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DETERMINATION OF THE AFFINITY OF IBUPROFEN TOWARD ALDEHYDE DEHYDROGENASE BY FLUORESCENCE QUENCHING

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ABSTRACT

The interaction of Ibuprofen with Aldehyde dehydrogenase (ALDH) was investigated by fluorescence spectroscopy. Spectrofluorimetric measurements revealed that Ibuprofen could not strongly quench the ALDH intrinsic fluorescence but produced a non-fluorescent complex. ALDH has one binding mode for Ibuprofen at its saturating concentration. The dissociation constant (K_d) value of the ALDH-Ibuprofen complex was 2.20 X 10^{-5} M with an association constant (K_a) of 1.54 X 10^{-8} M at 25°C, pH 7.0 indicating ALDH-Ibuprofen complex dissociation is more favourable than its association. The equilibrium constant of ALDH-Ibuprofen complex was not significantly affected by acidic pH of 5.0 and alkaline pH 9.0. The standard Gibbs free energy changes (ΔG) of dissociation of ALDH-Ibuprofen complex were not spontaneous but entropic driven. The thermodynamic data showed that the interaction between ALDH and Ibuprofen was through Van der Waals forces and Hydrogen bond. Speculatively, the affinity of Ibuprofen for ALDH could be used as a structural probe to examine the inhibitory mechanism for treatment of ALDH associated diseases.

Keywords: Ibuprofen; Aldehyde dehydrogenase; fluorescence quenching, Association constant

INTRODUCTION

Ibuprofen, (2RS)-1[4-(2-methyl propyl) phenyl] propionic acid or isobutyl phenyl propanoic acid (Fig 1), is a non-steroidal, orally administered and over the counter anti-inflammatory drug (NSAID) used for treating pain, fever, inflammation, painful menstrual periods, migraines, and rheumatoid arthritis (Beaver, 2003; Bushra and Aslam, 2010). It is one of the commonly and frequently prescribed NSAID (Bushra and Aslam, 2010). As with other NSAIDs, Ibuprofen is handy in the treatment of severe orthostatic hypotension (Zawada, 1982) and lowers the risk of Parkinson diseases (Chen *et al.*, 2005). It is sparingly soluble in water with a pKa of 5.3 (Herzfeld and Kummel, 1983).

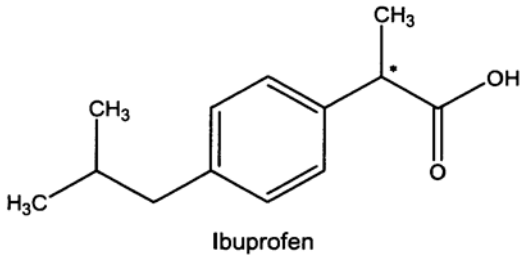

Fig 1: Structure of Ibuprofen

The effectiveness of Ibuprofen, like any other NSAIDS, is attributed to the inhibition of cyclooxygenase (COX) enzymes that convert arachidonic acid to prostaglandin H_2 (PGH_2) (Hamoya *et al.*, 2016; Bushra and Aslam, 2010). PGH_2, in turn, is converted by other enzymes to

several other prostaglandins (which are mediators of pain, inflammation, and fever) and to thromboxane A2 (which stimulates platelet aggregation, leading to the formation of blood clots). Ibuprofen self-medication has good tolerance and safety (Macesková, 2001). The risk of hepatotoxicity and gastrointestinal hemorrhage of Ibuprofen is linked to its concomitant usage with alcoholic drinks (Corelli, 2004). However, alcohol metabolism undergoes a two-step process by Alcohol dehydrogenase and aldehyde dehydrogenase.

Aldehyde dehydrogenases (ALDH) are involved in the $NAD(P)^+$-dependent oxidation of endogenous and exogenous aldehydes to their corresponding carboxylic acids or CoA esters (Vasiliou *et al*., 2004; Yoval-Sańchez and Rodríguez-Zavala, 2012; Morgan and Hurley, 2015). The corresponding acids are less toxic and easier to eliminate. ALDH superfamily contributes to numerous biological functions as well as to the cellular defense against aldehyde cytotoxicity. Aldehydes are generated from chemically diverse endogenous and exogenous precursors and aldehyde-mediated effects vary from homeostatic and therapeutic to cytotoxic and genotoxic. ALDHs are involved in the synthesis of critical carboxylic acids including retinoic acid, a key regulator of cell growth and development, and the neurotransmitter, γ- aminobutyric acid (Yoshida *et al*., 1992; Kurys *et al*., 1989). Increased ALDH (e.g., ALDH1A1) gene expression and unbalanced catalytic activity have been associated in a variety of disease states, including alcoholic liver disease, Sjögren−Larsson syndrome (SLS), type 2 hyperprolinemia, hyperammonemia, Parkinson's disease, and cancers (Marchitti *et al*., 2008; Rizzo and Carney, 2005; Smith *et al*., 2015).

