

Inhibition of (Na⁺/K⁺)-ATPase by Cibacron Blue 3G-A and Its Analogues

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Abstract. A specific feature of anthraquinone dyes (AD) is to mimic the adenine nucleotides ATP, ADP, NAD and NADH, enabling them to act as ligands in interaction with nucleotide-binding sites of several enzymes and receptors. In the present study, the interactions and/or inhibitory effects of eight AD, including Cibacron Blue 3G-A (Reactive Blue 2), Procion Blue MX-R (Reactive Blue 4) and Remazol Brilliant Blue R (Reactive Blue 19) on the activity of (Na⁺/K⁺)-ATPase were investigated. The AD used in this paper could be divided into two groups: i) AD1–AD4 that do not contain the triazine moiety; ii) AD5–AD8 that contain the triazine moiety. Interaction affinity between the respective dye and (Na⁺/K⁺)-ATPase was characterized by means of enzyme kinetics. All AD, excluding AD1 and AD2 (which were practically ineffective) exerted effective competitive inhibition to the (Na⁺/K⁺)-ATPase activity. Present study is devoted to elucidation of relationship between the inhibitory efficacy of AD against (Na⁺/K⁺)-ATPase activity, their acid-basic properties and their three dimensional structure. From the results obtained, the following conclusions could be driven:

1. Similarities in the mutual position of positively and negatively charged parts of ATP and AD are responsible for their interaction with ATP-binding site of (Na⁺/K⁺)-ATPase. This may be documented by fact that mutual position of 1-aminogroup of anthraquinone and –SO₃[–] group of benzenesulphonate part of respective AD plays crucial role for inhibition of this enzyme. Distances of these two groups on all effective AD were found to be similar as the distance of the 6-aminogroup of adenine and the second phosphate group on ATP molecule. This similarity could be responsible for biomimetic recognition of AD in ATP-binding loci of (Na⁺/K⁺)-ATPase.

2. The affinity of AD to ATP-binding site of (Na⁺/K⁺)-ATPase increases with increasing values of molar refractivity, i. e., with increasing molecular volume and polarizability.

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Introduction

P-type ATPases are often described as a target structure, the function of which is altered under several pathological insults (Ziegelhoffer et al. 2000; Lehotský et al. 2002; Matejovičová et al. 2002; Vrbjar et al. 2002; Das Evcimen et al. 2004; Vlkovičová et al. 2006). Therefore, understanding exact principles of their function has focused the interest of scientists to these enzymes. (Na⁺/K⁺)-ATPase is an enzyme responsible for active transport of Na⁺ and K⁺ across plasma membrane (for review, see Ziegelhoffer et al. 2000). ATP-binding site of this member of P-type ATPase family was extensively studied previously. Lysine 480 identified by Farley et al. (1984), Kirley et al. (1984) and Ohta et al. (1985) was often assumed to be important for binding of ATP to the high affinity binding site (Scheiner-Bobis et al. 1993). Scheiner-Bobis and Schreiber (1999) have assumed, that this lysine arrests β - or α -phosphate of ATP in a proper position prior to hydrolysis of γ -phosphate group. Other authors (Ziegelhoffer et al. 1983; Scheiner-Bobis et al. 1992; Breier et al. 1995, 1996; Gatto et al. 1999; Breier and Ziegelhoffer 2000) focused their interest to the role of the cystein SH-groups of (Na⁺/K⁺)-ATPase for binding and hydrolysis of ATP. Nevertheless, all details about the exact mechanism of recognition and binding of ATP in the ATP-binding site of (Na⁺/K⁺)-ATPase are not yet fully understood. Study of ATP-binding moieties of (Na⁺/K⁺)-ATPase with the aid of substances structurally different from ATP but mimicking ATP may be helpful in identification of crucial features on ATP molecule responsible for effective binding to the ATP-binding sites.

