP can vary across individuals and in different disease states, such as cancer.

between 55–140 mg/100 mL [14]. If we take the midpoint of 82.5 mg/mL, this results in a concentration of 20.6 μM. The formulation of FLAP given in Shwartz et al. [15] states that 50 mg/mL vials were diluted in 10 mL of ddH_2O to achieve a concentration of 4.5 mg/mL. 250 mL of saline was then added to the 10 mL to give a concentration of 0.17 mg/mL in a 260 mL solution. If we divide the concentration of 0.17 mg/mL by the molecular weight of flavopiridol hydrochloride (Mw = 438.3), we get the molar concentration of $0.17/0.4383 = 388$ μM. If we assume that this 260 mL solution completely mixes with, on average, 5 litres of blood in the human body, we get $260/5000 = 0.052 \times 388$ μM $= 20.18$ μM of flavopiridol.

Bovine. Certification analysis provided with purchased fetal bovine serum (FBS) from Sigma-Aldrich states that the total protein for FBS is between 30–45 mg/mL. If we take the midpoint of this range, we get a value of 37.5 mg/mL. If we assume that 60% of the total serum protein is albumin, we get a concentration of $37.5 \times 0.6 = 22.5$ mg/mL. If we divide this concentration by the molecular weight of albumin, we get $22.5/66.5 = 338.3$ μM of serum albumin in the bovine blood sample. In the cell assay, 10% FBS in used, which equates to 338.3 μM$\times 0.1 = 33.83$ μM. AGP concentration is given as 1–2% of the total serum protein concentration. If we use the midpoint of 1.5%, we get a concentration for AGP of 37.5 mg/mL$\times 0.015 = 0.5625$ mg/mL divided by the molecular weight of AGP: $0.5625/44 = 12.8$ μM. Again, in the cell assay, FBS was used at a 10% dilution, so the final concentration is 12.8 μM $\times$ 0.1 = 1.3 μM (to one decimal place). As stated by Bryd et al. [4], the concentration of FLAP in the fetal bovine serum assay was 0.18 μM.

3. Results

The binding affinities of BSA_{Aff}, $HSA_{Aff,}$ bAGP and hAGP were determined using CD (Fig. 2), ITC (Fig. 3) and fluorescence titration spectroscopy (Fig. 4). Using circular dichroism, spectral changes caused by the binding of both BSA_{Aff} and HSA_{Aff} could be observed (see Fig. 2, Top Left and Right). In order to observe a large enough signal change in the BSA_{Aff} titration, a high concentration of the albumin was required, namely 500 μM, compared to the usual concentrations of around 20 μM that are typically used in CD. This concentration increase shifted the drug-binding equilibrium of the BSA_{Aff}:FLAP to a more bound state, increasing the bound signal as a proportion of the total signal. It also sharpened the binding curve of drugs that bind in the lower medium scale of binding affinity (tens of micromolar affinity). As both the HSA_{Aff} and BSA_{Aff} binding curves have yet to plateau, this suggests that there is either weak, non-specific binding or the presence of multiple, varying affinity binding sites. A good estimate for the plateau is required for accurate curve fitting, or else a large error range is introduced