NSAIDs have been reported to regulate the induction of ALDH (Pappas *et al*., 1995). This obviously points to the connection between Ibuprofen and ALDH. However no adequate attention has been paid to the toxicity effect of Ibuprofen on ALDH by spectroscopy. Here, the interaction of Ibuprofen on ALDH was evaluated by fluorescence spectroscopic method. A fluorescence spectroscopic approach allows non-intrusive measurements of substances at low concentration under physiological conditions. Fluorescence technique is the simplest method to study the interaction of drugs and bio macromolecules because it has the advantage of high sensitivity, rapidity and ease of implementation (Oravcova *et al*., 1996; Royer, 2006; Ni *et al*., 2008a). It is an important method to sense changes in the local microenvironment of fluorescent chromophore and help understand biopolymer's binding mechanisms to drugs and provide clues to the nature of the binding phenomenon. This research provides reference to the study of the toxic interaction between ALDH and Ibuprofen.

MATERIALS AND METHODS

Materials

Baker's yeast Aldehyde dehydrogenase (ALDH; molecular weight 200,000 Dalton) was obtained from Millipore EMD Millipore Corporation, Billerica, MA, USA and was used without further purification. MES, HEPES, MOPS and Ethanol were purchased from Sigma Aldrich Chemicals Company (St. Louis, MO, USA). Pure (S)-(+)-Ibuprofen (99%) concentrate was obtained from Vitabiotics Nig. Ltd., Lagos, Nigeria. All other chemicals were commercial products of analytical grade unless specialized. All solutions were prepared with doubled distilled water. All glass Ostwald viscometer (VWR, USA) was used to measure the intrinsic and extrinsic relative viscosity. ALDH protein concentrations were measured by Bradford method (Bradford, 1976). Protein sample, ligand solutions and buffers were filtered through a Millipore membrane filter (0.45 micron syringe filters) immediately before use.

Methods

Fluorescence spectra were recorded with a Hitachi F-4500 Fluorescence Spectrophotometer (Hitachi Ltd., Tokyo, Japan) equipped with a refrigerated circulating water bath (Pharmacia Biotech). Read out was transmitted at 10Hz sampling rate to a Dell PC (Window XP). The equipment was furnished with a 150 W Xenon lamp and a 1 cm quartz cell. The spectra were recorded in the wavelength range of 300–500 nm upon excitation at 280 nm when ALDH samples were titrated with the ligand (Ibuprofen). Both excitation and emission bandwidths were set on 5 nm with a scan speed at 900nm/min with a high sensitivity and a response time of 1 sec. Titrations were performed

manually by using trace syringes. A 2.0 ml solution containing an appropriate concentration of ALDH (0.250 µM) in 25 mM MOPS pH 7.0 containing 0.1M NaCl was titrated manually by successive additions of stock solution of Ibuprofen to final concentration of 125 µM. The presence of this volume of ethanol in the assay mixtures had no effect on the fluorescence measurements. Also, respective blanks of the buffer were used for the correction of all fluorescence spectra. The Ibuprofen quenching effect on ALDH was investigated at its maximum emission range (340–350 nm). The experiments were repeated and found to be reproducible within the limits of experimental errors. The maximum emission intensities were used to calculate binding constants, occupation of binding sites and thermodynamic parameters. All UV-Vis absorbance spectral determinations were performed on a Shimadzu double beam UV-Visible spectrophotometer (UV-1800) equipped with a Pharmacia refrigerating circulator for temperature control (25 ± 0.1°C) unless otherwise stated. The pH was recorded using an Adwa AD1030 pH-meter (ADWA Instruments Inc., Romania). MES and HEPES were used to prepare buffers at pH 5.0 and 9.0, respectively.

