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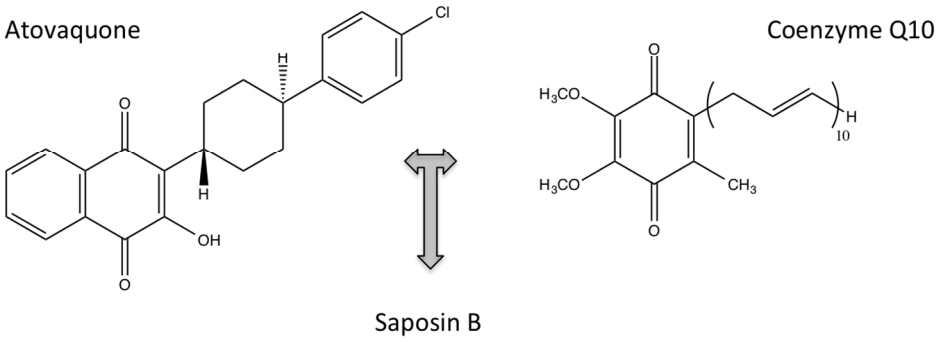

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The Antimalarial Drug Atovaquone Binds to Saposin B with Comparable Affinity to Coenzyme Q10

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Atovaquone is a front-line antimalarial drug that functions by competitively inhibiting binding of coenzyme Q10 to the cytochrome bc_1 complex. Atovaquone is administered orally, but has low solubility and is poorly absorbed with high variability in bioavailability. In vivo binding of human serum albumin has been cited as the major transporter in plasma. The research presented herein demonstrates that saposin B, a known binder/transporter of coenzyme Q10, also binds to atovaquone in a 1:1 ratio and with comparably high affinity at pH 5.5.

Introduction

Atovaquone (ATO) is a quinone based, orally administered, pharmaceutical effective against several disease-causing protozoa including *Plasmodium falciparum* (Malaria), *Pneumocystis jiroveci* (Pneumonia), and *Toxoplasma gondii* (Toxoplasmosis).[1]

The activity of ATO is tracked to its ability to competitively inhibit (K_i 9 nM) Coenzyme Q10 (CoQ10; ubiquinol) binding to the parasite cytochrome bc_1 complex, with a myriad of downstream consequences to the parasite's mitochondrial respiratory chain.[2]

Considerable work has been conducted in resolving issues pertaining to the poor bioavailability and high variance across patients of the poorly soluble ATO with diet upon ingestion playing a significant role (For a comprehensive summary on ATO (see a recent review by Biagini *et al*.).[1]

In human plasma, however, it appears that ATO is clearly bound (>99.5%) to plasma proteins with Zsila *et al*. specifically demonstrating that human serum albumin (HSA) has two high-affinity binding sites with K_a of ~ 2 x 10^6 M^{-1} as measured by circular dichroism spectroscopy.[3]

What is not clear, however, is what other proteins may play a role in the pharmacokinetics of ATO and whether such proteins may compete with HSA for binding, actively transport ATO in vivo, remove ATO from circulation, facilitate ATO degradation or be themselves functionally affected by ATO (a drug typically well tolerated with minimal side-effects).

Recent work by Yamamoto *et al*.[4] and from our laboratory[5] as well as others[6] has clearly shown that saposin B (sapB), a protein responsible for aiding in lysosomal sphingolipid hydrolysis,[1] is a human CoQ10 binding protein. Yamamoto also showed that sapB transports CoQ10 by demonstrating the presence of CoQ10 bound to sapB in human urine.[4] SapB is, in general, important in human health with the protein present in most tissues as well as blood plasma.[1] Deficiency of sapB is implicated in metachromatic leukodystrophy, a lysosomal storage disease that leads to a toxic build up of lipids with poor patient prognosis.[7]

While working on human sapB based recombinant expression[5] and its binding of CoQ10 it occurred to us that, given the mechanism of action of ATO and its similarity in structure to CoQ10, it might be expected that sapB would bind ATO, a result with possible implications for the pharmacokinetics of ATO noted above. To address this intriguing possibility, isothermal titration calorimetry (ITC) experiments of sapB binding to both ATO and CoQ10 were performed.