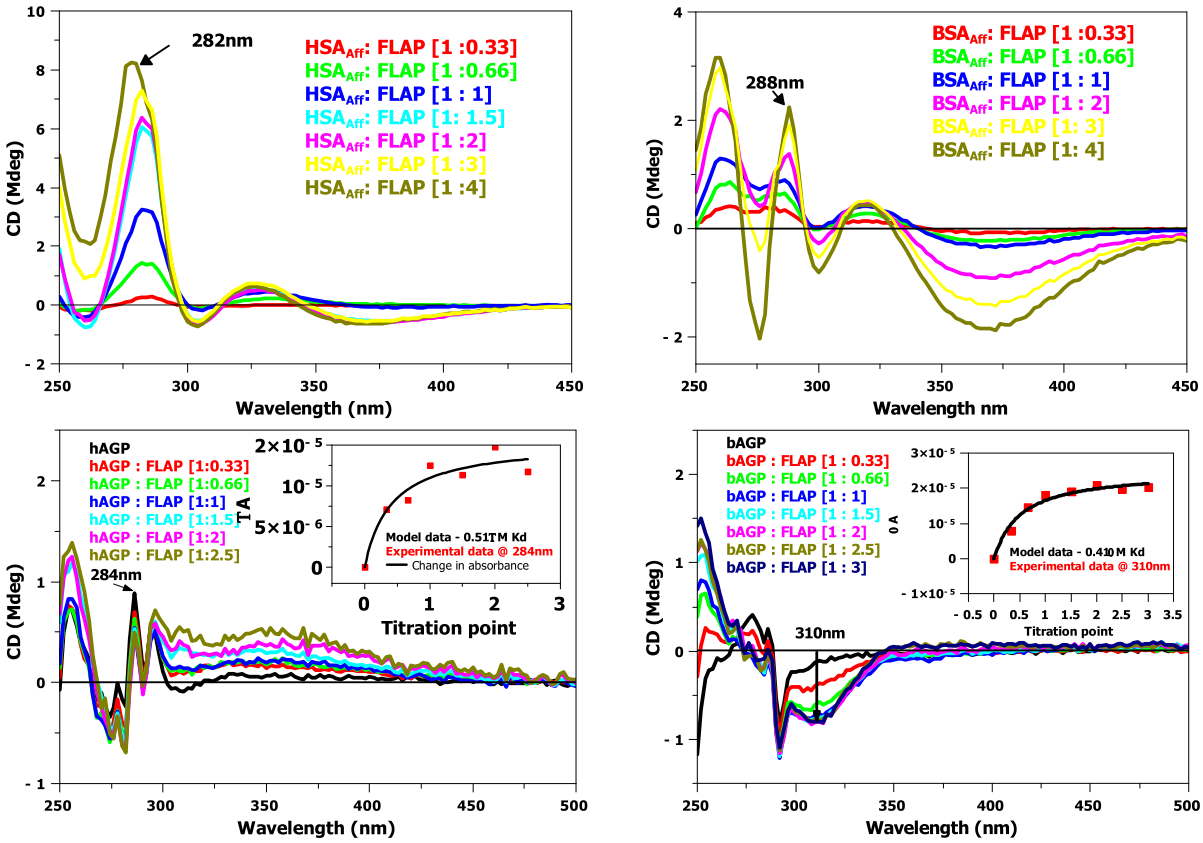

Fig. 2. Difference CD spectra of FLAP binding to aqueous human (top left) and bovine (top right) fatty acid free serum albumins (concentration of HS_{Aff} and $BS_{Aff} = 500\ \mu M$) and aqueous human (bottom left) and bovine (bottom right) alpha-1-acid glycoproteins (concentration of hAGP $= 20\ \mu M$ and bAGP $= 10\ \mu M$). The difference CD spectra were calculated by subtracting from the FLAP: Protein [n:1] mixtures the spectra of albumin [4] and the [n] equivalents of FLAP alone. Inserts show the raw data as red points fitted to model curves (black trace) of CD data at single wavelengths versus FLAP concentration using a non-linear regression analysis model.

into the binding affinity value [17]. Although the equation can be modified to accommodate multiple binding sites, without further information on the system, we run the risk of overanalysing the current data. Therefore, no attempt has been made, using single wavelength non-linear regression, to fit the binding curve and determine the binding affinities of HSA_{Aff} and BSA_{Aff}. A similar CD titration protocol was undertaken for human and bovine AGP with FLAP (see Fig. 2, Bottom Left and Right). This time, the spectra have a clear plateau using single, set wavelengths (284 and 310 nm, respectively). This allows for the accurate, one binding site modelling of the data to a binding affinity of approximately 0.5 μM.

ITC titration experiments were performed on the BSA_{Aff}, HSA_{Aff}, bAGP and hAGP (see Fig. 3). Although only small exothermic signals were obtained, they were large enough to be modelled using an independent, one binding site model. Binding affinities of 57.6, 34.1 and 2.9 μM were obtained for HSA_{Aff}, BSA_{Aff} and bAGP, respectively. No ITC titration was obtainable for hAGP, due to its aggregation upon titration of FLAP. hAGP's propensity to aggregate at higher than physiological concentrations has already been observed in ITC experiments and may be linked to its high level of glycosylation [5]. It should be noted that the ITC experiments had to balance a number of experimental variables, including