Statistical Analysis

All statistical and graphical analyses for ALDH-Ibuprofen quenching mechanism were performed using KaleidaGraph 4.5 software (Synergy software, Reading, PA, USA) for Macintosh Computer.

RESULTS AND DISCUSSION

The change in the intrinsic fluorescence of ALDH measured as a function of Ibuprofen concentration (0-125µM) at 298K and pH 7.0 after excitation at 280 nm is shown in Fig 2. ALDH was partially quenched. The change in maximum fluorescence (ΔFmax) of ALDH was 14% by addition up to 125µM of Ibuprofen. This is at the molar ratio up to 500. The natural fluorescence emission spectrum of ALDH displays a single peak with a maximum at 340 nm. Apparently, there was neither distinct blue shift nor red shift of ALDH fluorescence emission wavelengths as Ibuprofen interacted with the protein at high concentration. This suggests that the complex of Ibuprofen–ALDH did not alter the hydrodynamic volume of ALDH. With this, structurally, Ibuprofen might not be probing enough to alter structural integrity of ALDH. Emission spectrum is a function of the kind and structure of the ligand, and microenvironment of fluorescence chromophores (Sprakel *et al*., 2006).

Surprisingly, the data obtained from the change in fluorescence (ΔF) of ALDH due to interaction with increasing concentration of Ibuprofen showed hyperbolic kinetics following

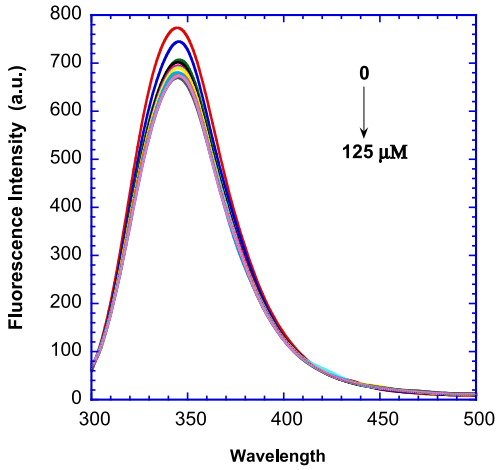

Fig 2: The effect of increasing concentration of Ibuprofen (0-125 µM) on the intrinsic fluorescence of ALDH. The initial concentration of ALDH was 0.250 µM. The fluorescence intensity was partially quenched at pH 7.0 and 25°C. The λex = 280 nm.

the Michaelis-Menten kinetic model (Fig 3). This showed saturating Ibuprofen quenching instead of non-saturating. There was non-dependent quenching co-operativity. All kinetic data were calculated in saturating condition. The hyperbolic quenching kinetics was not altered by change in pH and temperature.

Based on the partial quenching of ALDH by Ibuprofen, the relationship was treated as equilibrium of Enzyme-Inhibitor (EI) complex using previous assumption (Daba *et al*, 2013; Ohnishi *et al*, 1977).

$$EI \leftrightarrow E+I \qquad (1)$$

Where, EI, E and I are enzyme-inhibitor complex, enzyme and Inhibitor, respectively. In the condition of 0.250 µM of ALDH compared to 125

µM of Ibuprofen, E (ALDH) was considered negligible. It can be written as

$$[EI] = [E]_t [I] / (K_d + [I]) \quad (2)$$

where K_d is the dissociation constant of the EI complex and $[E]_t$, is the total enzyme concentration. By considering that ΔF is proportional to [EI] (Ohnishi *et al.*, 1977). The equation (2) was linearized to:

$$[I] / \Delta F = (Kd / \Delta F_{max}) + ([I] / \Delta F_{max}) \quad (3)$$

Where, ΔF_{max} is the maximum decrease in fluorescence observed when the enzyme is saturated by Ibuprofen. The validity of equation (2) is confirmed by the linearity of the Hanes-Woolf plot ([I]/ΔF vs. [I] plot. The result is presented in Fig 4. The Hanes-Woolf plot for Ibuprofen concentration range (0-125µ M) gave a K_d of 22 µM at pH 7.0 and 25°C. The pattern of linearity and the statistical value of standard deviation (S.D.) of the K_d (Daba *et al.*, 2013) clearly showed that Ibuprofen has one binding mode to ALDH.

