Identification of Hydroxyxanthones as Na/K-ATPase Ligands

Zhongbing Zhang, Zhichuan Li, Jiang Tian, Wei Jiang, Yin Wang, Xiaojin Zhang, Zhuorong Li, Qidong You, Joseph I Shapiro, Shuyi Si and Zijian Xie

Institute of Medicinal Biotechnology, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing, P.R. China (Z.Z., W.J., Z.L., S.S.); Department of Physiology, Pharmacology and Medicine, University of Toledo College of Medicine, Toledo OH 43614, USA (Z.L., J.T., J.S., Z.X.); Zhejiang Academy of Medical Sciences, Hangzhou, Zhejiang Province, P.R. China (Y.W.); and Department of Medicinal Chemistry, School of Pharmacy, China Pharmaceutical University, Nanjing, P.R. China (X.Z., Q.Y.).
Running Title: Na/K-ATPase Ligands

Address correspondence to:

Zi-Jian Xie, Ph.D.

Department of Physiology and Pharmacology, College of Medicine

University of Toledo Health Science Campus

3000 Arlington Avenue, Toledo, OH 43614-2598.

Telephone: 419-383-4182; FAX: 419-383-2871; E-mail: zi-jian.xie@utoledo.edu

1 text page, 0 table, 0 figure, 0 reference, and 151 words in the Abstract.

3 text pages, 0 table, 0 figure, 25 references, and 567 words in the Introduction.

4 text pages, 0 table, 0 figure, 16 reference, and 883 words in the Discussion.

ABBREVIATIONS: ERK, extracellular signal-regulated kinase; PAGE, polyacrylamide gel electrophoresis; MAPK, mitogen-activated protein kinase.
Abstract

We have screened a chemical library and identified several novel structures of Na/K-ATPase inhibitors. One group of these inhibitors belongs to polyphenolic xanthone derivatives. Functional characterization reveals the following properties of this group of inhibitors. First, like ouabain, they are potent inhibitors of the purified Na/K-ATPase. Second, their effects on the Na/K-ATPase depend on the number and position of phenolic groups. Methylation of these phenolic groups reduces the inhibitory effect. Third, further characterization of the most potent xanthone derivative, MB7, reveals that it does not change either Na\(^+\) or ATP affinity of the enzyme. Finally, unlike ouabain, the inhibitory effect of MB7 on Na/K-ATPase is not antagonized by K\(^+\). Moreover, MB7 does not activate the receptor Na/K-ATPase/Src complex and fails to stimulate protein kinase cascades in cultured cells. Thus, we have identified a group of novel Na/K-ATPase ligands that can inhibit the pumping function without stimulating the signaling function of Na/K-ATPase.
Introduction

The Na/K-ATPase, also known as the sodium pump, is a ubiquitous trans-membrane enzyme that transports Na\(^+\) and K\(^+\) across the plasma membrane by hydrolyzing ATP (Blanco and Mercer, 1998; Lingrel and Kuntzweiler, 1994; Skou, 1957; Sweadner, 1989). It belongs to the family of P-type ATPase that transits between E1 and E2 conformational states during pumping cycles. The functional enzyme is mainly composed of \(\alpha\) and \(\beta\) subunits. The \(\alpha\) subunit is the catalytic component of the holoenzyme as it contains both the nucleotide and the cation binding sites (Blanco and Mercer, 1998; Lingrel and Kuntzweiler, 1994; Sweadner, 1989). Interestingly, studies during the past few years have uncovered many non-pumping functions of the Na/K-ATPase such as signal transduction (Aizman et al., 2001; Aydemir-Koksoy et al., 2001; Cai et al., 2008; Haas et al., 2002; Kometiani et al., 1998; Nguyen et al., 2007; Tian et al., 2006; Wang et al., 2004; Yuan et al., 2005). Specifically, the signaling Na/K-ATPase resides in caveolae and interacts with a number of signaling proteins such as Src, the IP3 receptor and caveolin-1 (Cai et al., 2008; Tian et al., 2006; Wang et al., 2004; Yuan et al., 2005). While the interaction between the Na/K-ATPase and the IP3 receptor facilitates Ca\(^{2+}\) signaling (Tian and Xie, 2008), the dynamic association between the Na/K-ATPase and Src regulates cellular Src activity, and makes it possible for cardiotonic steroids to stimulate protein kinase cascades (Li and Xie, 2009).