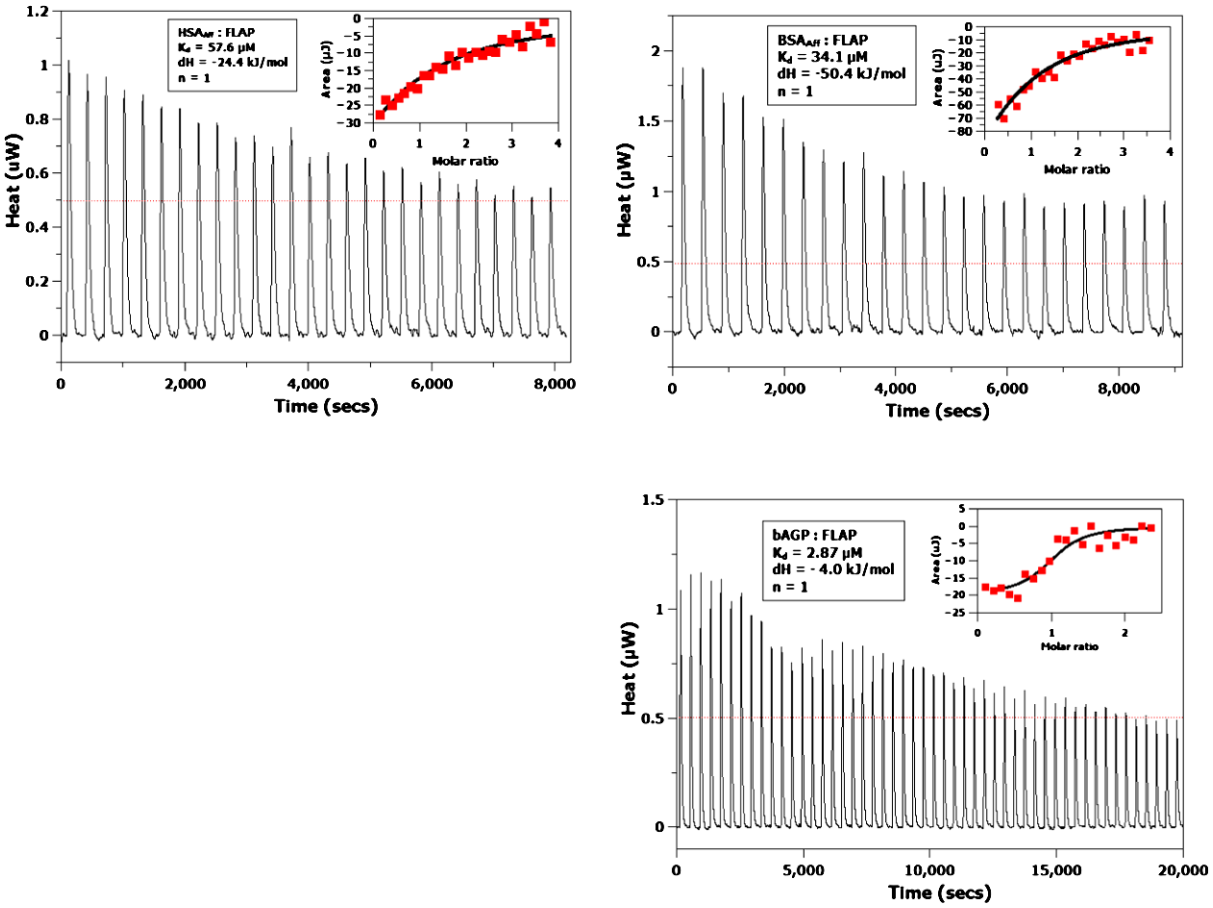

Fig. 3. ITC titrations of HSA_{Aff} (top left), BSA_{Aff} (top right) and bAGP (bottom right) with FLAP, respectively. Inserts give the raw data as red points fitted to a single binding site independent binding curve (black trace). The values obtained from the binding curves are given in the boxes. The red lines on the titrations are the raw injection data, which gives the experimental baseline from where the albumin and FLAP energies of injection were subtracted.

protein/drug concentration, precipitation, temperature, FLAP's hydrophobicity and the requirement of DMSO, whilst looking to obtaining as large an enthalpy change as possible.

Fluorescence titration experiments (see Fig. 4) are similar in format to circular dichroism titrations. The data was fitted to a one binding site, non-linear regression model at a single, maximal wavelength (HSA_{Aff} = 337 nm, BSA_{Aff} = 343 nm, hAGP = 334 nm and bAGP = 330 nm). Experimental binding affinities of 21.6, 14.6, 3.9 and 1.3 μM were determined for HSA_{Aff}, BSA_{Aff}, hAGP and bAGP, respectively. A full list of the experimental binding affinities determined are given in Table 2. The binding affinities are not overly high (i.e. in the nanomolar range) as to cause high drug retention. In fact, the values obtained (which were in the micromolar range) may help to solubilise and transport the drug. Contrary to previous hypotheses, I suggest that the high binding of flavopiridol to albumin and AGP is not the reason for its poor efficacy in human clinical trials [6].