The determination of Inhibition constant (K_i) was performed using the Cheng- Prusoff's equation (Cheng and Prusoff, 1973):

$$K_i = \frac{IC_{50}}{1 + ([L] / K_d)} \quad (4)$$

Where: K_i is the inhibition constant, IC_{50} is the concentration that inhibits 50% of the enzyme, L is the free Inhibitor, K_d is the dissociation constant. The K_i value was 5.84 µM (Table 1)

Temperature Dependence of K_d

The respective K_d values of the ALDH-Ibuprofen at the same concentrations range (0-125 µM) were estimated from the Hanes-Woolf plots at 20˚C, 25 ˚C, 30 ˚C and 35˚C. The K_d values of ALDH-Ibuprofen dissociations increased slightly with increasing temperatures. The thermodynamic parameters were calculated from Van't Hoff equation (Daba *et al.*, 2013).

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$$\ln K_d = \frac{\Delta H}{RT} - \frac{\Delta S}{R} \quad (5)$$

Here, K_d is the effective dissociation constant at the corresponding temperature and R (8.314 $J.mol^{-1}K^{-1}$) is the universal gas constant. The enthalpy change ΔH and entropy change ΔS were obtained from the slope and intercept of the fitting curve of lnK_d versus 1/T, respectively. The free energy change was obtained from the following relationship

$$\Delta G = \Delta H - T\Delta S = -RT \ln K_d \quad (6)$$

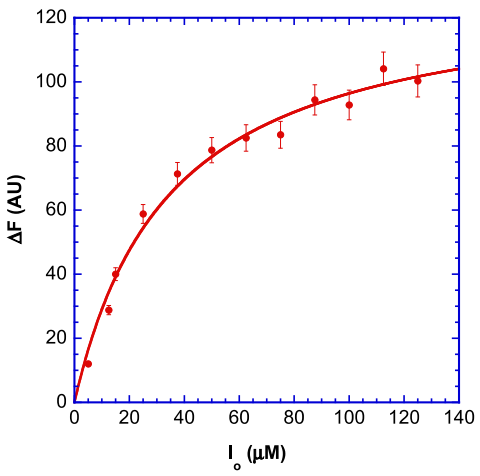

Fig 3: The change in the fluorescence intensity (ΔF) with increasing concentrations of Ibuprofen. The fluorescence data was collected at pH 7.0 and 25°C. The λex = 280 nm while λem = 340 nm.

Here, there was a good linear relationship between lnK_d and reciprocal absolute temperature, 1/T (Fig 5). The thermodynamic values (ΔH and ΔS) were obtained from the slopes and the ordinates at the origin of the fitted lines. The linear plots of ln Kd versus 1/T (see eqn 5) produced positive values for ΔH (21.32 kJ mol−1) and negative ΔS (-17.33 J mol−1 K−1) at pH 7.0. Also, positive ΔG value,

26.48 kJ mol^{-1} was obtained from eqn. (6) (Table 2). The positive values of ΔG and ΔH mean that the dissociation process of Ibuprofen-ALDH complex is non-spontaneous and is endothermic (Table 2). However, it is entropic driven. The result shown from the effect of pH on the thermodynamic parameter clearly showed that optimum condition (pH 9) of ALDH dictates the dissociation of Ibuprofen-ALDH complex not the pI of Ibuprofen (Table 2).

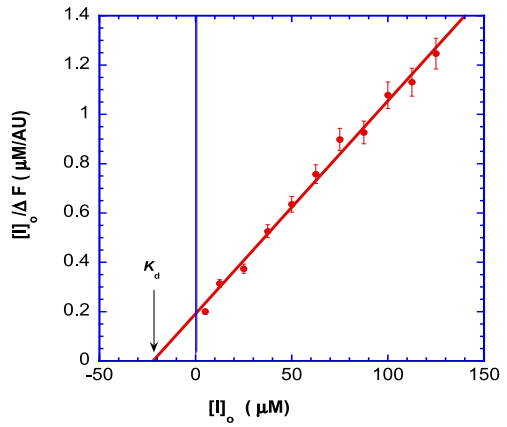

Fig 4: The Hanes-Woolf plots of Ibuprofen quenching the fluorescence of ALDH at pH 7.0 and 25□C at λ_{ex} = 280 nm. The x-intercepts of the linear equations give -K_d values in this study.