Our results indicate that sapB binds ATO with two independent classes of binding sites, one strong and one weak, with the strong binding site having comparable affinity to that

of CoQ10. In either case, the stoichiometry of the strong class of binding sites was noted as 1:1 sapB to ATO or CoQ10.

Results and Discussion

The human sapB protein used in the studies herein was obtained by recombinant expression in *E. coli* as previously reported and has been extensively investigated and validated for CoQ10 binding in aqueous isopropyl alcohol (IPA).[5] Given the lack of aqueous solubility of CoQ10 (and ATO), solubility studies were necessary to facilitate mixing of the quinone with the sapB protein in aqueous buffer. We established that use of 10 % (v/v) isopropyl alcohol (IPA) in MES buffer (pH 5.5) did not result in sapB unfolding while dissolving the CoQ10. Unfortunately, when translating this work to ATO, we observed that ATO was not sufficiently soluble in the 10 % IPA/MES. Use of 4 % THF (or 10 % DMF) at pH 5.5, however, did allow suitable solubility of both CoQ10 and ATO to enable ITC measurements and some comparison to be made between the quinones and their sapB binding behaviour. To verify that sapB maintained its characteristic helical conformation for the ITC binding studies, circular dichroism (CD) studies were performed.

The results of these studies are shown in Figure 1 and demonstrate that the introduction of 4 % THF solvent did not significantly impact the secondary structure of sapB. The spectra shown in Figure 1 were of samples runs in phosphate buffer with THF since MES and DMF absorb strongly in the spectral window explored (see experimental procedure).

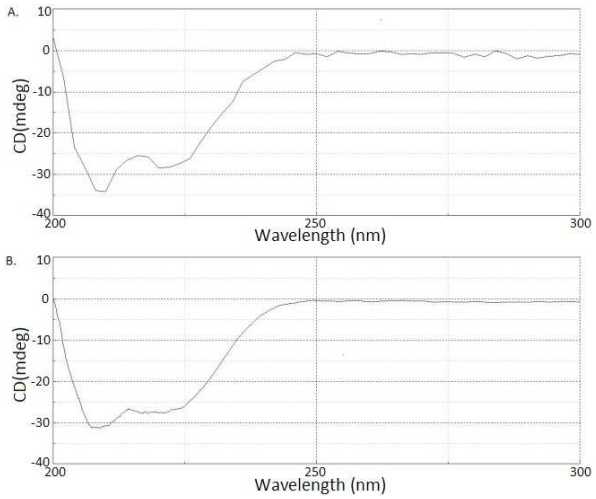


Figure 1. CD data for sapB. (A) Performed in 20 mM phosphate buffer, pH 5.5, and (B) performed in 20 mM phosphate buffer, pH 5.5, 4 % THF. Both samples contained 20 µM sapB.

ITC was performed initially for CoQ10 with sapB in MES buffer, pH 5.5. The pH was chosen based on the lysosomal pH (5.5) where sapB primarily functions. We have also demonstrated preferential binding at pH 5.5 over pH 7 or 9 previously.[5] Figure 2 shows the raw ITC results (panels A and C) with the corresponding integrated heats (µW) for each injection *vs* the molar ratio of CoQ10 to sapB (panels B and D). The heats at the end of these titrations were in excess of the heat of dilution, indicating an additional weak association event. Accordingly, a model of two sets of independent binding sites, one strong and one weak, was used to fit the data. Because of this weak binding event, the thermodynamic parameters cannot be individually determined and only the product of the three parameters, $n_2.K_2.\Delta H_2$ can be reported using the TA Instrument curve fitting software (see Table 1). However, excellent fits were achieved for the strong binding sites and the derived thermodynamic parameters are compiled in Table 1. Interestingly, whether 4 % THF or 50 % DMF is used to solubilize CoQ10, the ITC data show an endothermic binding event for the first class of strong binding sites with a stoichiometry of approximately one CoQ10 per sapB monomer and a dissociation constant in the range of 37-59 nM (Table 1).