Cibacron Blue 3G-A and other anthraquinone dyes (AD) are generally known as potent nucleotide-mimetic ligands (Dean and Watson 1979; Subramanian et al. 1984). They are structurally unrelated to ATP and other nucleotides. The nucleotide mimetic properties of AD were utilized in the purification of several enzymes that contain binding site for nucleotides by bio-affinity separation techniques (Kulbe and Schuer 1979; Gemeiner et al. 1981; Kopperschlager and Johansson 1982; Scawen et al. 1982; Mislovičová et al. 1988; Kroviarski et al. 1988; Žúbor et al. 1993). AD were also found to inhibit numerous nucleotide-dependent and nucleotide-utilizing enzymes such as lactate dehydrogenase (Ďurišová et al. 1990; Boháčová et al. 1998), formate dehydrogenase (Labrou and Clonis 1995), NADP-dependent carbonyl reductase (Higuchi et al. 1993), hexokinase (Puri and Roskoski 1994), protein kinase CK2 (De Moliner et al. 2003) and the (Na⁺/K⁺)-ATPase (Schonfeld et al. 1984; Ďurišová et al. 1990; Dočolomanský et al. 1994). AD were also found to alter several physiological processes like: i) cardiac contractility (Mantelli et al. 1993) *via* blockade of adenosine receptors; ii) effect of ATP on mitochondrial K⁺ uniport (Beavis et al. 1993) *via* blockade of ATP-binding site of mitochondrial ATP sensitive K⁺ channel. Moreover, they may antagonize the putative P2x (Michel and Humphrey 1993) as well as the P2y (Mantelli et al.

1993) purine receptors. Danieluk et al. (1999a,b) described ATP sensitive binding of Cibacron Blue 3G-A on the annexin VI from porcine liver that also binds ATP to a new type of ATP-binding site (Bandorowicz-Pikula et al. 2003).

The present paper is dealing with the AD structural features decisive for their inhibitory effects on the (Na⁺/K⁺)-ATPase activity. We were aiming to find quantitative structure-activity relationships based on the presumption that interaction between AD and the enzyme is of an electrostatic nature.

Materials and Methods

Reagents and chemicals

(Na⁺/K⁺)-ATPase from dog kidney medulla was prepared by the method of Jørgensen (1988) using centrifugation with the zonal rotor of a UP65 ultracentrifuge (Germany). The purity of the preparation was checked by means of the Fast system SDS electrophoresis (Pharmacia, Sweden) on 8–24% gradient polyacrylamide gels as well as by estimation of the specific activity of (Na⁺/K⁺)-ATPase. The resulting enzyme preparation exhibited only two electrophoretically distinguished protein bands (M1 ~110 and M2 ~35 kDa), which corresponded to the α and β subunits of the (Na⁺/K⁺)-ATPase. Specific activity of the ATPase preparation was about 25 μ mol Pi/min·mg protein. Cibacron Blue F3G-A (Reactive Blue C.I.2, AD7 in Fig. 1), Remazol Brilliant Blue R (C.I. 19, AD3 in Fig. 1) and Procion Blue MX-R (Reactive Blue C.I. 4, AD4 in Fig. 1) were obtained from Sigma (St. Louis, MO, USA). The sodium salt of 1-amino-4-bromo-2-(9,10-anthraquinone)-sulfonic acid was prepared at the Research Institute of Organic Synthesis (AD1 in Fig. 1; Pardubice, Czech Republic). Other dyes (AD2, AD5, AD6 and AD7 in Fig. 1) were prepared in our laboratory as described previously (Dočolomanský et al. 1994). All other chemicals were purchased from Serva (Heidelberg, Germany) and Sigma (St. Louis, USA). All commercially available chemicals were of analytical grade.

Estimation of (Na⁺/K⁺)-ATPase activity

(Na⁺/K⁺)-ATPase activity was determined as the difference between the amounts of orthophosphate liberated by splitting of ATP (0.125–4.000 mmol/l) in the presence of NaCl (100 mmol/l), KCl (10 mmol/l) and MgCl₂ (4 mmol/l), and in the presence of 4 mmol/l of MgCl₂ only. Enzyme reaction was running for 10 min at 37°C, in 0.5 ml of 50 mmol/l imidazole-HCl buffer, pH 7.0, in the presence of 1–2 mg of enzyme protein. It was started by simultaneous addition of different concentrations of ATP and AD, and stopped by ice-cold trichloroacetic acid (12%). The liberated orthophosphate was estimated by means of the method of Taussky and Shor (1953). The protein content in the membrane fractions was determined according to Markwell et al. (1978). Parameters of the enzyme kinetics were obtained by non-linear regression of the initial reaction velocities V as a function of concentration of ATP using the PC software Sigma Plot 2.01. Computations were

based on the Michaelis-Menten relationship

$$V_0 = V_{\max} c (c + K_m)^{-1} \quad (1)$$

where V_0 and V_{\max} represent the initial velocities of $(\text{Na}^+/\text{K}^+)\text{-ATPase}$ reaction at concentration of ATP c and $c \rightarrow \infty$, respectively, and K_m is the Michaelis-Menten constant.