By determining the protein concentrations for a bovine cell assay and human physiological conditions (see Table 1, human = 600 μM albumin and 20.6 μM AGP, compared to 10% fetal bovine serum cell assays = 33.83 μM albumin and 1.3 μM AGP), we see that the two situations

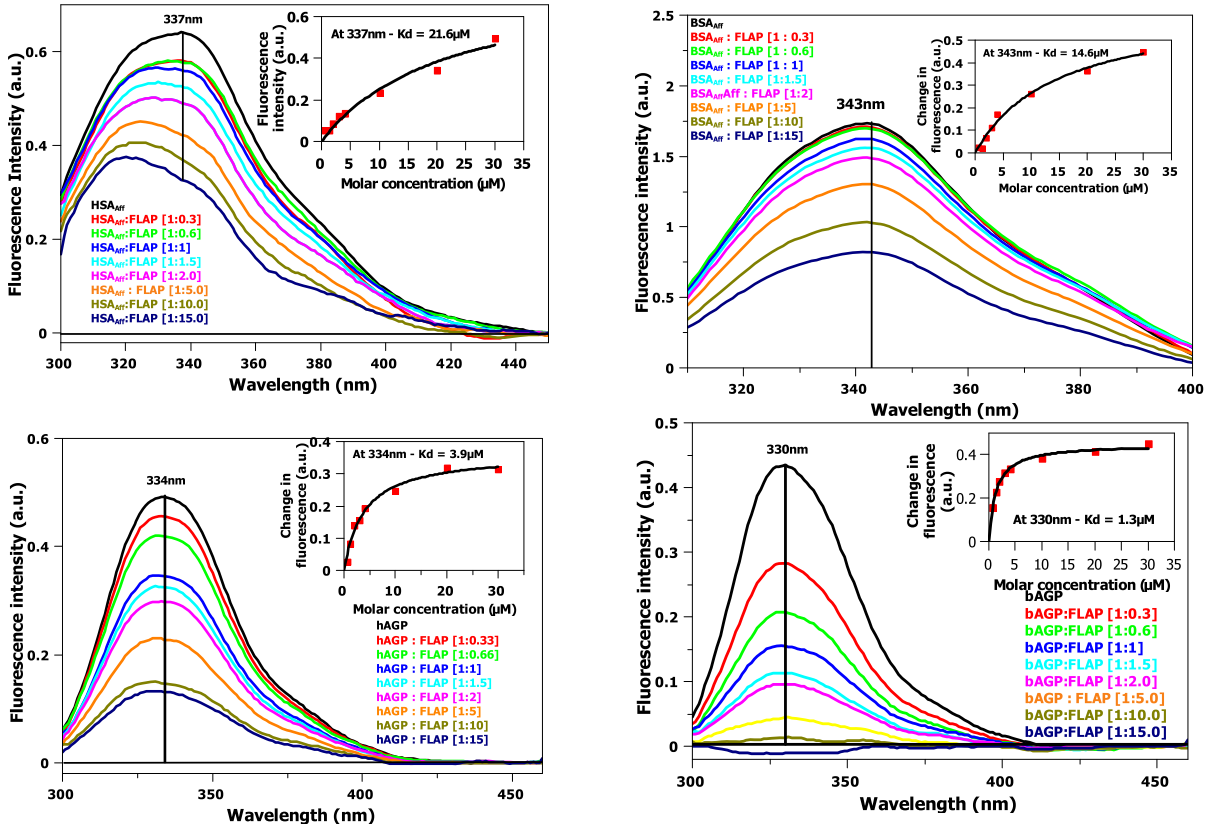

Fig. 4. Fluorescence titration of FLAP with HSA_{Aff} (top left) and BSA_{Aff} (top right) and hAGP (bottom left) and bAGP (bottom right). Corrections of the excitation and emission light intensities of the albumin due to flavopiridol UV absorption have been made. Concentration of HSA_{Aff}, BSA_{Aff}, hAGP and bAGP = 2 μM. Excitation wavelength = 285 nm, cell pathlength 1 cm.