The sign of the heats in the ITC figures are given relative to the TA Instruments isothermal titration calorimeter. Negative and positive measured heats correspond to endothermic and exothermic binding reactions, respectively. Thus, after subtracting the control heats (shown in red in Figure. 2, panels A and C and Figure. 3, panel A), endothermic events for the first class of strong binding sites (positive values of the integrated heats in Figure. 2, panels B and D and Figure 3, panel B) are observed and reported in Table 1 as ΔH_1. The second class of weak binding sites shows positive heats (or exothermic binding reactions) with undefined binding parameters.

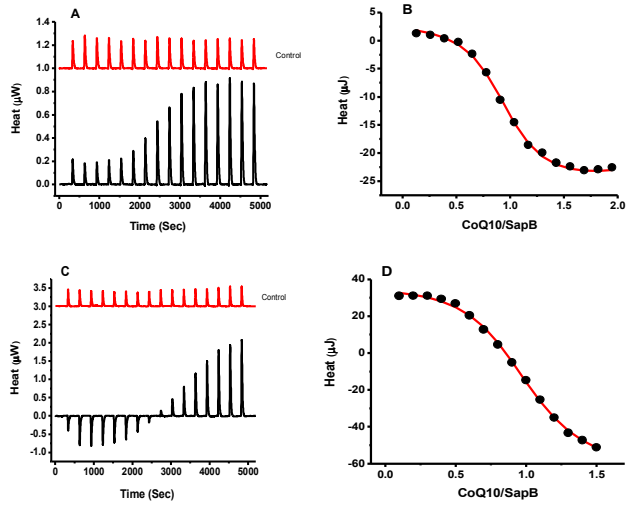


Figure 2: Calorimetric titration of coQ10 with sapB. (A) and (C) are raw ITC data; (B) and (D) are plots of the integrated heat versus the coQ10/sapB molar ratio. Conditions: 15 µM sapB, 120 µM CoQ10 in 50 mM MES and 4 % THF by volume (panels A and B) and 25-27 µM sapB, 154 µM CoQ10 in 50 mM MES and 50 % DMF by volume (panels C and D). The ITC titrations were carried out with a total of 16 injections, 3 µL each, pH 5.5 and 25.00 °C.

Table 1: Best fit parameters for ITC measurements (N = 4) of sapB binding to CoQ10 and to ATO at pH 5.5 and 25.00 °C.

	15 ! M sapB, 120 ! M CoQ10 in 50 mM MES and 4% THF, pH 5.5	25-27 ! M sapB , 154 ! M CoQ10 in 50 mM MES and 50% DMF, pH 5.5	15-20 ! M sapB , 150 ! M ATO in 50 mM MES and 10% DMF, pH 5.5
$K_1(M^{-1})$	(2.41 ± 0.15) ! 10^7	(1.7 ± 0.54) ! 10^7	(1.11 ± 0.37) ! 10^7
$!H_1^0$ (kJ/mol)	10.37 ± 0.73	90.48 ± 4.63	17.97 ± 8.06
n_1	1.1 ± 0.1	1.06 ± .05	1.16 ± 0.15
$!G_1^0$ (kJ/mol)	- 42.13 ± 0.14	- 41.27 ± 0.78	- 40.12 ± 0.81
$!S_1^0$ (J/mol.K)	176.08 ± 0.74	441.9 ± 4.69	134.63 ± 2.73
$n_2.K_2.!H_2$	(- 2.41± 0.98) ! 10^8	(- 3.82 ± 1.32) ! 10^8	(-4.38 ± 2.14) ! 10^8

The reported quantities are apparent average values and standard errors from replicate determinations (N = 4) are indicated.

