Molecular Mechanisms Linking Sodium to Hypertension: Report of a Symposium

Bruce P. Hamilton, Mordecai P. Blaustein

ABSTRACT

There is abundant clinical and epidemiologic data linking excess body sodium with hypertension. The mechanism(s) at the molecular level to explain this relationship are unknown. Recent studies by multiple investigators, have identified several ion transport mechanisms in the vascular wall that interact to control vascular tone and contractility. These new data include 1) biochemical, pharmacologic, and molecule structural studies, 2) experiments in transgenic and knockout mice, and 3) results in clinical hypertension. The overall results provide compelling evidence for the concept that salt-dependent hypertension involves the secretion of endogenous ouabain (EO), an adrenal steroid synthesized with the same initial steps as aldosterone and secreted by the zona glomerulosa. Circulating EO inhibits arterial smooth muscle Na+ pumps with alpha 2 subunits. These are functionally coupled to the type 1 Na/Ca exchanger (NCX1). Thus when α2 Na pumps are inhibited in arterial smooth muscle, the resulting subplasma membrane increase in Na^+ concentration triggers, via NCX1 Ca^{2+} entry, a rise in cytosolic Ca^{2+} concentration and increased myogenic tone and contractility. The ultimate result is a rise in peripheral vascular resistance—the hemodynamic hallmark of hypertension. The elucidation of this pathway has facilitated the development of pharmacologic agents that have therapeutic potential for hypertension and other cardiovascular diseases. These include agents that compete with EO for binding to the Na^+ pump and inhibitors of NCX1.

Key Words: sodium, hypertension, sodium pump, ouabain, sodium/calcium exchanger

The American Federation of Medical Research sponsored a symposium entitled "Molecular Mechanisms: Linking Sodium to Hypertension" at Experimental Biology 2005 and the XXXV International Congress of Physiological Sciences. The meeting took place in San Diego, California, and the symposium was held on Saturday, April 2, 2005. The symposium brought together for the first time key investigators from diverse laboratories that have identified several steps involving humoral and molecular mechanisms that may explain how excess salt can produce sustained increases in peripheral vascular resistance. The authors of this article were co-chairmen.

A large amount of epidemiologic and clinical data link either increased dietary intake of salt or sodium retention by the kidneys with the development of elevated blood pressure (BP).[1,2]

Furthermore, methods of decreasing the body load of salt either by dietary salt restriction or by natriuretic agents have been a cornerstone of the treatment of hypertension for many decades.[3]

Blood pressure is dependent on both cardiac output (CO) and total peripheral vascular resistance (TPR), and at constant CO, mean BP = CO $\times$ TPR. Acute plasma volume expansion, as occurs with sodium loading, elevates BP by increasing CO. However, with sustained volume expansion, CO falls to near-normal levels, whereas TPR rises to maintain the BP elevation. An elevated TPR and a near-normal CO are the hemodynamic hallmarks of essential hypertension.[1] The rise in TPR has been attributed to autoregulation,[4,5] an intrinsic property of the vascular bed to regulate blood flow depending on the metabolic need of tissues. Increased activity of the sympathetic nervous system and several other mechanisms have also been proposed, but the mechanism of this "autoregulation" is still controversial,[1,6] and the mechanism of the elevated BP (ie, the pathogenesis of essential hypertension) at the molecular level has remained unexplained.[7]

Recent studies by a number of investigators working in different countries have identified several ion transport mechanisms in the vascular wall that interact to control vascular tone and contractility in response to hormones and neurotransmitters. The new data include (1) the results of molecular structural, biochemical, and pharmacologic studies; (2) experiments in transgenic and knockout mice; and (3) data from human subjects with hypertension. Taken together, the results provide compelling evidence for the view that salt-dependent hypertension involves the secretion of endogenous ouabain (EO), an adrenal steroid secreted by the zona glomerulosa. Circulating EO inhibits arterial smooth muscle Na^+ pumps with α2 subunits. The Na^+ pumps with α2 subunits are func-

From the Division of Endocrinology, Diabetes & Nutrition (B.P.H.), Department of Medicine, Baltimore VA Medical Center and University of Maryland School of Medicine, Baltimore, MD; Departments of Physiology and Medicine (M.P.B.), University of Maryland School of Medicine, Baltimore, MD.

Address correspondence to: Dr. Bruce P. Hamilton, Medical Service, VA Medical Center, 10 N. Greene Street, Baltimore, MD 21201; e-mail: bruce.hamilton@med.va.gov.

DOI 10.2310/6650.2006.05054

tionally coupled to the type 1 Na/Ca exchanger (NCX1). Thus, when these Na^+ pumps are inhibited in arterial smooth muscle, the resulting rise in local sub–plasma membrane Na^+ concentration ($[Na^+]_{SPM}$) promotes Ca^{2+} entry. The myocyte cytosolic Ca^{2+} concentration ($[Ca^{2+}]_{CYT}$) then rises, increases myogenic tone and contractibility, and thereby results in the elevation of BP.