Table 2

The binding affinity of FLAP to bovine and human blood serum albumin (fatty acid free) and alpha-1-acid glycoprotein (AGP)

	CD[1]	ITC	Fluorescence[3]	AUC[4]	Used in Model
Human Albumin (HSA_{Aff})	Not determined – see results	Binding affinity (k_D) 57.6 (46.3–76.4)[2]	Binding affinity (k_D) 21.6 (±6.5)		25
Human AGP	Binding affinity (k_D) 0.51 (±0.16)	N/A – due to aggregation at high AGP concentrations	Binding affinity (k_D) 3.9 (±0.5)		1
Bovine Albumin (BSA_{Aff}) – AUC used BSA_{FA}	Not determined – see results	Binding affinity (k_D) 34.1 (26.0–49.7)[2]	Binding affinity (k_D) 14.6 (±2.4)	Binding affinity (k_D) 20	25
Bovine AGP	Binding affinity (k_D) 0.41 (±0.20)	Binding affinity (k_D) 2.9 (1.05–3.95)[2]	Binding affinity (k_D) 1.3 (±0.1)		1

HSA_{Aff}, BSA_{Aff}, hAGP and bAGP model binding site number (n) = 1.
[1]The Chi^2/doF with 95% confidence level hAGP: FLAP = 2.38×10^{-3}, bAGP: FLAP = 1.65×10^{-3}.
[2]Range denoted in brackets show the binding affinities within a 95% confidence level.
[3]The Chi^2/doF with 95% confidence level HSA_{Aff}: FLAP = 7.12×10^{-4}, BSA_{Aff}: FLAP = 2.89×10^{-4}, hAGP: FLAP = 2.44×10^{-4}, bAGP: FLAP = 1.00×10^{-4}.
[4]The SEDFIT models fitted the experimental data with a high degree of accuracy, giving root mean standard deviations (RMSD) of 3.621×10^{-3}, 5.179×10^{-3} and 5.318×10^{-3}, for FLAP, BSA_{FA} and 1:1 molar ratio of FLAP:BSA_{FA}, respectively.

Table 3
Simulation of the percentage of bound FLAP, with different albumin binding affinities

	[Albumin], μM	[AGP], μM	Kd, AGP	Kd, Albumin	% FLAP bound
Bovine cell assay, 2 binding site model	33.83	1.3	1	10	82
				25	72
				50	66
Human physiological Conditions, 2 binding site model	600	20.6	1	10	99
				25	97
				50	96

From the table, we can see that 72% of FLAP bound in the cell assay, compared to 98% in the human clinical trial. The human trial uses a bloodstream concentration of FLAP of 20.18 μM, while the bovine assay uses 0.18 μM.