To examine whether an association between ATO and sapB is possible, ITC binding experiments were carried out at pH 5.5 in MES buffer and 10 % DMF by volume. Here again, an endothermic reaction is observed and a good fit of the ITC data (Figure 3B) is achieved using a model with two sets of independent binding sites. As with CoQ10, both strong and weak binding classes of sites are reported with ATO (Figure 3, Table 1) with a stoichiometry of 1 molar equivalent of ATO per protein monomer for the strong binding sites. Significantly, the dissociation constant of the ATO-sapB complex is on the same order of magnitude as that of CoQ10-sapB, suggesting a similar binding affinity of these two ligands to sapB. On a side-note, the anti-malarial artemisinin was also screened for binding by sapB and was not bound.

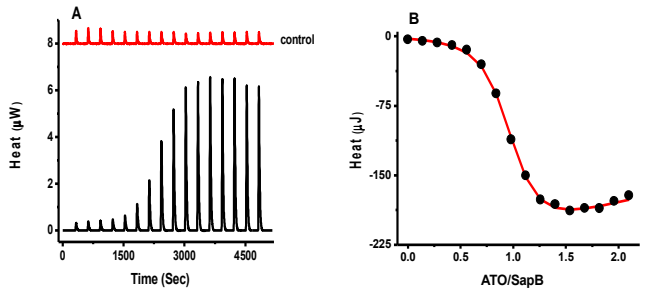
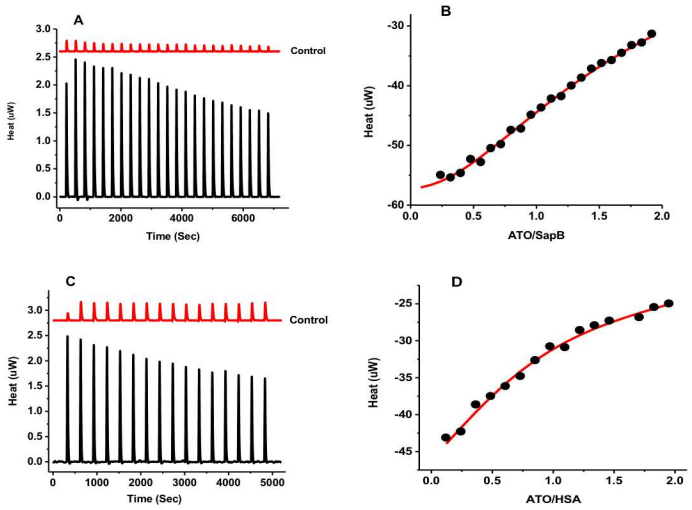


Figure 3: Calorimetric titration of ATO with sapB. (A) Raw ITC data; (B) plot of the integrated heat versus the ATO/sapB molar ratio. Conditions: 15-20 μM sapB, 150 μM ATO in 50 mM MES and 10 % DMF by volume, 16 injections, 3 μL each, pH 5.5 and 25.00 °C.

The aforementioned results raised questions related to whether sapB could bind ATO at pH 7.4 and how this binding compares to that reported for HSA.[3] To answer these questions, ITC was again used to examine such putative binding for both sapB and HSA to ATO at pH 7.4. The results are shown in Table 2 and Figure 4 below and demonstrate that both proteins bind ATO at pH 7.4 with similar binding affinities (on the order of 10^5 M^{-1} for both proteins).

The ITC data in Figure 4 show a stoichiometry of ~ 0.5 ATO molecules per sapB monomer or per HSA protein. Interestingly, the enthalpy change at pH 7.4 is negative, suggesting an exothermic binding reaction. Earlier



measurements using circular dichroism[3] reported a binding affinity of HSA to ATO on the order of 10^6 M^{-1}, a value 10-fold lower than the ~ 10^5 M^{-1} measured here by ITC. Of note, fluorescence quenching experiments of ATO binding to HSA corroborated the ITC results reprorted herein and gave similar stoichiometry and affinity constant values {i.e. n = (0.51 ± 0.08) and K_a = (7.15 ± 1.04) × 10^4 M^{-1}}. The detailed thermodynamic parameters obtained by ITC are reported in Table 2.