Data obtained by direct measurement of inhibition of the $(\text{Na}^+/\text{K}^+)\text{-ATPase}$ reaction by the AD were processed by means of the Eq. (2) derived from the Dixon's graphical method for evaluation of enzyme inhibition

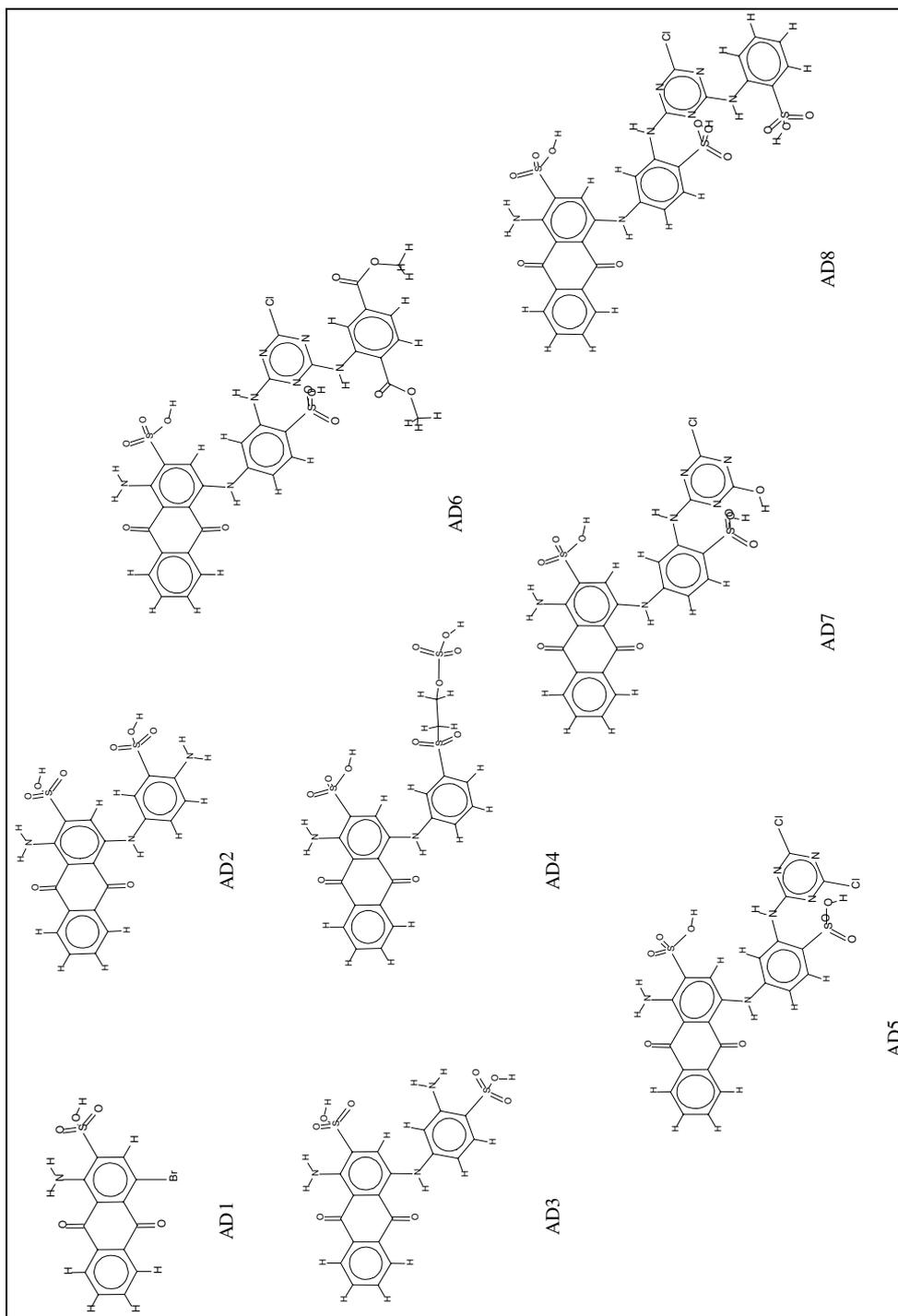
$$V = V_0 [1 + (i/\text{IC}_{50})]^{-1} \quad (2)$$

where V and V_0 represent the initial velocity of enzyme reactions at concentration of inhibitor i and $i = 0$, respectively. IC_{50} is the median inhibition concentration.

Others methods

Three dimensional structures of the respective AD and ATP were computed using Desktop molecular modeling software (Oxford Electronic Publishing, UK). The apparent acid dissociation constants $\text{p}K_a^{\text{app}}$, reflecting the acid-basic properties of single AD, were estimated by means of a titration described previously (Dočolomanský et al. 1994). Calculation of physico-chemical constant, i.e. AD structure-related descriptors, was based on generally accepted assumption that they can be expressed as sum of atomic and group increments. Using ACD/ChemSketch 4.04 (Advanced Chemistry Development Inc., Toronto, Canada), we estimated the following descriptors: V_M , molar volume; R_M , molar refractivity; d , crystal density. Detail information about descriptors computation can be found in Österberg and Norinder (2001) or in the ADC web site (<http://www.acdlabs.com/products>).