The four symposium speakers and their topics were John Hamlyn, University of Maryland School of Medicine, Baltimore, Endogenous Ouabain; Jerry B. Lingrel, University of Cincinnati College of Medicine, Cincinnati, Na^+ pump isoforms and their roles in hypertension; Patrizia Ferrari, Passis-Sigma Tau, Milan, Italy, ouabain antagonists; and Takahiro Iwamoto, University of Fukuoka, Fukuoka, Japan, Na^+/Ca^{2+} exchange and exchanger inhibitors.

ENDOGENOUS OUABAIN: BIOSYNTHESIS AND STRUCTURE

Cardiotonic steroids, such as digitalis glycosides and bufodienolides, have had a long history of use in cardiovascular disease. Synthesis of digitalis glycosides by specific plants is well known, whereas bufodienolides are endogenous to certain amphibians. The conservation of a specific high-affinity receptor for cardiotonic steroids on PM Na^+ pumps (Na,K–adenosine triphosphatase [ATPase]) that regulate intracellular Na^+ in most mammalian cells has fueled efforts to identify similar endogenous digitalis-like compounds in mammals.

Work from Hamlyn's laboratory has demonstrated the presence in human plasma of the cardiac glycoside ouabain or a closely related isomer, EO,[8] and three additional specific inhibitors of the sodium pump.[9] EO was the most polar and had the most powerful activity in a functional assay measuring Na^+ pump–mediated ion fluxes. The presence, in mammals, of steroids that resemble digoxin, 19-norbufalin and marinobufagenin, has been reported by other groups.[10,11] It appears, therefore, that mammals may have both endogenous cardenolide-like and bufodienolide-like inhibitors of the sodium pump. Although these agents may share structural similarities, their selectivities and physiologic roles may be quite different.

EO has been purified from the tissue and/or plasma of three different species (humans, rats, cows), and its structure has been studied in four different laboratories by analytic methods (mass spectroscopy and nuclear magnetic resonance [NMR]).[12,13] It is a polyhydroxylated steroid containing an unsaturated lactone ring and is identical or closely related to the plant-derived glycoside ouabain. The structural features of EO (Figure 1) differ from the classic hepatic, adrenal, and sex steroids that contain A/B and C/D rings fused in either a trans-trans-trans configuration (eg, corticosteroids) or a cis-trans-trans configuration (bile salts). The unique cis-trans-cis configuration of EO, not previously described in mammals, supports high-affinity binding to the Na^+ pump and ensures specificity. The potency to mass ratio of EO is similar to that of other adrenocortical steroids, such as aldosterone; they have similar molecular weights (300–600) and similar affinity for their respective receptors (low nanomolar range). In addition, the sugars at C3 in both EO and ouabain are deoxyhexoses. In ouabain, the sugar moiety is rhamnose, which dramatically increases the tightness of binding to the Na^+ pump. Rhamnose has not been specifically identified as the sugar moiety of human EO, but there is good evidence that mammals can synthesize rhamnose from glucose.

The population mean value for plasma EO as determined by specific radioimmunoassay lies between 200 and 300 pmol/L. A dietary source has been ruled out. The highest levels of EO occur in adrenal tissue. In fact, a large body of evidence indicates that EO is of adrenal origin: there is a fivefold venous to arterial gradient across the adrenal gland in dogs and humans,[14] circulating EO is increased by administration of angiotensin II (A-II) and adrenocorticotropic hormone (ACTH),[15] and plasma EO declines significantly following acute adrenalectomy.[8] Finally, adrenocortical cells in culture secrete EO, and in

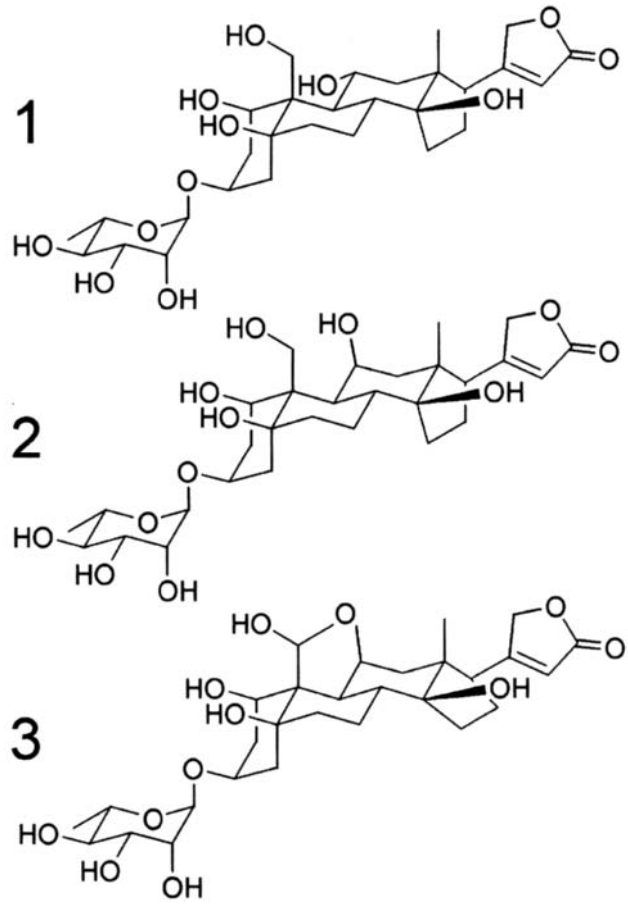


FIGURE 1 Structures of ouabain (1), the 11β isomer of ouabain (2), and the 11 to 19 hemiketal (3). Reproduced with permission from Hamlyn JM et al.[17] Copyright 2003 New York Academy of Sciences, U.S.A.