Cardiotonic steroids (CTS) include plant-derived digitalis drugs such as digoxin
and ouabain, and vertebrate-derived aglycones such as bufalin and marinobufagenin (MBG) (Akera and Brody, 1976; Schoner and Scheiner-Bobis, 2007). Clinically, these steroids can be used to treat congestive heart failure because they have well-documented inotropic effects on the heart (Akera and Brody, 1976; Repke et al., 1996). Although CTS have been considered as drugs since their discovery, recent studies have identified several of them, including ouabain and MBG, as endogenous steroids (Bagrov and Fedorova, 1998; Hamlyn et al., 1991). It is known that the Na/K-ATPase serves as a receptor for these steroids. While binding of CTS to the Na/K-ATPase inhibits the pumping function, it stimulates the signaling function of Na/K-ATPase (Li and Xie, 2009). For example, binding of ouabain to the Na/K-ATPase/Src receptor complex stimulates Src kinase. The activated Src, in turn, trans-activates receptor tyrosine kinases such as the EGF receptor (EGFR) and converts the tyrosine kinase signal to stimulation of serine/threonine kinases, lipid kinases and lipases as well as increased production of reactive oxygen species (ROS) (Li and Xie, 2009; Liu et al., 2000). Interestingly, while inhibition of the Na/K-ATPase by CTS is essential for these drugs to increase cardiac contractile function (Altamirano et al., 2006; Reuter et al., 2002), stimulation of protein kinases and subsequent increases in the production of ROS by these steroids also cause cardiac hypertrophy and fibrosis in animal studies (Ferrandi et al., 2004; Kennedy et al., 2006).

Because CTS affect both ion pumping and signal transducing functions of the
Na/K-ATPase, we were prompted to search for new Na/K-ATPase ligands that only regulate the ion pumping function of Na/K-ATPase. To achieve this goal, we have developed a high throughput screen assay and tested a chemical library of drug-like small molecules prepared from either Chinese herb medicine or bacterial metabolites. We report here the identification of a novel class of chemicals that are different from CTS, inhibiting the Na/K-ATPase without activating protein kinases in cultured cells.
Materials and Methods

**Materials**: ATP and ouabain were obtained from Sigma (St. Louis, MO). Biomol Green was purchased from BIOMOL (Plymouth Meeting, PA). ERK/MAPK (Phospho-Thr202/Tyr204) phosphorylation/translocation cell-based assay kit was purchased from Cayman Chemical Company (Ann Arbor, MI). Purified recombinant Src was obtained from Upstate Biotechnology (Lake Placid, NY). Polyclonal anti-Tyr(P)418-Src was obtained from Invitrogen (Camarillo, CA). Anti-c-Src (B-12) monoclonal antibody was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Fresh pig kidneys were purchased from a slaughterhouse, and stored at -80°C until used for enzyme preparation.

**High throughput screen assay**: The chemical library used for screening in the present study contained 2600 structurally diverse, drug-like, naturally occurring organic compounds or their semi-synthetic derivatives, mainly of plants and bacterial origin. The purity of these compounds was above 95% unless otherwise indicated. Stock compounds were prepared in 96-well plates at 10 mg/ml in DMSO.

The purified Na/K-ATPase was prepared from pig kidney as described before (Xie et al., 1996). The specific activities of the Na/K-ATPase of various kidney preparations were in the range of 900–1,200 μmol/mg/h, which was greater than 95% of
the total ATPase activity. The high throughput screen is conducted in a 96-well format with the final reaction volume of 100 μl containing the following components: 100 mM NaCl, 20 mM KCl, 1 mM MgCl₂, 1 mM EGTA, 20 mM Tris-HCl (pH 7.4) and 0.2 μg of purified Na/K-ATPase. After compounds were added, the mixtures were incubated at 37°C for 15 min and reaction was initiated by adding 2 mM ATP·Mg²⁺ mixture. Reactions were carried out for 15 min and then stopped by the addition of 100 μl ice-cold 8% trichloroacetic acid. Reaction mixtures were cleared by centrifugation, and assayed for released phosphate using the BIOMOL GREEN™ Reagent according to the manufacturer’s instructions. In addition, the control Na/K-ATPase activity was measured in the presence and absence of 1 mM ouabain, and taken as 0 and 100%, respectively. Furthermore, 5 μM ouabain and 0.1% of DMSO were included in each plate as a positive and a vehicle control, respectively. Control experiments showed that ATP hydrolysis catalyzed by the Na/K-ATPase was in linear range within 30 min of incubation under the above experimental conditions.