Figure 1. The structures of the anthraquinone dyes: **AD1** – 1-amino-4-bromo-2-(9,10-anthraquinone)-sulfonic acid; **AD2** – 1-amino-4-(4-amino-3-sulfanilino)-2-(9,10-anthraquinone)-sulfonic acid; **AD3** – 1-amino-4-(4-amino-4-sulfanilino)-2-(9,10-anthraquinone)-sulfonic acid; **AD4** – 1-amino-4-[4-(1-sulfonyl-ethyl-2-sulfoxy)]-2-(9,10-anthraquinone)-sulfonic acid; **AD5** – 1-amino-4-[3-(3,5-dichloro-2,4,6-triazinylamino)-4-sulfanilino]-2-(9,10-anthraquinone)-sulfonic acid; **AD6** – 1-amino-4-{3-[3-(2,5-dimethoxycarbonylanilino)-5-chloro-2,4,6-triazinylamino]-4-sulfanilino}-2-(9,10-anthraquinone)-sulfonic acid; **AD7** – 1-amino-4-[3-(3-chloro-5-hydroxy-2,4,6-triazinylamino)-4-sulfanilino]-2-(9,10-anthraquinone)-sulfonic acid; **AD8** – 1-amino-4-{4-[3-chloro-2,4,6-triazinylamino-5-(3-sulfanilino)-3-sulfanilino]}-2-(9,10-anthraquinone)sulfonic acid. AD1–AD4 in contrast to AD5–AD8 do not contain triazine moiety. AD4, AD5 and AD8 are commercially available and are classified as C.I. 19, C.I. 4 and C.I. 2.



Results and Discussion

Characterization of anthraquinones

Structures of the anthraquinone dyes used in this study are shown in Fig. 1. Among them the derivatives AD4, AD5 and AD8, i.e., Remazol Brilliant Blue R (Reactive Blue 19), Procion Blue MX-R (Reactive Blue 4) and Cibacron Blue 3G-A (Reactive Blue 2), are also available commercially. The purity of derivatives was controlled by TLC chromatography (Beissner and Rudolph 1978; Federici and Stadtman 1985) and always exceeded 95%. The structural analyses of the compounds synthesized have been described in a previous paper (Dočolomanský et al. 1994).

Among the compounds used in the present study, the AD4–AD8 may be considered as reactive AD, and four of them contain reactive chlorine located on triazine moieties (in position 3 on AD5–AD8 as well as in position 5 on AD5). The reactive moiety of derivative AD4 represents an ethylenedisulfonate group. In comparison to these five derivatives, derivatives AD1–AD3 may be considered as less reactive under physiological conditions. Acid-basic behavior of anthraquinone derivatives was characterized by pK_a^{app} (Dočolomanský et al. 1994; Boháčová et al. 1998) that describe the net exchange of protons between the anthraquinone molecule and the surrounding aqueous solution (Table 1). In a previous paper (Boháčová et al. 1998), the distance A_{AD} between 1-NH₂ group located on anthraquinone moiety and —SO₃H group located on diaminobenzene part (in AD4

Table 1. Values of IC₅₀ for AD-induced inhibition of (Na⁺/K⁺)-ATPase and applied physicochemical descriptors

| | A_{AD} | A | pK_a^{app} | $\text{PH} - pK_a^{\text{app}}$ | R_{M} | IC ₅₀ [$\mu\text{mol/l}$] |
|-----|-----------------|-------|---------------------|---------------------------------|----------------|---|
| AD1 | 0.000 | 1.097 | 7.216 | −0.216 | 80.58 | 350.0 ± 120.3 |
| AD2 | 0.820 | 0.277 | 7.235 | −0.235 | 115.19 | 420.0 ± 185.2 |
| AD3 | 1.0350 | 0.062 | 7.208 | −0.208 | 115.19 | 89.8 ± 5.1 |
| AD4 | 1.059 | 0.038 | 7.196 | −0.196 | 131.39 | 13.9 ± 0.6 |
| AD5 | 1.032 | 0.065 | 7.069 | −0.069 | 142.95 | 25.3 ± 1.7 |
| AD6 | 1.042 | 0.055 | 6.673 | 0.327 | 188.73 | 30.4 ± 1.8 |
| AD7 | 1.041 | 0.056 | 6.796 | 0.204 | 139.68 | 34.3 ± 2.1 |
| AD8 | 1.032 | 0.065 | 6.828 | 0.172 | 186.76 | 4.8 ± 0.2 |