bovine cells, the site has been localized to the zona glomerulosa.[16]

The mammalian biosynthesis of cardiac glycosides almost certainly proceeds via a pathway similar to that of plants, using the same steroid intermediates found in plants and adrenal zona glomerulosa cells. The initial steps in EO and aldosterone synthesis appear to be common events and occur in the same cells. The branch point from the aldosterone pathway and distal steps in the EO pathway are under investigation.[17] The plant-derived cardiac glycoside ouabain has an 11α-hydroxyl group, whereas there is evidence from mass spectrometry and NMR of an 11β-hydroxyl in EO: that is, the mammalian compound appears to be a ouabain isomer. If the synthesis of EO diverges from the aldosterone pathway at corticosterone, there would be an 11β-oriented hydroxyl (ie, ouabain isomer), and this, in vivo, would form an additional ring structure via the 11 to 19 hemiketal (see Figure 1). In contrast, a branch point at 11-deoxycorticosterone would allow an 11α-hydroxyl EO (ie, ouabain per se).[17]

The physiologic role of EO has been studied using cells, intact physiologic preparations, whole animals, and humans. The shared biosynthetic pathway with aldosterone means that the secretion of both steroids is augmented by the same stimuli, for example, ACTH, A-II, and plasma K^+. These stimuli are recruited physiologically by Na^+ depletion and low Na^+ states and therefore were vitally important in the preservation of the species during mammalian evolution. Under contemporary dietary conditions, Na^+ is in excess of physiologic needs. As a consequence, factors such as A-II, aldosterone, and EO that augment Na^+ retention are suppressed and play no obviously important role in the renal handling of Na^+. Instead, their primary physiologic roles are coordinated, and they exert subtle effects on K^+ balance: EO shifts K^+ from the tissues to the circulation, whereas aldosterone provides a stimulus to renal K^+ excretion. The primary teleologic roles for EO and aldosterone become most apparent during low dietary Na^+ intakes and a decline in effective circulating volume. Robust homeostatic increases in A-II, aldosterone, and EO occur to preserve venous return so that CO and BP are maintained. Several critical hormone-regulated mechanisms are involved: (1) enhancement of renal tubular reabsorption of Na^+ and water (aldosterone); (2) stimulation of thirst (A-II) and an increase in cardiac, venous, and arterial contractility (A-II and EO); and (3) augmentation of the activity of central and peripheral sympathetic nerves projecting to the heart, kidney, and vasculature (A-II and EO). The same neurohormonal systems are directly linked to some common disease states. The renin-angiotensin-aldosterone system and EO are typically stimulated in response to the low CO state of congestive heart failure, with additional maladaptive adverse effects.[18] Circulating EO is abnormally elevated in approximately 50% of patients with essential hypertension, and the levels are correlated with BP and cardiac hypertrophy.[19] In more advanced hypertension, EO levels are positively and significantly correlated with peripheral resistance and are inversely correlated with the stroke index (Figure 2).[20] Thus, there is mounting evidence that EO is directly involved in the pathogenesis of hypertension.[21–24] Support comes from the observation that prolonged administration of ouabain to achieve circulating levels comparable to those measured in essential hypertension induces hypertension in rats (Figure 3).[25]

ROLE OF NA^+ PUMP α SUBUNIT ISOFORMS IN VASCULAR CONTRACTILITY AND HYPERTENSION

The plasma membrane Na^+ pump (Na,K-ATPase) is a transmembrane protein that transports three Na^+ out of the cell and two K^+ in using adenosine triphosphate (ATP) hydrolysis as the driving force. The electrochemical gradient established by the Na^+ pump is essential for maintenance of cell-specific functions, such as muscle contraction and nerve transmission. The ion gradient also drives