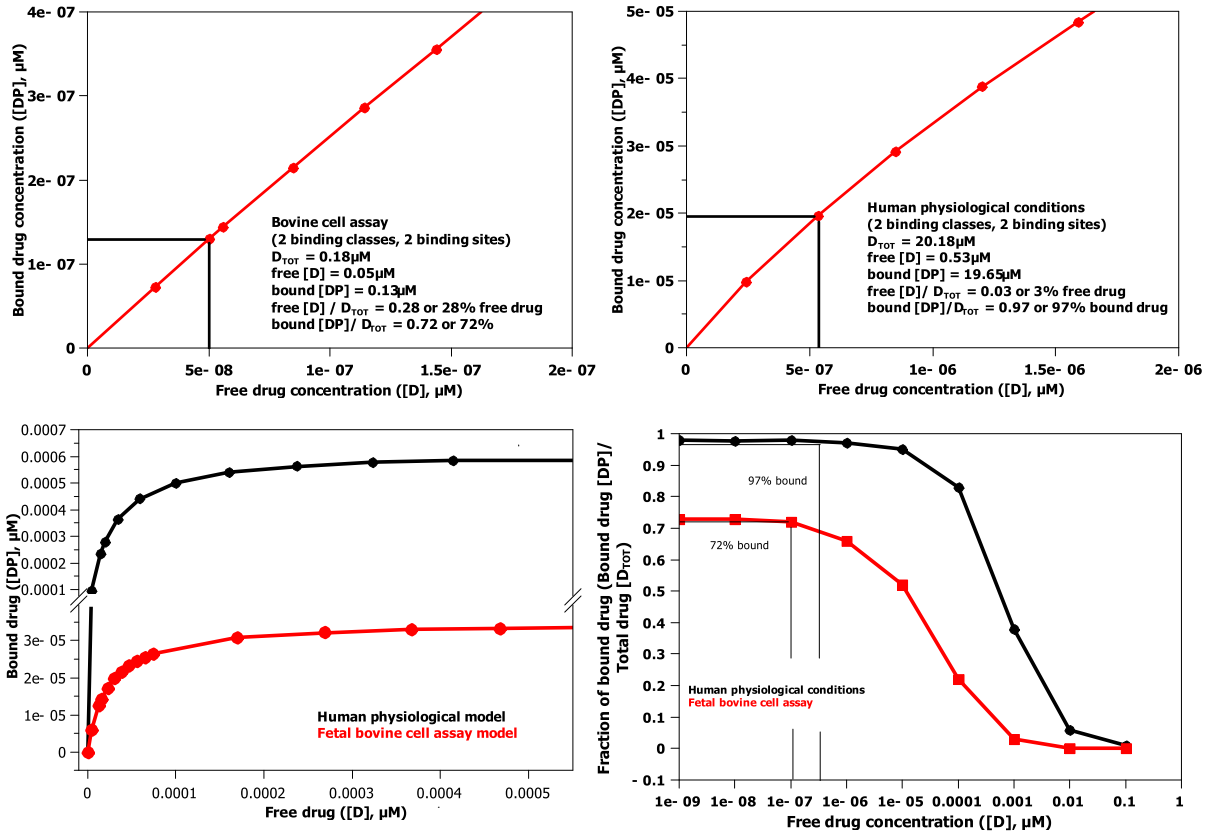

Fig. 5. The model binding curves (bottom left and magnified top left and right) and model log binding curves (bottom right) for the binding models shown in Table 3. Fetal bovine serum cell assay (red trace) and human physiological conditions (black trace). The human and bovine cell assays use the albumin and AGP concentrations described in Table 1.

are fundamentally different in terms of protein concentrations. By creating two theoretical models, the amount of free and bound drug can be determined. The models were developed from standard binding curves (see Table 3 and Fig. 5, Bottom Left). From these curves, we can build log models (Fig. 5, Bottom Right). We can take the model further by allowing two independent binding sites in the model (see Equation (2)) and replacing the protein concentration with real values for human physiological and bovine

Table 4
The free and bound concentrations of FLAP in the fetal bovine serum cell assay and human physiological conditions

FLAP	Human assay (20180 nM)	Human assay (300 nM mean peak steady state Christian et al. [6])	Bovine assay
Free, nM	532.0	6.6	50.2
Bound, nM	19648.0	293.4	129.8
[Total], nM	20180.0	300.0	180.0

cell assays (Table 1). By inputting these protein concentrations and the experimental binding affinities (see Table 2), we get the bound FLAP percentages as shown in Table 3. We see that in human physiological conditions, using 1 and 25 μM binding affinities for AGP and albumin, 97% of flavopiridol is bound. This leaves just 3% (or 545 nM using the calculated initial drug concentration of 20.18 μM) available for cell uptake. This is in comparison to the bovine cell assay, in which 72% (0.13 μM) of the 0.18 μM drug total is bound, leaving 28% (or 50.2 nM, see Table 4) free. Upon first examination, the human drug concentration is over 10 times higher than the bovine, but the human drug concentration is dependent on two factors that are not present for the bovine cell assay. First, the assumptions made in the human physiological model rely on the complete mixing of the drug in the blood. This is in contrast to the static bovine cell assay in a cell petri dish that is already fully mixed. Second, and probably more importantly, there is also rapid renal clearance of flavopiridol [3,13]. Renal excretion of flavopiridol is an exponential decay, causing the initial drug concentration (calculated here as 20.18 μM, see Table 1) to decrease rapidly to a lower level. As shown by Tan et al. [18], after 2 hours, the drug concentration is 1 μM and, after 10 hours, it is down to a steady state of around 300 nM. As the drug's efficacy is recorded over 24 hours [4], the steady state value is likely a better reflection of the total blood drug concentration. Using the value of 300 nM for total drug concentration gives a free drug concentration of 4.3 nM, which is over 10 times lower than the bovine cell assay value (50.2 nM).

AGP has a much larger initial effect on the amount of drug bound in the model, because its binding affinity is 25 times greater than albumin, when the drug level, as in both human and bovine cell assays, is comparable to or lower than the AGP concentration level (bovine assay $=$ 1.3 μM AGP to 0.18 μM FLAP, human conditions $=$ 20.6 μM AGP and 20.18 μM FLAP). By modeling just the AGP binding site, I determined that 81% of FLAP is bound in human physiological conditions and 55% of FLAP is bound in the bovine cell assay. Serum albumin is still important, binding approximately 20% of the FLAP in these conditions (human: 81% AGP + 16% albumin $=$ 97%, bovine: 55% AGP + 18% albumin = 72%). The reason albumin still has a significant effect is because, although its affinity is lower, its concentration is approximately 30 times higher Another important consideration is the difference in protein concentrations of the bovine cell and human assays. As the human protein concentrations are approximately 20 times the bovine cell assays (20.6 μM and 600 μM for human AGP and albumin, respectively, compared to 1.3 μM and 33.83 μM for the bovine cell assay), the drug-protein equilibrium shifts to the bound state at the higher concentration. This is why human physiological conditions generally have a higher bound percentage (e.g. 97% drug bound in the human model, compared to 72% drug bound in the bovine for a two binding site $K_D = 1$ μM and 25 μM model.) The log curve graphs shown in Fig. 5 (Bottom Right) show the relationship between free drug concentration and the drug bound percentage.