Cell culture: The pig kidney epithelia cells (LLC-PK1 cells) and human non-small lung cancer cells (A549 cells) were obtained from ATCC and maintained in Dulbecco’s modified Eagle’s medium (DMEM) in the presence of 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin in a 5% CO₂ humidified incubator. To eliminate the confounding effect of growth factors in the serum, cells were serum starved for 24 h before experiments unless otherwise indicated.
Western blot analysis: Cells were washed with PBS and solubilized in ice-cold RIPA buffer containing 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 1 mM NaF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 50 mM Tris-HCl (pH 7.4) as previously described (Wang et al., 2004). Cell lysates were then cleared by centrifugation at 14,000 rpm, and supernatants were used for protein assay and subjected to Western blot analysis. Samples were separated on SDS-PAGE (50 μg/lane) and transferred to a cellulose membrane. Membranes were blocked with 3% non-fat dried milk for total Src and ERK or 1% BSA plus 1% non-fat dried milk for phosphorylated Src and ERK in TBST (Tris-HCl 10 mM, NaCl 150 mM, Tween 20, 0.1%; pH 7.5) for 1 h at room temperature and then probed with specific antibodies. Protein signals were detected using an ECL kit and quantified using an imaging densitometer.

Assay for the activation of receptor Na/K-ATPase/Src complex: The activity of the receptor Na/K-ATPase/Src complex was assayed as we previously described (Tian et al., 2006). Briefly, the purified Src (4.5 U) was incubated with 2 μg of the purified Na/K-ATPase in PBS for 30 min at 37°C. Afterward, the Na/K-ATPase/Src complex was exposed to ouabain or MB7 for 10 min. Reaction was initiated by addition of 2 mM ATP/Mg$^{2+}$, continued for 5 min at 37°C, and stopped by addition of the SDS sample buffer. The activation of Src was measured by Western blot using the anti-pY418
antibody. To control for gel loading and transfer, total Src was also probed.

Confocal Imaging and Immunocytochemistry: LLC-PK1 cells grown on coverslips were serum starved for 24 h and treated with MB7 or ouabain for various times. Immunostaining of p-ERK was performed using the commercial ERK/MAPK (Phospho-Thr202/Tyr204) Phosphorylation/Translocation Cell-Based Assay Kit according to the manufacturer’s instructions. The signals were detected by a Leica confocal microscope as previously described (Tian et al., 2006). Leica confocal software was used for data analysis.

Data Analysis: Data are given as mean ± S.E. Statistics were performed by one way ANOVA followed by Tukey’s multiple comparison post hoc analysis. Student’s t-test was used for comparisons between two groups. Significance is accepted at p<0.05.
RESULTS

High throughput screening of Na/K-ATPase inhibitors: To screen a chemical library for Na/K-ATPase inhibitors, we developed a 96-well format assay. As depicted in Fig. 1A, ouabain, as a positive control, produced a dose-dependent inhibition of the Na/K-ATPase. On the other hand, DMSO, the vehicle, showed no effect on Na/K-ATPase activity when used at a concentration below 0.1% of reaction volume (data not shown). The apparent IC$_{50}$ for ouabain was about 1 μM, comparable to what was reported (Bottinger and Habermann, 1984). Thus, we used the same assay and tested a total of 2600 compounds at the final concentration of 10 μg/ml. This concentration was adapted because a majority of compounds has a molecular mass around 200, thus being adopted around 50 μM, 50 times the IC$_{50}$ of ouabain. Ouabain (5 μM) was used as a positive control whereas 0.1% DMSO was used as a negative control in each 96-well plate. Assays were conducted in duplicate, and the compound that produced 25% or more inhibition of the Na/K-ATPase was identified as a positive hit. Under these experimental conditions, we found and confirmed a total of 13 positive compounds (Table 1) that include several well-known Na/K-ATPase inhibitors such as oligomycin and cardiotonic steroids.