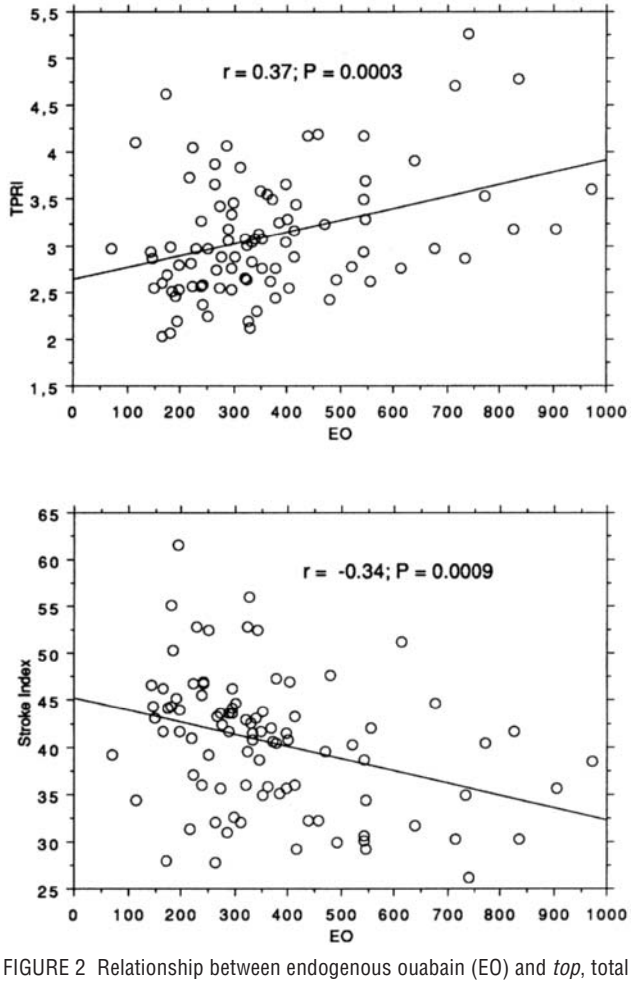


FIGURE 2 Relationship between endogenous ouabain (EO) and *top*, total peripheral resistance index (TPRI) and *bottom*, stroke index. EO is expressed in pmol/L, TPRI in dyn.s/cm^{-5}/m^2, and stroke index in mL/m^2. Reproduced with permission from Pierdomenico SD et al.[20]

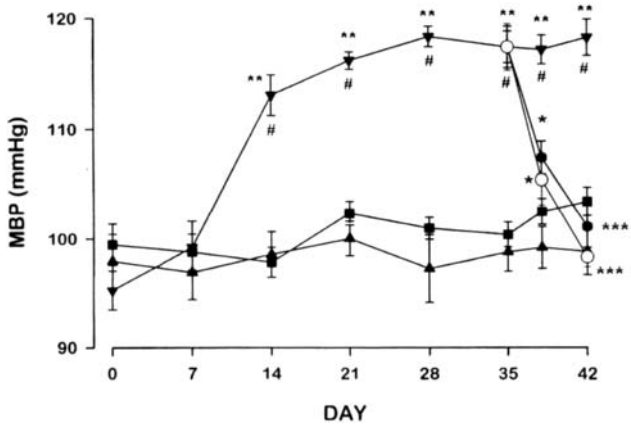


FIGURE 3 Ouabain-dependent hypertension in the rat. Rats were infused with either vehicle (■), ouabain 15 μg/kg/d (▼), or digoxin 30 μg/kg/d (▲) for 35 days. From days 35 to 42, three groups of ouabain-infused rats also received an infusion of digoxin 30 μg/kg/d (●), digitoxin 30 μg/kg/d (0), or vehicle (▼). Mean blood pressures (MBP) were obtained by tail cuff. $*p < .05$ vs ouabain; $**p < .001$ vs digoxin; $***p < .001$ vs ouabain; $\#p < .005$ vs vehicle. Chronic infusion of ouabain but not digoxin produced significant hypertension that was antagonized by secondary infusion of digoxin or digitoxin. Reproduced with permission from Manunta P et al.[25]

many plasma membrane transport processes, including Na^+-coupled Ca^{2+} transport by the Na^+/Ca^{2+} exchanger (NCX) and uptake of nutrients, such as glucose in kidney and gut, via Na^+ cotransporters. The Na^+ pump is a dimer composed of an α and a β subunit in a 1:1 ratio: the catalytic α subunit contains the Na^+, K^+, and ATP binding (and hydrolytic) sites, as well as a binding site for cardiac glycosides, such as ouabain, which inhibit the pump. The β subunit modulates cation affinity and is necessary for proper folding and translocation of the Na^+ pump to the plasma membrane. There are four isoforms of the α subunit (α1, α2, α3, and α4), each with unique kinetic properties and tissue distribution that suggest different and tissue-specific functional roles.