FLAP is a drug designed to treat CLL [3], but within the required dose calculation, little account has been taken of the albumin and AGP concentration changes that occur in a cancer patient. Cancer generally causes a 2–10 fold increase in AGP levels (even higher if the patient has undergone surgery)

Table 5
A table modeling the effect of changes in albumin and AGP concentrations, as seen in cancer, and how that affects the levels of free FLAP

[AGP], μM	[Albumin], μM	Percentage Unbound, %	Unbound [FLAP], nM
20.6 normal	600 – 100%	2.64	532
	450 – 75%	3.22	650
	300 – 50%	4.21	850
41.2 2-fold	600 – 100%	1.83	369
	450 – 75%	2.10	424
	300 – 50%	2.49	502
103 5-fold	600 – 100%	0.89	180
	450 – 75%	0.95	192
	300 – 50%	1.02	205
206 10-fold	600 – 100%	0.47	95
	450 – 75%	0.48	98
	300 – 50%	0.50	101

and a concomitant decrease in albumin levels to 70–80% of usual levels [10,12]. These serum protein concentration changes caused by cancer can be introduced to the model (see Table 5) and have a profound effect on the free and bound drug concentrations. From Table 5, we can see that a 10 fold increase in AGP concentration causes a 5 fold decrease in initial FLAP concentration levels, which is significant, while a 50% decrease in albumin levels has an approximate 1.6 fold increase in flavopiridol concentrations. As the first step to obtaining experimental data to validate the models produced, an AUC experiment was undertaken using BSA_{FA} and FLAP (see Fig. 6). All components were recorded separately and then later in a 1:1 molar ratio. First, all components were visible by AUC, including flavopiridol (Mw = 438.3 at 0.13 S), albumin monomers (at 3.9 S) and a small component of dimers (at 6.3 S). As the absorption spectrum at 280 nm was used to record the standard processed data, it was possible to determine a value for the amount of free flavopiridol. This was accomplished by determining the percentage difference, which was achieved by dividing the area under the free FLAP peak when it was in a 1:1 complex with BSA_{FA} (green trace) with the area of the free 25 μM flavopiridol (black trace). A value of 57.3% was determined (see Table 6). This value was in agreement with the expected model value for a one binding site protein with a binding affinity of 20 μM, resulting in a free FLAP value of 58.4%.

4. Discussion

A number of conclusions can be drawn from this paper. First, flavopiridol does not bind to bovine or human albumin and AGP with an overly high binding affinity. In fact, the mid-range binding affinity (in the micromolar range) of both proteins may actually be beneficial for the drugs' solubilisation and transport. As a result, further drug modification of flavopiridol, to reduce its binding affinity to albumin or AGP, would not be beneficial. Second, it is the free drug concentration, not the total drug concentration, that is routinely recorded in clinical papers, which is important for the drug's efficacy at its target [11,15]. Third, although it does not make a significant difference in the case of flavopiridol, it is still common practice to use fetal bovine serum in cell assays in drug development for historical reasons. Species specific differential binding affinity, as seen with the drug ET-743, can be significant [1,19]. All cell