Identification of hydroxyxanthones as a new class of Na/K-ATPase ligands: Among the thirteen positive hits, many are polyphenolic compounds including six
hydroxyl xanthone derivatives (MB1 to MB7) (Table 1). Structurally, they are similar to the well-characterized polyphenolic compounds such as quercetin (Fig. 1B) (Kuriki and Racker, 1976; Robinson et al., 1984). Thus, we further explored the inhibitory properties of these hydroxyxanthones. Because MB7 was the most potent inhibitor of this group (Table 2), it was used in the following studies. In experiments depicted in Fig. 1A, we compared the dose-response curves of MB7, ouabain and quercetin. Like ouabain, MB7 exhibited dose-dependent inhibition of the Na/K-ATPase. The apparent IC\textsubscript{50} (1.6 ± 0.1 μM) is comparable to that of ouabain. In comparison, quercetin is less effective as an inhibitor of Na/K-ATPase. Unlike ouabain and MB7, it only produced a partial inhibition of the Na/K-ATPase. The maximal inhibition was achieved when 5 μM quercetin was applied (Kuriki and Racker, 1976).

To determine whether MB7 produces a reversible inhibition of Na,K-ATPase, the purified Na,K-ATPase was treated with 10 μM MB7 at 37°C for 15 min, washed twice by centrifugation, resuspended, then assayed for Na/K-ATPase activity. After washed enzyme was incubated in assay medium for 15 and 60 min, the recovery of the activity reached 67±5% and 94±6%, respectively.

To explore whether MB7 inhibits the Na/K-ATPase by altering substrate affinity, we measured the Na/K-ATPase as a function of substrate concentration in the presence or the absence of MB7. As shown in Fig. 2, MB7 had no effect on either Na\textsuperscript{+} or ATP.
affinity of the Na/K-ATPase. Interestingly, increases in K\(^+\) failed to antagonize MB7-induced inhibition of the Na/K-ATPase (Fig. 2B), which is in contrast to ouabain-induced inhibition (Myers et al., 1979). Since MB7 and quercetin are both polyphenolic compounds, we further explored the effect of DMSO on MB7-induced inhibition of Na/K-ATPase. It is known that high concentrations of DMSO inhibit Na/K-ATPase by stabilizing the enzyme at the E2 conformation, which antagonizes the inhibitory effect of quercetin on the Na/K-ATPase (Foster and Ahmed, 1982; Robinson et al., 1984). Indeed, as shown in Table 3, DMSO produced a dose-dependent inhibition of Na/K-ATPase. Under such conditions, the maximal effect of 10 \(\mu\)M quercetin on the Na/K-ATPase was significantly reduced. For example, addition of 5% DMSO abolished the inhibitory effect of quercetin (Table 3). On the other hand, the inhibitory effect of 5 \(\mu\)M MB7 on the Na/K-ATPase was not affected by DMSO.

To explore the structure-activity relationship, we compared the dose-response curves of xanthone, six hydroxyxanthones and several methylated hydroxyxanthone derivatives. As depicted in Table 2, while xanthone failed to inhibit Na/K-ATPase activity, an increase in the number of phenolic groups increased the efficacy and potency of hydroxyxanthones (e.g., comparing MB2 with MB5). Consistently, full or partial methylation was able to reduce the inhibitory effect of hydroxyxanthones on the Na/K-ATPase (e.g., comparing MB5 with MB8). Finally, it appears that when phenolic groups were positioned near the oxygen in the pyrone ring (i.e., at position 4 and 5), they
had more effect on the potency of these compounds (e.g., comparing MB3 with MB5).

**MB7 fails to activate the receptor Na/K-ATPase/Src complex:** We have demonstrated that the Na/K-ATPase interacts with Src kinase to form a functional receptor complex for ouabain to activate protein kinase cascades (Tian et al., 2006). To test whether MB7 works as ouabain, capable of activating protein kinases, we first measured the effect of MB7 and ouabain on Src activity using the reconstituted Na/K-ATPase/Src complex. Because 1 μM ouabain, causing 50% inhibition of Na/K-ATPase, was effective in stimulating the Na/K-ATPase-associated Src (Fig. 3), we tested the effect of 1 and 10 μM MB7. At such concentrations, MB7 would produce 31 and 100% inhibition of Na/K-ATPase (Fig. 2). As depicted in Fig. 3, addition of MB7 failed to stimulate Na/K-ATPase-associated Src in the test tube. These findings suggest that MB7 may inhibit the ATPase activity without stimulating the receptor function of Na/K-ATPase.