The plasma membrane NCX is regulated by the Na^+ pump via the pump's influence on the Na^+ electrochemical gradient across the plasma membrane. Inhibition of the Na^+ pump by cardiac glycosides (eg, ouabain) raises $[Na^+]_{CYT}$, and this, in turn, increases $[Ca^{2+}]_{CYT}$. The increase in cytosolic and sarcoplasmic/endoplasmic reticulum (S/ER) Ca^{2+} produces a positive inotropic response of the heart and thereby confers the therapeutic effect of these agents used in the treatment of congestive heart failure.[24]

Most cells express α1 Na^+ pumps, as well as pumps with one of the other α subunit isoforms; α1 has a higher affinity for Na^+ than α2 or α3. In rodents, α1 has an unusually low affinity for ouabain, whereas α2 and α3 have a high affinity; in humans, for example, all three isoforms have a similar high affinity for ouabain. The isoforms also have a different distribution in the plasma membrane: whereas α1, in most types of cells, is fairly uniformly distributed in the plasma membrane, α2 and α3 are confined to plasma membrane microdomains that overlie S/ER.[26] The NCX, too, is confined to plasma membrane microdomains that overlie the S/ER.[26] In many types of cells, α1 Na^+ pumps predominate (eg, they comprise about 70 to 80% of the pumps in the heart and vascular smooth muscle) and serve as a "housekeeper" to maintain the low global $[Na^+]_{CYT}$.

The role of the Na^+ pump α2 isoform in ouabain-induced cardiac intropy was investigated in mice expressing a mutated ouabain-resistant α2 isoform[27] and in mice with a single null mutation in either the α2 or α1 isoform gene.[24] These α2 or α1 heterozygotes express ≈50% of the normal complement of α2 or α1, respectively.

Cardiac contractility was augmented in the hearts of α2, but not α1, heterozygotes; in contrast, the hearts of the α1 heterozygotes were "hypocontractile." Nevertheless, low-dose ouabain induced a cardiotonic effect in the hearts of the α1 heterozygotes.[24] Moreover, ouabain had no cardiotonic effect on the isolated hearts from the ouabain-resistant (targeted) α2 mice.[27] These results clearly demonstrate that the α2 isoform of the Na^+ pump mediates the ouabain-induced cardiotonic effect in mice.[27]

BP also was measured in the α2 targeted mice. Although chronic ouabain infusion induced hypertension in wild-type mice, it did not elevate BP in mice with a ouabain-resistant α2 isoform.[27] Thus, ouabain-induced hypertension is mediated by the α2 isoform in mice. In unpublished studies, Lingrel and colleagues demonstrated that ACTH injection rapidly (within 48 hours) induces hypertension in mice. ACTH hypertension could not, however, be induced in the α2 targeted (ouabain resistant) mice. This seminal observation raises the possibility that EO may be the "missing hormone" in ACTH-induced hypertension. It is consistent with the evidence that ACTH stimulates the secretion of EO.[15]

The proximity of the α2 pumps to the NCX on plasma membrane microdomains that overlie S/ER suggested that ouabain might regulate cell Ca^{2+} signaling by inhibiting only α2 pumps controlling the $[Na^+]_{CYT}$ primarily in the space between these plasma membrane microdomains and the adjacent junctional S/ER. Thus, α2 Na^+ pumps would be expected to regulate local $[Na^+]_{SPM}$ and, via NCX, not only the local $[Ca^{2+}]_{SPM}$ in the junctional space but also the $[Ca^{2+}]$ in the S/ER that plays a key role in signaling.

This hypothesis was tested in astrocytes cultured from normal (wild type) mice and mice with a null mutation in one (heterozygote) or both (knockout) α2 genes. The results[28] correlate with the structural evidence that α2 Na^+ pumps and NCX are confined to plasma membrane microdomains that overlie the S/ER. In the cells from α2 heterozygotes, $[Ca^{2+}]_{CYT}$ was slightly increased, the S/ER Ca^{2+} stores were augmented, and Ca^{2+} signaling was amplified without elevation of bulk $[Na^+]_{CYT}$. These effects are, presumably, the result of an undetected increase in $[Na^+]_{SPM}$ in the tiny space between the plasma membrane and S/ER. In cells from α2 knockout (homozygous) mice,

even global $[Na^+]_{CYT}$ was increased slightly, and resting $[Ca^{2+}]_{CYT}$ and Ca^{2+} signaling were further amplified.[28]