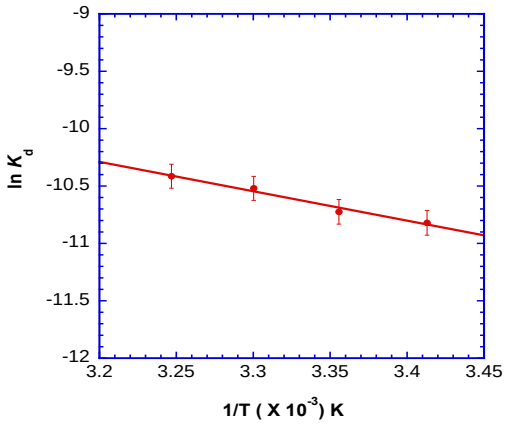

Fig 5: Van't Hoff plots of Ibuprofen quenching on the dissociation constants the fluorescence of ALDH with increasing temperature at pH 7.0 at λex = 280 nm. The standard enthalpy changes (ΔH) of ALDH binding Ibuprofen was obtained from the slope of the linear equations.

Temperature Dependence of Association constant

Quenching of intrinsic fluorescence by various ligands is often used to assess the binding parameters (Ni *et al*., 2008b). The binding constant, K_a, and the number of binding sites, n, of Ibuprofen for ALDH, were calculated using the following equation, the Scatchard equation:

$$\log\left(\frac{F_o - F}{F}\right) = \log K_a + n \log[Q] \quad (7)$$

Where, F_0 and F are the fluorescence intensities in the absence and presence of quencher (Q), respectively. *K*a and n can then be readily obtained from the slope and the intercept, respectively (Fig. 4). The binding constant K_a between Ibuprofen and ALDH was >1.0 X 10^2 L mol^{-1}, representing a low ligand−protein interaction (Table 3). Furthermore, there was one available binding site for Ibuprofen in a tetrameric nature of ALDH. This is in agreement with the dissociation constant data analysis. Importantly, it was found that the value of K_a increased as the temperature increased. The formation of Ibuprofen-ALDH complex is exothermic (-28.48 kJ mol^{-1}) and entropic driven (-130.95 $Jmol^{-1}$ K^{-1}). The enthalpic contribution was too low for covalent bond formation (200−400 kJ mol^{-1}). This ruled out the possibility of Ibuprofen forming covalent bonding with ALDH. There are essentially four types of non-covalent interactions that play a key role in binding ligands to proteins. These are hydrogen bonds, Van der Waals forces, electrostatic and hydrophobic bonds interactions (Li *et al.*, 2007; Shohrati *et al.*, 2007). The signs and magnitude of thermodynamic parameters, enthalpy change (ΔH) and entropy change (ΔS) for protein reactions are the main evidence for confirming the binding force. Ross and Subramanian (1981) characterized this sign and magnitude of the thermodynamic parameter associated with various individual kinds of interaction that may take place in protein association process as thus: (a) ΔH > 0 and ΔS > 0, hydrophobic force; (b) ΔH < 0 and ΔS < 0, Van der Waals force and hydrogen bond; (c) ΔH < 0

and ΔS > 0, electrostatic interactions. The interaction between ALDH and Ibuprofen is Van der Waals force and Hydrogen bond.

The equilibrium binding constant between K_d and K_a could create sufficient balance sheet for Ibuprofen to be carried by ALDH prior to intoxication, therapeutic and/or detoxification

Table 1: Effect of pH and temperature on Dissociation constant and Inhibition constants of Aldehyde dehydrogenase – Ibuprofen system

Temperature	*pH 5.0*		*pH 7.0*		*pH 9.0*	
	K_d	K_i	K_d	K_i	K_d	K_i
(K)	[μM]	μM	μM	μM	μM	μM
293	23 ± 4.4	6.99	20 ± 3.4	4.97	19 ± 5.7	5.01
298	26 ± 6.7	8.09	22 ± 2.7	5.84	22 ±4.4	6.59
303	29 ± 5.9	10.36	27 ± 4.1	7.28	25 ± 6.4	7.67
308	34 ± 7.2	12.83	30 ± 3.9	8.52	27 ± 5.8	7.99