To verify the above findings, we measured the effect of MB7 on Src and ERKs in cultured cells. Ouabain was again used as a positive control. As shown in Fig. 4A, while 100 nM ouabain stimulated Src in A549 cells as previously reported (Wang et al., 2009), MB7 from 0.1 to 10 μM failed to do the same. To further confirm these findings, we treated LLC-PK1 cells with either 100 nM ouabain or different concentrations of MB7. We previously showed that ouabain stimulated Src and subsequently the ERK
cascade in LLC-PK1 cells (Haas et al., 2002; Tian et al., 2006). Indeed, we found that ouabain increased the cellular amount of active ERKs as detected by immunostaining (Fig. 4B). However, under the same experimental conditions, MB7 (from 0.1 to 10 μM) failed to affect cellular ERK activity.
DISCUSSION

In this study, we adapted a simple but effective high throughput screen assay and identified 13 Na/K-ATPase inhibitors that represent several structurally divergent classes of compounds. Moreover, we were able to differentiate the newly identified inhibitors from other known inhibitors such as ouabain by assessing their effects on substrate dependence of the Na/K-ATPase. Finally, we found that the newly identified xanthone derivatives, unlike ouabain, only inhibit the ATPase activity, but do not activate the receptor Na/K-ATPase/Src complex. Thus, we have identified a new class of Na/K-ATPase ligands.

Xanthone derivatives as a new class of Na/K-ATPase inhibitors: According to the Albers-Post reaction scheme, the Na/K-ATPase transits from the E1 to the E2 state via multiple conformational changes. While Na$^+$ favors the E1 state, K$^+$ promotes the conversion of E2P to the E2 state. Over the years, several classes of organic Na/K-ATPase inhibitors have been identified (Fahn et al., 1966; Yoda and Yoda, 1982). They inhibit the Na/K-ATPase by stabilizing the enzyme at different conformational states. For example, ouabain binds and stabilizes the Na/K-ATPase at the E2P. Increases in K$^+$ compete with the binding of ouabain to the Na/K-ATPase and reduce ouabain-induced inhibition. In contrast, K$^+$ fails to antagonize the inhibitory effect of MB7 on the Na/K-ATPase.
Hydroxyxanthones and flavonoids have similar chemical structures since they both contain the benzopyrone structure (Fig. 1B). Moreover, quercetin and myricetin are well known Na/K-ATPase inhibitors (Suolinna et al., 1975). However, unlike MB7, quercetin only produces a partial inhibition of Na/K-ATPase (Fig. 1). Mechanistically, stabilization of Na/K-ATPase by DMSO at E2 conformation blocks quercetin (Robinson et al., 1984), but not MB7-induced inhibition of Na/K-ATPase (Table 3). Thus, the fusion of a benzene ring to benzopyrrole may alter the characteristics of interaction between hydroxyxanthones and the Na/K-ATPase.

Structurally, the data presented in Table 2 point out the importance of phenolic groups in xanthone derivative-induced inhibition of the Na/K-ATPase. The parent compound xanthone has no phenolic group attached to benzene rings and it had no detectable inhibition of the Na/K-ATPase. While tetrahydroxyxanthone (MB7) is the most potent inhibitor, dihydroxyxanthones barely affect the ATPase activity. Moreover, the position of phenolic groups also affects the potency of the compounds. For example, while both MB7 and MB6 contain 4 phenolic groups, MB7 is more potent than that of MB6.

Because the Na/K-ATPase shares many common features with other P-type ATPases, it is possible that the newly identified xanthone derivatives may also affect other ion pumps. This issue remains to be resolved. In addition, it would be of interest to characterize other positive hits. These compounds have different chemical structures from the known Na/K-ATPase inhibitors and hence may give us another new class of
Na/K-ATPase inhibitors working through a different mechanism.