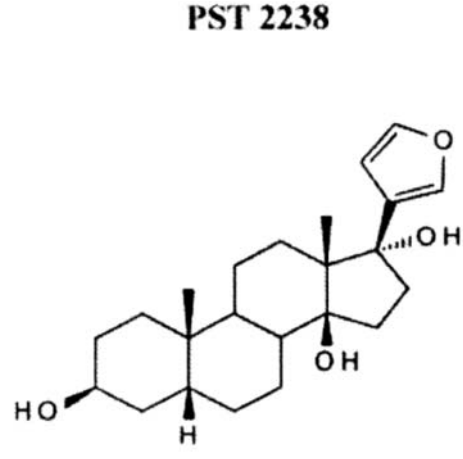
OUABAIN ANTAGONISTS: EFFECTS ON BLOOD VESSELS AND ROLE IN HYPERTENSION THERAPY

A renal defect in Na^+ excretion is a principal mechanism for the rise in BP in experimental[28,29] and genetic[30] rat models and in at least some forms of monogenic human hypertension.[31,32] For many years, the Milan group has directed its attention to the molecular defects leading to this renal Na^+ abnormality and the question of whether similar mechanisms are involved in human essential hypertension.[30,33,34] These studies would not only identify new targets for innovative treatment but also genetic markers that would characterize hypertensive patients responsive to this treatment. In clinical studies of human hypertensive patients, and using Milan hypertensive strain (MHS) rats, an animal model with some pathophysiologic similarities to a subset of human hypertension, two molecular-genetic mechanisms have been identified: (1) mutation of genes coding for the cytoskeleton protein adducin[31,33] and (2) increased circulating levels of EO. Both mechanisms reportedly lead to increased function of renal Na,K-ATPase, the transport system that drives Na^+ from the luminal to the interstitial side of the renal tubular cell. Although there is considerable evidence that these two mechanisms may play roles in clinical hypertension and cardiovascular complications, there is also controversy. For example, the role of the human α-adducin (ADD1) TRP allele has been confirmed in many studies involving linkage with appropriate deoxyribonucleic acid (DNA) markers, BP, and the cardiovascular complications of hypertension.[33,34] Nevertheless, some Japanese studies have reported the absence of such an association.[35,36]

EO,[32,33] in addition to an effect on renal tubular cells and a direct effect on the myogenic tone of small resistance arteries, appears to have importance in the development of cardiac and renal complications associated with hypertension. This has been demonstrated in rat models[34] and in human hypertensive patients.[19,37] Data suggesting that EO at subnanomolar concentrations stimulates both renal tubular Na^+ reabsorption[38] and at the same time increases myogenic tone and vascular resistance by inhibiting α2 Na^+ pumps[23] are apparently contradictory and the subject of debate. Also, in normotensive rats and mice, infusion of ouabain to achieve subnanomolar concentrations not only elevates BP[25] and increases myogenic tone,[23] it also stimulates renal Na,K-ATPase activity.[38] The renal effect implies that nanomolar EO (or ouabain) stimulates the α1 Na,K-ATPase in tubular cells even though rodent α1 has an unusually low affinity for ouabain. Conversely, the vascular effect of EO (or low-dose ouabain) involves inhibition of the high–ouabain affinity α2 Na,K-ATPase isoform in vascular smooth muscle.[23] These latter effects of nanomolar ouabain are mimicked by reduced expression of α2[23] and are prevented by mutation of the α2 ouabain binding site.[27] To explain their paradoxical findings, the Milan group hypothesized that EO acts not only as a classic Na^+ pump inhibitor but also, at very low concentrations, as a signal transducer. EO would thus induce tyrosine phosphorylation of the renal α1 Na^+ pump via a Src-EGRr–dependent pathway, with activation of function, increased Na^+ reabsorption, volume expansion, and resulting hypertension.[34,38,39] However, it is the volume expansion, per se, that apparently increases the plasma EO level.[40] These issues therefore still need to be resolved.

The hypothesis that ouabain-like compounds might play a key role in the pathogenesis of salt-dependent hypertension[8,21,25] fostered the search for new and effective ouabain antagonists. Rostafuroxin (PST 2238) is the first such agent developed. Rostafuroxin is a novel digitoxigenin derivative (Figure 4) that in vitro can displace ^3H-ouabain–specific binding from purified Na,K-ATPase. Rostafuroxin does not inhibit the Na^+ pump directly and has no cardiotonic effects.[41]

With respect to modulation of renal Na^+ pump expression (kidneys normally express, almost exclusively, α1 Na^+ pumps), rostafuroxin was tested in cultured normal rat kidney cells either transfected with the mutated variant of rat α-adducin or incubated with nanomolar concentrations of ouabain. In both of those conditions, rostafuroxin selectively counteracted the Na^+ pump overexpression.[41] In addition, rostafuroxin, in a nanomolar dosage range, normalized the BP in several salt-dependent hypertension animal models and in normal Sprague-Dawley rats chronically infused with low-dose ouabain (Figure 5).[41] In the latter animals, rostafuroxin also counteracted the activation of the renal Na^+ pump and cardiac hypertrophy.[42] Rostafuroxin had no effect on normotensive control Sprague-



17β-(3-furyl)-5β-androstan-3β, 14β, 17α-triol

FIGURE 4 Structure of rostafuroxin (PST 2238), 17β-(3-furyl)-5β-androstan-3β, 14β, 17α-triol. This is a digitoxigenin derivative that in vitro displaces ^3H-ouabain–specific binding from purified Na^+, K^+–adenosine triphosphatase.