A_{AD} – distance between 1-NH₂ group located on anthraquinone moiety and —SO₃H group located on diaminobenzene part (in AD4 on sulfonyl-ethylene-sulfonate side chain). A – represents the difference between A_{ATP} and A_{AD} . A_{ATP} – distance between the 6-aminogroup located on adenosine part of ATP and second phosphate group amounting to 1.097 nm. A_{ATP} and A_{AD} were accounted using Desktop Molecular Modeling software (Oxford Electronic Publishing, UK). pK_a^{app} – apparent acid dissociation constant estimated according to Dočolomanský et al. (2004). R_{M} – molar refractivity computed with the aid of ACD/ChemSketch 4.04 (Advanced Chemistry Development Inc. Toronto, Canada).

on sulfonyl-ethylene-sulfonate side chain was shown to be essential for effective inhibition of lactate dehydrogenase. We assumed this distance to be mimicking the distance between the 6-aminogroup located on adenosine part of NADH and the first phosphate group. A similar distance on AD molecules may be important also for simulation of ATP molecule. Based on the idea of Scheiner-Bobis and Schreiber (1999) that ATP is fixed in ATP-binding moiety through β -phosphate prior hydrolysis of γ -phosphate, we compared A_{AD} with the distance $A_{ATP} = 1.097$ nm between the 6-aminogroup of ATP and β -phosphate (Table 1). With the exception of AD1 (not containing any —SO₃H group on another part as anthraquinone) and AD2 (0.82 nm), these distances were found to be in the interval 1.00–1.06 nm for all AD and thus were rather similar to the distance A_{ATP} . The measure of AD similarity with ATP was expressed as the difference $A = A_{ATP} - A_{AD}$.

Values of pK_a^{app} for all AD were found to be in the range of 6.6–7.3. AD with pK_a^{app} exceeding 7.0 will be predominantly in protonized form, i.e. with positive charge(s) located on the nitrogen atom(s) at neutral medium (pH 7.0) applied for estimation of (Na⁺/K⁺)-ATPase activity. AD with pK_a^{app} below 7.0 will be predominantly in an unprotonized form. We applied ratio $pH - pK_a^{app}$ for digitalization of these feature. AD structure-related descriptors (see Material and Methods) are also documented in Table 1.

Effect of AD on (Na⁺/K⁺)-ATPase activity

AD1 and AD2 did not induce considerable inhibitory effects on (Na⁺/K⁺)-ATPase activity (Fig. 2) in concentration ranges of 0–50 μ mol/l. All other AD were found to inhibit the (Na⁺/K⁺)-ATPase activity in a concentration-dependent manner (Fig. 2). Respective IC₅₀ values of this inhibition are summarized in Table 1. The concentration range in which AD1 and AD2 could induce a considerable inhibition may be estimated as 0.5 mmol/l (Table 1). Thus AD1 and AD2 that differ from other AD in the aspect of A_{AD} and A are considerably less potent inhibitors of (Na⁺/K⁺)-ATPase (with about ten times lower affinity). On the other hand, from the data in Table 1 may be deduced that minimal structure of AD necessary for effective inhibition of (Na⁺/K⁺)-ATPase represents 1-amino-4-(3-amino-4-sulfanilino)-2-(9,10-anthraquinone)-sulfonic acid. By means of model compounds with substructures corresponding to Cibacron Blue 3G-A similar minimal structures were identified to be necessary for inhibition of hexokinase (Puri and Roskoski 1994). Moreover, similar minimal structures were described for lactate dehydrogenase (Boháčová et al. 1998), i.e., for enzyme utilizing NAD⁺ nucleotide coenzyme. In the case of formate dehydrogenase, it is precisely the anthraquinone moiety of the dyes that, by means of electrostatic interactions, is capable of recognizing the NADH-binding site of the enzyme (Labrou and Clonis 1995). All the above facts indicated that binding of AD to the ATP-binding sites of (Na⁺/K⁺)-ATPase, hexokinase and NAD⁺/NADH-binding sites of lactate dehydrogenase (and also formate dehydrogenase) could be based on a common principle.