MB7 does not activate the receptor Na/K-ATPase/Src complex: We have demonstrated that the Na/K-ATPase binds Src both in vitro and in vivo (Chen et al., 2009; Liang et al., 2006). This association regulates cellular Src activity by keeping Src in an inactive state (Tian et al., 2006). Moreover, formation of the Na/K-ATPase/Src complex produces a functional receptor for ouabain to stimulate the pump-associated Src, which subsequently assembles and activates multiple downstream protein kinase cascades including the ERKs. In contrast to ouabain, binding of MB7 to the Na/K-ATPase/Src complex in vitro failed to activate Src (Fig. 3). Consistently, it had no effect on cellular Src and ERK activity when MB7 was applied to cultured cells (Fig. 4). These findings indicate that MB7, unlike ouabain, fails to alter conformation of the Na/K-ATPase in a way so that the Src kinase domain can be released from the Na/K-ATPase. This is not surprising because MB7 inhibits the Na/K-ATPase in a different way from that of ouabain. As discussed above, many types of inhibitors have been identified over the years. In view of our new findings, it would be of great interest to test how other Na/K-ATPase inhibitors such as oligomycin and quercetin affect receptor function of the Na/K-ATPase.

Perspectives: The name xanthone designates a group of secondary metabolites normally found in a small group of plants, fungi and lichens. The xanthones from plants
appear to be associated mainly with the families *Polygalaceae, Guttiferae, Moraceae* and *Gentianaceae*. These plants have been widely used in traditional Chinese medicine. For example, Yuanzhi, the root of *Polygala sibirica* L., is extensively used for a variety of medical conditions (Morimura, 2003; Yong Jiang, 2001). As phenolic compounds, xanthones have been described for their antioxidant properties (Minami et al., 1995; Sato et al., 1992; Wang et al., 1997). These properties have been implicated in their anti-inflammatory and chemopreventive actions (Pinto et al., 2005). One of the xanthones, dimethylxanthenone-4-acetic acid, is currently undergoing clinical trials as an antitumor agent (McKeage et al., 2009). Based on our new findings, it will be of great interest to test whether MB7 is able to increase cardiac contractile function. If this can be demonstrated, these hydroxyxanthones could be of great value because they do not stimulate the receptor Na/K-ATPase/Src complex. It has been well-documented in the literature that stimulation of Na/K-ATPase-mediated signal transduction by either endogenous or exogenous CTS alters cardiac growth and induces cardiac fibrosis (Ferrandi et al., 2004; Kennedy et al., 2006). Therefore, MB7 and its analogs may improve contractile function without precipitating cardiac hypertrophy and fibrosis seen under clinical conditions of congestive heart failure.
References


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FOOTNOTES

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S.S. and Z.X. are co-corresponding authors.
Legends for figures

Figure 1.  A: Concentration curves of ouabain, MB7 and quercetin on the Na/K-ATPase.
The purified Na/K-ATPase was incubated with different concentrations of compounds for
15 min, then assayed for ouabain-sensitive ATPase activity as described in “Materials and
Methods”.  The data are combined from 3-5 separate experiments and are presented as
mean ± SE. The IC50 for ouabain and MB7 are 1 ±0.1 μM and 1.6 ±0.1 μM, respectively.
B: The chemical structures of xanthone and quercetin.

Figure 2.  Effects of MB7 on Na+, K+ and ATP dependence. Na/K-ATPase activity was
measured as in Fig. 1 as a function of Na+, K+ or ATP concentration. The data are
combined from 4 to 6 separate experiments and are plotted as Mean ± SE. MB7 was used
at 1.5 μM. The apparent Km values for Na+ are 11±1 and 9±1 mM for control and
MB7-treated Na/K-ATPase, respectively. The apparent Km values for K+ are 0.6±0.1 and
0.5±0.1 mM for control and MB7, respectively. The Km values for ATP are 0.25±0.02
mM and 0.22±0.02 mM for control and MB7-treated Na/K-ATPase, respectively.

Figure 3.  Effects of MB7 and ouabain on the receptor Na/K-ATPase/Src complex. The
purified Na/K-ATPase and purified Src were incubated in the presence of either ouabain
(1 μM) or MB7 (1 and 10 μM) as indicated for 15 min and assayed for Src activation as
described in “Materials and Methods”. Values are Mean ± SE of three independent
experiments. ** p<0.01 compared with control.
Figure 4. Effects of MB7 and ouabain on Src and ERKs. A: A549 cells were treated with ouabain or MB7 for 10 min, and cell lysates (50 μg/lane) were separated by SDS-PAGE and analyzed for Src activation as in Fig. 3. The values are mean ± SE from four to six separate experiments. B: LLC-PK1 cells were treated with MB7 or ouabain for 10 min and were immunostained with the ERK/MAPK (Phospho-Thr202/Tyr204) Phosphorylation/Translocation Cell-Based Assay Kit according to the manufacturer’s instructions. The images were collected as described under “Materials and Methods.” The scale bar represents 50 μm. Representative images from three experiments are shown. The quantitative data of p-ERK fluorescence intensity are collected from 40 different fields in three independent experiments and expressed as mean ± SE. *, p<0.05 versus control.
Tables