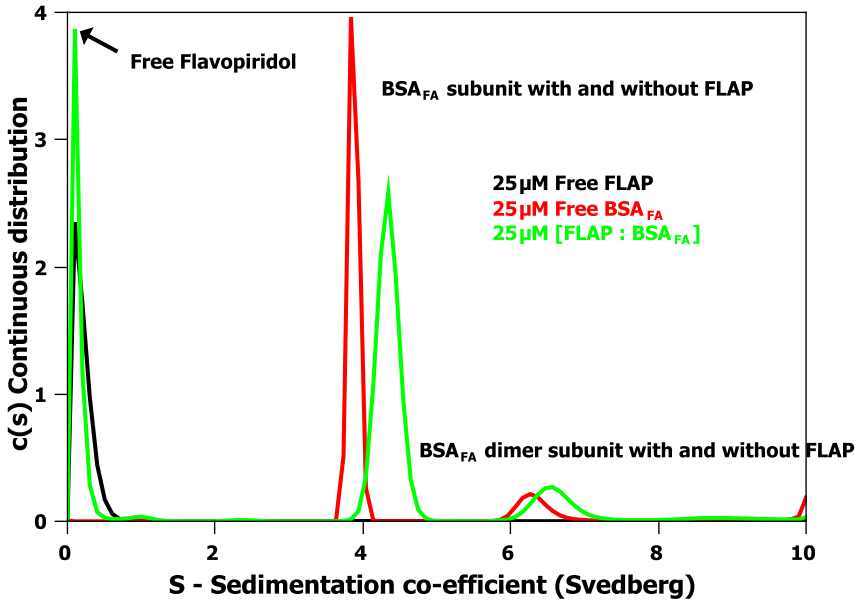

Fig. 6. Equilibria Analytical ultracentrifuge data of 25 μM FLAP (black), 25 μM BSA_{FA} (red) and a mixture of 25 μM FLAP: 25 μM BSA_{FA} (green) in ddH_2O. The sedimentation coefficient distribution was obtained by SEDFIT Analysis [9] of the absorbance data at 280 nm of a sedimentation velocity experiment at 129,024 g (RCF) and 20°C.

assays should be performed with human serum, which is now commonly available and only slightly more expensive than fetal bovine serum. Fourth, due to flavopiridol's poor clinical effect in human clinical trials, the dose schedule has now been modified and increased in concentration and duration [3]. While this methodology shows some response improvement, it has also brought about an increase in side effects due to greater toxicity levels. As stated previously, albumin and AGP levels may alter radically in disease states, including cancer. However, little account has previously been taken in accommodating these changes in patients. Measuring patient AGP and serum albumin concentrations, which is relatively easy to do in the clinic, would allow for individual modelling of a patient's drug dose. The tailoring of a drug dose to individual patients would help produce maximal drug efficacy with minimal toxicity side effects. The model and protocol set out in this paper can be used with any drug or combination of drugs (commonly used in cancer treatment). This methodology may help better link clinical drug trials to experimental biochemical binding affinity data, becoming an integral part of drug development.

5. Future work

This paper is a first step toward combining experimentally determined binding affinities with theoretically determined binding models, to give insights into pre-clinical fetal bovine serum assays in comparison to human patients. This method can be further validated using different drug targets and complimentary experimental techniques such as ultrafiltration and equilibrium dialysis (LC-MS). Serum albumin and alpha-1-acid glycoprotein have been implicated in the binding of flavopiridol, although other proteins such as lipoproteins, immunoglobulins and erythrocytes could also be involved [16]. The theoretical models produced could also be expanded in the future to include these proteins.

Table 6
The location and area of the analytical centrifugation (AUC) peaks as shown on Fig. 6

	FLAP Location (S)	Percentage area	Local concentration (integrated area)
FLAP	0.13	0.99	0.96
BSA_{FA} monomer	0	0	0
BSA_{FA} dimmer	0	0	0
	Total		
RMSD of model to theoretical data	3.621×10^{-3}		
	BSA_{FA} Location (S)	Percentage area	Local concentration (integrated area)
FLAP	0	0	0
BSA_{FA} monomer	3.88	84.8	0.75
BSA_{FA} dimmer	6.33	12.4	0.11
	Total	97.2	
RMSD of model to theoretical data	5.179×10^{-3}		
	BSA_{FA}:FLAP Location (S)	Percentage area	Local concentration (integrated area)
FLAP	0.14	31.6	0.55
BSA_{FA} monomer	4.34	55	0.96
BSA_{FA} dimmer	6.59	9.9	0.17
	Total	96.5	
RMSD of model to theoretical data	5.318×10^{-3}		
Free FLAP area / Total FLAP area			**0.55/0.96 = 57.3%**
1 binding site model with a $K_D = 20\ \mu M$			14.6 μM/25 μM = 58.4%

The area of the free FLAP in the FLAP: BSA_{FA} complex has been divided by the 25 μM free FLAP peak to give the percentage of free FLAP in the complex. The figure was then been modeled to determine the binding affinity of the complex. The RMSD of the model to the theoretical data gives an indication of the goodness of fit.

Declarations of interest

The author declares that there are no competing interests with the manuscript.

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Supplementary data

Supplementary data is available at: http://dx.doi.org/10.3233/BSI-170165.

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