Inhibition of (Na⁺/K⁺)-ATPase activity by AD is competitive in respect to ATP as it is shown for AD3, AD4, AD7 and AD8 in Fig. 3 (AD induced significant

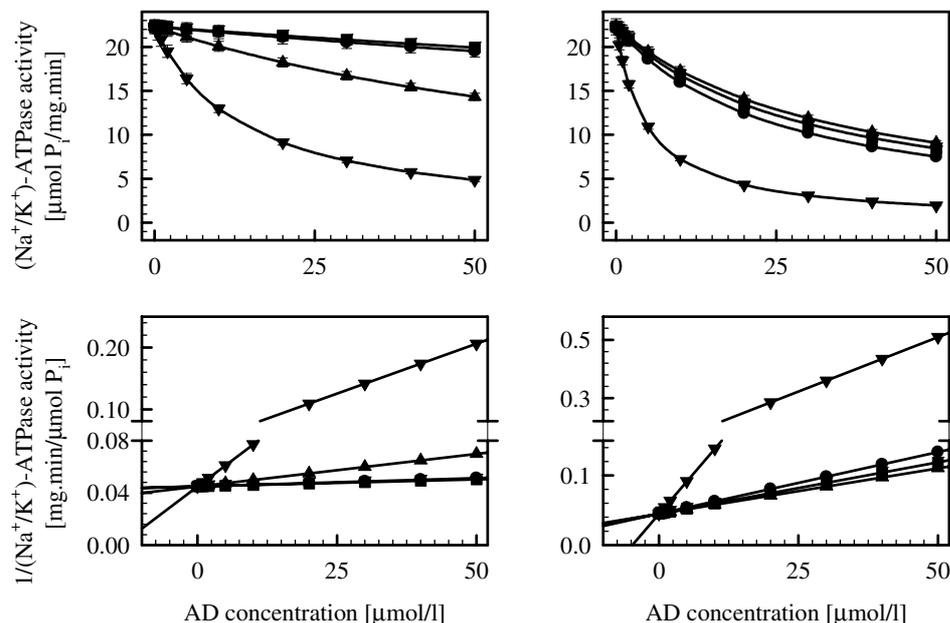


Figure 2. Inhibitory effects of AD1–AD4 (left panels) and AD5–AD8 (right panels) on $(\text{Na}^+/\text{K}^+)\text{-ATPase}$ activity in direct plots (upper panels) and in Dixon linearization plots (lower panels). Symbols: ● AD1 and AD5; ■ AD2 and AD6; ▲ AD3 and AD7; ▼ AD4 and AD8. Data represent mean \pm S.E.M. from six independent measurements. Values of IC_{50} obtained by nonlinear regression according to Eq. (2) are given in Table 1.

increases in K_m value without any change of V_{\max} value). Thus, effective AD inhibited $(\text{Na}^+/\text{K}^+)\text{-ATPase}$ activity by binding to the loci on $(\text{Na}^+/\text{K}^+)\text{-ATPase}$ molecule that bind ATP. It may be speculated that AD1 and AD2 that are weak inhibitors of $(\text{Na}^+/\text{K}^+)\text{-ATPase}$ activity and could not mimic ATP by similarities in the respective A_{AD} and A_{ATP} do not inhibit the enzyme specifically in this loci. Another anthraquinone – emodin has recently been found to depress $(\text{Na}^+/\text{K}^+)\text{-ATPase}$ activity in the small intestinal mucosa of mice after its application using intragastric gavage seven times during one week (Zhang et al. 2005). In the structure of this compound, any similar distance as A_{AD} could not be found. Thus, AD1, AD2 and probably emodin represent another type of anthraquinone exerting lower level of ATP mimic properties in $(\text{Na}^+/\text{K}^+)\text{-ATPase}$ ATP-binding site than AD3–AD8.

Quantitative relationship between AD structure-related descriptors and AD-induced inhibitory effect

The values of IC_{50} for inhibition of $(\text{Na}^+/\text{K}^+)\text{-ATPase}$ by AD were correlated with descriptors quantifying their physico-chemical properties (summarized in Table 1). IC_{50} values in simple interrelations with A and R_M show increased and decreased