Dawley rats infused with saline. In genetic MHS rats, which show both adducin polymorphisms and increased levels of EO, rostafuroxin reduced the BP and normalized the up-regulated α1 Na^+ pump expression, but the doses necessary to control cardiac hypertrophy were 10- to 50-fold higher than in ouabain-hypertensive rat. This suggests that rostafuroxin is more selective for mechanisms supported by high levels of EO.[42,43] Rostafuroxin is currently being studied in phase II trials in Europe for the treatment of essential hypertension. It lowers BP in about 40% of hypertensives with an efficacy that seems to correlate with the levels of circulating EO.[43]

ROLE OF THE VASCULAR NA^+/CA^{2-4+} EXCHANGER IN SALT-DEPENDENT HYPERTENSION

The Na^+/Ca^{2+} exchanger catalyzes the bidirectional exchange of Ca^{2+} for Na^+ ions across the plasma membrane,[44] that is, the NCX may mediate Ca^{2+} efflux/Na^+ influx ("forward" mode) or Na^+ efflux/Ca^+ influx ("reverse" mode) exchange. The direction of net Ca^{2+} flux is determined by the magnitude and orientation of membrane potential and the transmembrane Na^+ and Ca^{2+} ion gradients. In arterial smooth muscle cells, the NCX is thought to play an important role in the regulation of $[Ca^{2+}]_{CYT}$ during the contraction-relaxation cycle.

Three isoforms of the exchanger have been identified; they are encoded by distinct genes in mammals (*NCX1*, *NCX2*, and *NCX3*). Recent genetic engineering and pharmacologic studies indicate that the Ca^{2+} influx mode of vascular NCX1 (splice variant 1.3, NCX1.3) is involved in the pathogenesis of salt-dependent hypertension (Figure 6).[22] NCX1 transcripts are widely expressed in heart, arteries, kidney, brain, and other organs. Expression of *NCX2* and *NCX3* genes is restricted to the brain and skeletal muscles.

In the heart, NCX1 is involved in excitation-contraction coupling, where it is the dominant myocardial Ca^{2+} efflux system. During diastole, NCX1 extrudes the Ca^{2+} that enters cardiac myocytes through voltage-gated Ca^{2+} channels during contraction and thus returns the cardiac cells to the resting state.

The role of NCX1 has been studied in mutant mice that lack the *NCX1* gene.[45] Homozygous *NCX1*-deficient mice die in utero. Their hearts do not beat, and the cardiac myocytes exhibit apoptosis. No forward-mode (Na^+ entry, Ca^{2+} exit) or reverse-mode (Na^+ exit, Ca^+ entry) NCX activity was detected in null mutant hearts. In heterozygous mice, NCX protein expression and NCX activity are decreased by about 50% in the heart, kidney, aorta, and smooth muscle cells.[45] It is especially noteworthy that these animals are resistant to salt-induced hypertension. Whereas administration of deoxycorticosterone acetate and salt markedly elevated BP in uninephrectomized, normal (wild type) mice, BP rose slowly and only to a small extent in uninephrectomized NCX1 heterozygotes. In addition, tension development of aortic rings in Na^+-free solution was markedly impaired in the NCX1 heterozygotes. Conversely, mice that overexpress NCX1.3 in smooth muscle were very salt sensitive and developed hypertension on a high-salt diet without uninephrectomy or deoxycorticosterone acetate.[22] These findings indicate that NCX1 is required for heart beat and cardiac myocyte survival in embryos and that it plays a critical role in Na^+-dependent Ca^{2+} handling in the heart and vascular smooth muscle.

Several NCX inhibitors with therapeutic potential are under investigation. They have, in common, a benzyloxyphenyl structure, which suggests that this portion of the molecule is essential for binding to the exchanger (Fig-

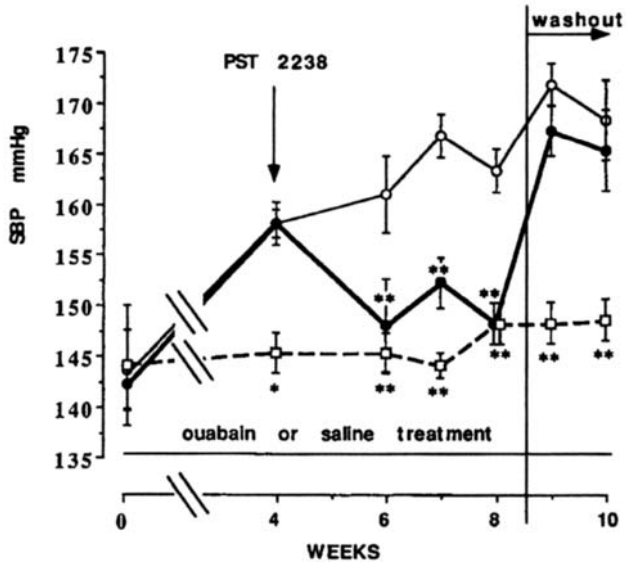


FIGURE 5 Systolic blood pressure (SBP) in adult Sprague-Dawley rats receiving ouabain (50 μg/kg/d) (○●) or saline (□) for 10 weeks via osmotic minipumps. At week 4, one group of the ouabain-infused rats was treated with oral rostafuroxin (PST 2238) (●) 0.1 mg/kg/d, whereas the other ouabain group (○) received only vehicle. A washout period of 2 weeks from the PST 2238 treatment started at the end of week 8. Seven rats in each group. $*p < .05$; $**p < .01$ significantly different from ouabain infusion–only rats (○). Reproduced with permission from Ferrari P et al.[41]