Figure 4. Calorimetric titration of ATO with sapB and human serum albumin (HSA). (A) and (C) are raw ITC data; (B) and (D) are plots of the integrated heat versus the ATO/sapB or ATO/HSA molar ratio. Conditions: 20 μM protein, 150 μM ATO in 20 mM phosphate and 30 % DMF by volume, pH 7.4 and 25.00 °C. The ITC titrations were carried out with either 24 injections of 2 μL each (panel A) or 16 injections of 3 μL each (panel C).

	20 ! M sapB, 150 ! M ATO in 20 mM phosphate and 30% DMF, pH 7.4	20 ! M HSA, 150 ! M ATO in 20 mM phosphate and 30% DMF, pH 7.4
$K_1(M^{-1})$	(8.02 ± 2.18) X 10^4	(1.06 ± 0.09) X 10^5
$!H_1^0$ (kJ/mol)	-84.34 ± 3.84	-101.02 ± 0.85
n_1	0.62 ± 0.17	0.56 ± 0.07
$!G_1^0$ (kJ/mol)	-27.99± 0.27	-28.67 ± 0.22
$!S_1^0$ (J/mol.K)	188.99 ± 3.85	242.66 ± 0.88
$n_2.K_2.!H_2$	(-1.11 ± 0.21) ! 10^6	(-7.66 ± 2.51) ! 10^7

Table 2. Best fit parameters for ITC measurements (N = 2) of sapB and HSA binding to ATO at pH 7.4 and 25.00 °C. The reported quantities are apparent average values and standard errors from replicate determinations are indicated.

Thus, under our experimental conditions, the ITC and fluorescence results indicate that at pH 7.4, sapB exhibits

similar affinity for ATO compared to HSA, raising the possibility that sapB may bind and transport ATO in serum.

In conclusion, we demonstrate that sapB, a protein distributed across human tissues and in blood serum, binds ATO with affinities that suggest a possible role in the pharmacokinetics of the drug. Questions as to whether sapB-ATO might be observed in human urine or human serum or if sapB might facilitate hydrolysis of ATO in lysosomes are intriguing. Furthermore, if sapB is significantly bound to ATO when administered in high doses, could this affect normal sapB function as it pertains to lipid hydrolysis?

Experimental Procedures

ATO, CoQ10 (Sigma-Aldrich, St. Louis, MO), 2-(N-Morpholino)ethanesulfonic acid (Research Organics, Cleveland, OH) was purchased and used as supplied. Typically, a stock solution of 400 µM sapB was prepared in either 50 or 100 mM MES, pH 5.5 and 30 % DMF and then diluted down to a final working solution of 15-27 µM in either 50 mM MES, 4 % THF by volume or 50 mM MES, 10 % or 50 % DMF by volume. The stock solutions of ATO and CoQ10 were initially prepared in 100 % THF or 100 % DMF and diluted down to a final working solution of 120-154 µM CoQ10 or ATO in 50 mM MES and either 4 % THF, 10 % or 50 % DMF. The stock solutions for the pH 7.4 measurements were prepared similarly and the experiments were carried out at final working solutions of 20 mM phosphate and 30 % DMF.

SapB Expression and Purification

A single colony of BL21 Gold (DE3) *E. coli* containing a pET27b+_sapB construct was picked and grown in 5 mL of LB media supplemented with 5 µL of kanamycin (35 mg/mL). The cells were incubated at 37 °C while shaking at 250 rpm over 16 hours. A 2 L flask containing 500 mL of LB media with 500 µL of kanamycin (35 mg/mL) was inoculated with 5 mL of the overnight culture. The cells were allowed to incubate at 37 °C while shaking at 250 rpm until an OD_{600} of 0.6 was reached. Protein expression was induced via the addition of isopropyl β-D-1-thiogalactoside to a final concentration of 0.01 mM. The temperature was reduced to 30 °C and the cells were incubated at 250 rpm for an additional 20 hours. The cells were pelleted by centrifugation (8,500*g*, 4 °C, 60 minutes) and the supernatant containing sapB was decanted and filtered through a 0.45 µm filter while on ice.