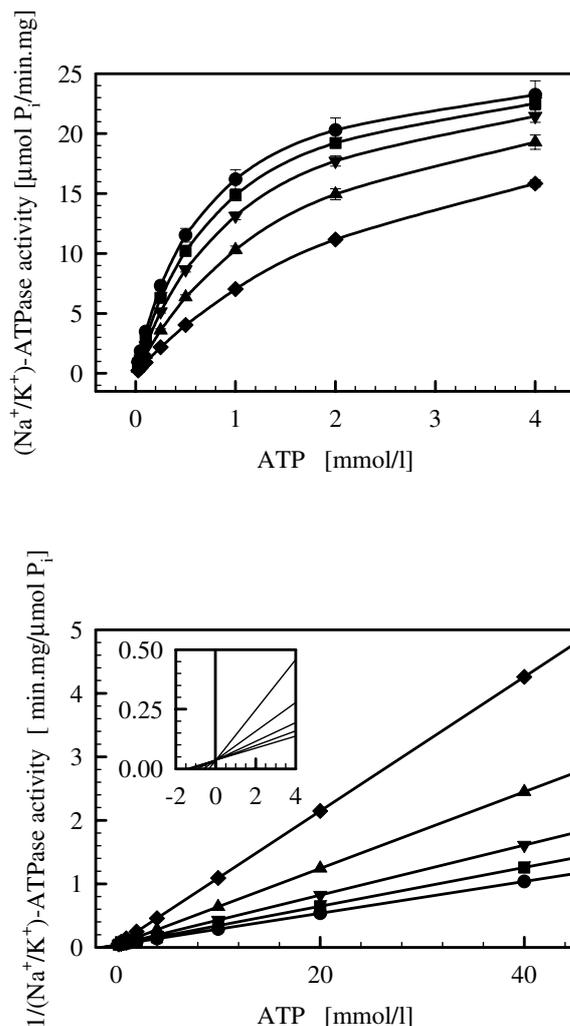


Figure 3. Kinetic evaluation of AD3-induced (■), AD4-induced (▲), AD7-induced (▼) and AD8-induced (◆) inhibition of $(\text{Na}^+/\text{K}^+)\text{-ATPase}$ activity (at a concentration of $2.5 \mu\text{mol/l}$) in comparison with the control experiment (●). The stimulation of ATPase activity by ATP is shown in direct Michaelis-Menten plot (upper panel) and in double reciprocal Lineweaver-Burke plot (lower panel). Data represent means \pm S.E.M. from six independent experiments. AD did not change the value of $V_{\text{max}} = 27.2 \pm 1.0 \text{ mmol P}_i/\text{min}\cdot\text{mg}$ protein that was obtained for the control. In contrast, significant increase in K_m values from $0.68 \pm 0.03 \mu\text{mol/l}$ (for the control) to $0.83 \pm 0.04 \mu\text{mol/l}$ (for AD3), $1.64 \pm 0.09 \mu\text{mol/l}$ (for AD4), $1.07 \pm 0.06 \mu\text{mol/l}$ (for AD7) and $2.87 \pm 0.12 \mu\text{mol/l}$ (for AD8) was obtained by nonlinear regression of experimental data according to Eq. (1).

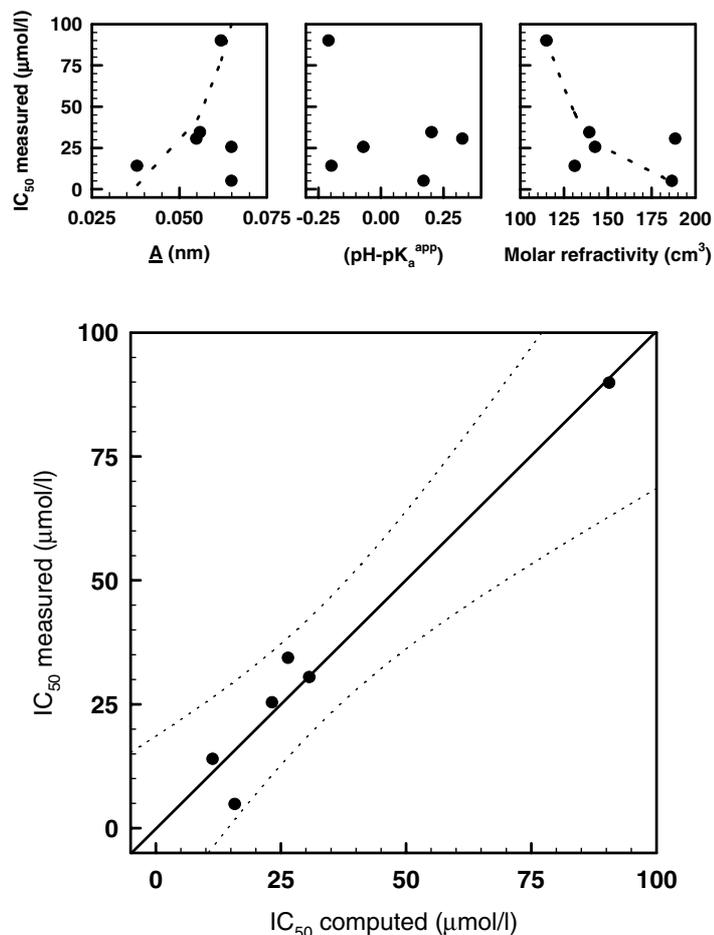


Figure 4. Multiple linear regression of relations between IC_{50} values for AD and the physico-chemical descriptors (summarized in Table 1). Simple plots were done for IC_{50} versus A (panel A), IC_{50} versus $\text{pH} - \text{p}K_a^{\text{app}}$ (panel B) and IC_{50} versus R_M (panel C). Multiple nonlinear regression done for IC_{50} and A , $\text{pH} - \text{p}K_a^{\text{app}}$ and R_M according to Eq. (3) yields QSAR model (Eq. (4)). Comparison between data obtained by measurement and computed by QSAR model is shown on panel D.