Table 2: The pH-dependence on the relative thermodynamic parameters of the dissociation Aldehyde dehydrogenase – Ibuprofen system

	pH 5.0			**pH 7.4**			**pH 9.0**		
T (K)	ΔH^0 ($kJmol^{-1}$)	ΔS^0 ($Jmol^{-1}$)	ΔG^0 ($kJmol^{-1}$)	ΔH^0 ($kJmol^{-1}$)	ΔS^0 ($Jmol^{-1}$)	ΔG^0 ($kJmol^{-1}$)	ΔH^0 ($kJmol^{-1}$)	ΔS^0 ($Jmol^{-1}$)	ΔG^0 ($kJmol^{-1}$)
293			26.03			26.39			26.45
298	19.21	-23.29	26.15	21.32	-17.33	26.48	17.77	-29.62	26.60
303			26.27			26.57			26.74
308			26.38			26.65			26.89

Table 3: The pH-dependence on the relative thermodynamic parameters of the Association of Aldehyde dehydrogenase – Ibuprofen system

	pH 5.0			**pH 7.4**			**pH 9.0**		
T (K)	ΔH^0 ($kJmol^{-1}$)	ΔS^0 ($Jmol^{-1}$)	ΔG^0 ($kJmol^{-1}$)	ΔH^0 ($kJmol^{-1}$)	ΔS^0 ($Jmol^{-1}$)	ΔG^0 ($kJmol^{-1}$)	ΔH^0 ($kJmol^{-1}$)	ΔS^0 ($Jmol^{-1}$)	ΔG^0 ($kJmol^{-1}$)
293			10.27			9.89			8.49
298	-28.36	-104.51	10.79	-28.48	-130.95	10.54	-24.78	-113.55	9.06
303			11.31			11.20			9.63
308			11.83			11.85			10.20

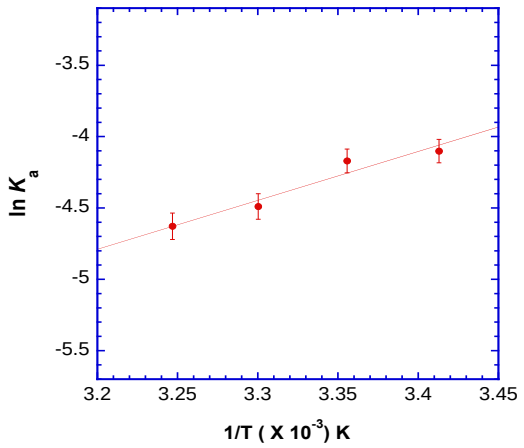

Fig 6: Van't Hoff plots for the interaction between Ibuprofen bound to ALDH. The temperatures used were between temperatures of 20 °C, 25 °C, 30 °C and 35 °C.

Effect of pH

ALDH must acquire a unique conformation in order to be functionally effective. This is crucial for enzyme catalysis. pH change tends to alter the ionization of the ligand functional groups, the polarity of the solvent environment and conformation of enzyme and hence could affect the association constant of ligand binding (Daba *et al.*, 2013). This we assumed will consequently affect the energetics of binding. The influence of acidic pH (5.0) and alkaline pH (9.0) on the interaction between Ibuprofen and ALDH was explored. The result is shown in Table 3. The stoichiometric of binding was not altered. The bonding was non-spontaneous at pH 9.0 which is the optimum pH of ALDH and essentially the bonding did not change. The bonding did not change as at pH of 5.0 The net free energy change at this pH was remarkable. However it was more favourable at alkaline pH of 9.0 compared to pHs 5.0 and 7.4. The reason for this is not immediately clear but pH 5.0 is outside the ALDH enthalpy of ionization and its optimum pH. The lowering the pH to 5 from physiological pH 7.4 increases the rate of agonist-induced conformational change is consistent with the hypothesis that acidification, and thus presumably protonation of one or more amino acids. This might lessen the responsiveness of ALDH for Ibuprofen and thus perhaps reflecting the lower stability of the ALDH. The binding stoichiometric between ALDH-acetaminophen was not affected by the change in pH either to 5 or 9.

CONCLUSION

Studies on ALDH fluorescence quenching by Ibuprofen have been presented. The results show that Ibuprofen is not a strong quencher of ALDH intrinsic fluorescence. The association/dissociation of Ibuprofen-ALDH was sensitive to pH and temperature but not affected by concentration of Ibuprofen. The bonding is predominantly Van der Waals and hydrogen bond. These changes could indicate that the biological activity of ALDH would be slightly weakened by the presence of the drug.

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