The protein was purified via immobilized metal affinity chromatography using a 5 mL crude Co^{2+} HiTrap™ TALON® column on an Akta Prime (GE Akta) FPLC. Buffer A consisted 20 mM sodium phosphate, 500 mM NaCl, buffered at pH 7.4 (PBS). Buffer B contained PBS with 250 mM imidazole at pH 7.4. The sample was loaded using a 150 mL superloop (GE). The protein was bound using 100 % buffer A at a flow rate of 2 mL/min. The elution was 90 % buffer A and 10 % buffer B at a flow rate of 2 mL/min. The protein was analysed by SDS-PAGE and MALDI-ToF MS. Protein concentrations were assayed using NanoDrop.

Protein Characterization

Circular Dichroism (CD) spectroscopy was performed using pure protein dialyzed into 20 mM sodium phosphate (pH 5.5), with four changes over 24 hours. The sapB concentration used for these experiments was 20 µM and the CD was run with the following parameters: 2 nm data pitch, 0.5 nm bandwidth, 0.5 s response, 50 nm/min scanning speed, room temperature, standard sensitivity. THF was added up to 4% in the phosphate buffered sample and the CD was repeated with the following conditions: 2 nm data pitch, 0.5 nm bandwidth, 0.5 s response, 20 nm/min scanning speed, room temperature, standard sensitivity. Both phosphate buffered samples had a scan range from 200 to 350 nm. Concentrations were determined by a Bradford assay (Bio-Rad).

Isothermal Titration Calorimetry

ITC experiments were carried out on a TA Instruments low volume Nano ITC with gold cells and an active cell volume of 185 µL. The association constant (K_a), stoichiometry (n) and the enthalpy change (ΔH^0) of binding were simultaneously and directly determined in a single ITC experiment and the Gibbs free energy of binding (ΔG^0) and the entropy change (ΔS^0) of the reaction were calculated using the relationships $\Delta G^0 = -RT\ln K$ and $\Delta G^0 = \Delta H^0 - T\Delta S^0$. The theoretical background and other practical aspects of ITC are discussed in detail elsewhere.[8-11] In this study, all titrations were performed at 25.00 °C in 50 mM MES buffer and either 4 % THF, 10 % or 50 % DMF by volume, pH 5.5 with a stirring rate of 250 rpm and a titrating syringe volume of 50 µL. Typically, an automated sequence of 16 injections, each of 3 µL ATO or CoQ10 was titrated into the sample cell containing sapB spaced at 5 min intervals to allow complete equilibration were performed with the equivalence point coming at the area midpoint of the titration. The data were collected automatically and analyzed using the NanoAnalyze software from TA Instruments and a mathematical model involving two classes of independent binding sites. All experiments were repeated four to eight times to ensure reproducibility with a background correction in the absence of sapB to account for the heat of dilution. Standards errors from replicate determinations are indicated in the tables and conditions for each experiment are given in the figure captions.

Fluorescence Spectroscopy

Fluorescence quenching measurements were performed at 25.0 °C in 14 mM phosphate buffer and 30 % DMF, pH 7.40 on a Varian Cary Eclipse fluorimeter with excitation at 280 nm. The bandwidths of the excitation and emission monochromators were 5 nm. Several injections of 3 µL each of an ATO solution at 845 μM in 100 % DMF were made to a 33.8 μM protein solution in phosphate buffer at pH 7.4. The fluorescence quenching data were fitted to the following binding equation:

$$I = I_o - \frac{(I_o - I_\infty)}{2n[P]_o} \cdot \left[1/K + [L]_o + n[P]_o - \sqrt{(1/K + [L]_o + n[P]_o)^2 - 4n[L]_o[P]_o}\right]$$

where K is the association constant, $[P]_o$ and $[L]_o$ are the protein and ligand (ATO) concentrations, and I_o and I_∞ are the relative fluorescence intensities in the absence of ATO and in the presence of ATO when the sites are fully saturated, respectively.

Notes and references

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