trends, respectively, both displaying an exponential shape (Fig. 4A and C). However, these trends are rather weak without real confidence. IC_{50} as a function of $(\text{pH} - \text{p}K_a^{\text{app}})$ did not show any monotonous trend (Fig. 4B).

On the other hand, when IC_{50} values were fitted with all three independent variables together according to Eq. (3), a rather good agreement between experi-

mental and measured data was obtained:

$$IC_{50} = a + 10^{[P_1 A + P_2 (\text{pH} - \text{p}K_a^{\text{app}})^2 + P_3 R_M]} \quad (3)$$

where P_1 – P_4 are parameters obtained by regression procedure.

This fitting procedure yields the following numerical model:

$$IC_{50} = (10.4 \pm 3.7)10^{\{[(392.6 \pm 74.9)A] + [(332.1 \pm 70.5)(\text{pH} - \text{p}K_a^{\text{app}})^2] - [(0.169 \pm 0.035)R_M]\}} \quad (4)$$

The comparison of the IC_{50} values computed in this way and those obtained experimentally is depicted in Fig. 4C. When the expressions in brackets in Eq. (4) were positive, the increase in descriptor value influenced the IC_{50} value positively, i.e., affinity of AD to ATP-binding site of (Na⁺/K⁺)-ATPase negatively. In contrast, when they were negative, the increase in descriptor value influenced the IC_{50} value negatively and affinity of AD to ATP-binding site positively. The model indicates that IC_{50} value was elevated with increased values of A , and $(\text{pH} - \text{p}K_a^{\text{app}})^2$, and with decreased value of R_M . These trends for A and R_M are consistent with the trends of dependency shown in Fig. 4A and C. Therefore we may conclude that the difference $A = A_{\text{ATP}} - A_{\text{AD}}$ represents the descriptor with important meaning for affinity of AD in ATP-binding site of (Na⁺/K⁺)-ATPase. Similar models as Eq. (4) could be obtained when values R_M were replaced by values of the V_M (computed for each derivative by ACD/ChemSketch 4.04 software) and molecular weight M_W . This may be explained by strong intercorrelation between R_M , V_M and M_W for the AD. However, the best fitting was obtained in a model when R_M was applied. The numerical model (Eq. (4)) indicated that increase in R_M induced a decrease in IC_{50} , i. e. increase in AD affinity to ATP-binding site of (Na⁺/K⁺)-ATPase. Meaning of the descriptor R_M could be expressed as V_M corrected by the refractive index and represents size and polarizability of a fragment or molecule (van de Waterbeemd et al. 1997). R_M was originally proposed by Pauling and Pressman (as cited in Bolger 2002) as a parameter for the correlation of dispersion forces involved in the binding of haptens to antibodies. In the present study, we determined it from the refractive index, M_W and d . Since refractive index does not vary much for organic molecules, it could be expected that the term is dominated by the M_W and d . The descriptor d informs about packing of molecules in the crystal structure. Higher density indicates better packing and stronger intermolecular interaction (Kupsakova et al. 2004).

Concerning the meaning of $\text{p}K_a^{\text{app}}$ for prediction of AD affinity to ATP-binding site of (Na⁺/K⁺)-ATPase, we would like to stress that situation is not unambiguous. A better fit was obtained when the difference $(\text{pH} - \text{p}K_a^{\text{app}})$ was applied in the second power as $(\text{pH} - \text{p}K_a^{\text{app}})^2$. This indicates that in the fitting procedure positive values of descriptor $(\text{pH} - \text{p}K_a^{\text{app}})$ and/or in quadratic expressions are more favorable for prediction of IC_{50} value. Therefore, it could be assumed that the best effects may be expected for a substance with a $\text{p}K_a^{\text{app}}$ corresponding mostly to pH of solution for ATPase reaction measurement. Thus, from our data it could not

be predicted if AD have to be in protonized or nonprotonized form for an effective interaction with ATP-binding site of (Na⁺/K⁺)-ATPase. From this reason the application of other AD with higher differences in pK_a^{app} values would be necessary.

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