Table 1  Na/K-ATPase inhibitors identified by high throughput screen*

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<th>Name</th>
<th>Formula</th>
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<td>C_{22}H_{22}N_{2}O_{8}.HCl</td>
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*These inhibitors produced at least 25% inhibition of Na/K-ATPase at 10 μg/ml.
Table 2  Structure and Activity Relationship of Xanthone Derivatives

![Chemical Structure](image)

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<tr>
<td>2027BA2</td>
<td>OH H OCH₃ H H H OCH₃ OH</td>
<td>&gt;100</td>
</tr>
<tr>
<td>C-017</td>
<td>OCH₃ OCH₃ OCH₃ H H H OCH₃ H</td>
<td>&gt;100</td>
</tr>
</tbody>
</table>

* The concentration curves of each compound were constructed as in Fig. 1 and the IC₅₀ values were calculated from three independent experiments.
Table 3  Effects of DMSO on MB7 and Quercetin

<table>
<thead>
<tr>
<th>Compound (DMSO, Quercetin, MB7)</th>
<th>Inhibition of Na/K-ATPase (%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1% DMSO</td>
<td>0</td>
</tr>
<tr>
<td>1% DMSO</td>
<td>8 ± 1</td>
</tr>
<tr>
<td>5% DMSO</td>
<td>30 ± 3</td>
</tr>
<tr>
<td>Quercetin 10 μM ( plus 0.1% DMSO )</td>
<td>46 ± 4</td>
</tr>
<tr>
<td>( plus 1% DMSO )</td>
<td>17 ± 3</td>
</tr>
<tr>
<td>( plus 5% DMSO)</td>
<td>3 ± 1</td>
</tr>
<tr>
<td>MB7 5 μM ( plus 0.1% DMSO )</td>
<td>96 ± 2</td>
</tr>
<tr>
<td>( plus 1% DMSO )</td>
<td>96 ± 2</td>
</tr>
<tr>
<td>( plus 5% DMSO )</td>
<td>94 ± 3</td>
</tr>
</tbody>
</table>

* The data are from 3-5 separate experiments and are presented as Mean ± SE. The % inhibition of quercetin and MB7 was calculated using the corresponding DMSO activity as 100%.
Fig. 1

A

\[
\text{Na,K-ATPase activity (\%) }
\]
\[
\text{Log C (\mu M)}
\]

- **Ouabain**
- **MB7**

B

- **Xanthone**
- **Quercetin**
Fig. 2

A

[Graph showing Na,K-ATPase activity (%) vs. NaCl concentration (mM) for Control and MB7 conditions.]

B

[Graph showing Na,K-ATPase activity (%) vs. KCl concentration (mM) for Control and MB7 conditions.]

C

[Graph showing Na,K-ATPase activity (%) vs. ATP concentration (mM) for Control and MB7 conditions.]
### Fig. 3

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Ouabain</th>
<th>MB7</th>
</tr>
</thead>
<tbody>
<tr>
<td>μM</td>
<td>1</td>
<td>1</td>
<td>10</td>
</tr>
</tbody>
</table>

**pY418-Src**

![Image of Western Blot](image-url)

![Bar Graph](image-url)

Relative level (% of control):

- Control
- Ouabain
- MB7

**Control**

- 1 μM

**Ouabain**

- 1 μM

**MB7**

- 10 μM

**Statistical Significance:**

- **p** < 0.01

![Graphical Representation](image-url)
**Fig. 4**

**A**

- **Control** vs. **Ouabain (0.1 μM)**

  - pY418-Src
  - Src

- **Control** vs. **MB7** (1, 10 μM)

  - pY418-Src
  - Src

**B**

- **Control**
- **Ouabain (0.1 μM)**
- **MB7** (0.1, 1, 10 μM)

**Graphs**

- **Src activity (% of control)**
- **Relative level (% of control)**

* indicates statistical significance.