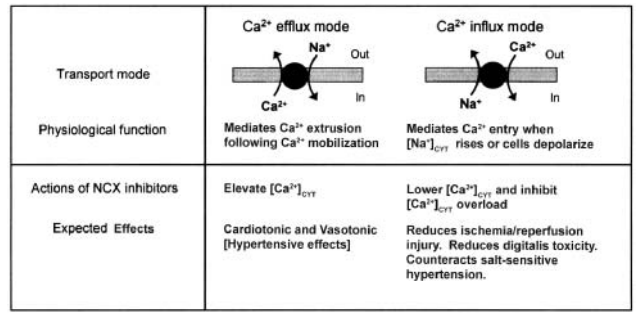


FIGURE 6 Physiologic and pharmacologic implications of the Na^+/Ca^+ exchanger (NCX) and NCX inhibitors SEA0400 and the other benzyloxyphenyl NCX inhibitors discussed. Although the mechanism is unknown, all of these inhibitors block the Ca^{2+} influx mode more effectively than the Ca^{2+} efflux mode. Adapted from Iwamoto T[46] with kind permission of Springer Science and Business Media.

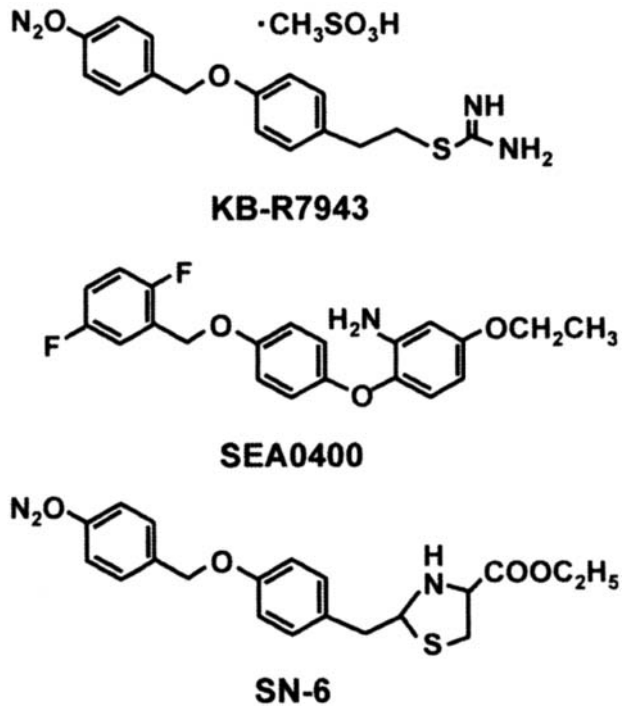


FIGURE 7 Chemical structures of benzyloxyphenyl NCX1 inhibitors. Reproduced from Iwamoto T[46] with kind permission of Springer Science and Business Media.

ure 7).[46] SEA 0400 is a newly developed, potent inhibitor of NCX, with unique selectivity for Ca^{2+} influx mode activity.[47] KB-R7943[48] was also developed as a prototype for a selective NCX inhibitor, but it has nonspecific actions against ion channels, the norepinephrine transporter, and other receptors and is consequently less specific for NCX1 than SEA0400. SN-6 is a derivative of KB-R7943,[49] and M-244769[48] is a potent NCX inhibitor with low cell toxicity, but its detailed profile is still under investigation.

SEA0400 preferentially inhibits the Ca^{2+} influx/Na^+ efflux (ie, the reverse mode) exchange mediated by NCX in neuronal cells and cardiac myocytes (see Figure 6).[46,50] This apparent selectivity is due to the binding of SEA0400 to the internal Na^+-inactivated state of the NCX.[51]

SEA0400 normalized BP in animals with ouabain-induced hypertension and in the several salt-dependent hypertension models in which it has been tested (deoxycorticosterone acetate–treated rat and mouse, the Dahl salt-sensitive rat, the spontaneously hypertensive rat [SHR], and the smooth muscle–specific NCX1.3 overexpression mouse, all on high-salt diets).[22] In contrast, SEA0400 had a negligible effect on BP in the normotensive rat and mouse and in salt-insensitive rat hypertensive models (SHR on a standard-diet, stroke-prone SHR, and 2 kidney-1 clip hypertension).[22] Also, SEA0400 has no effect on the elevated BP of salt-loaded transgenic mice that overexpress, in smooth muscle, a NCX1.3 mutant with greatly reduced affinity for SEA0400.[22] The latter observation clearly indicates that the BP-lowering effect of SEA0400 is due to specific inhibition of NCX1.3. These results suggest that selective inhibitors of NCX1.3 may have therapeutic potential in ischemic heart disease, arrhythmias, heart failure, and salt-dependent hypertension.

SUMMARY

A polyhydroxylated steroid, either the cardiac glycoside ouabain or a ouabain isomer (EO), is synthesized in the same cells of the adrenal zona glomerulosa using the same initial synthetic steps as aldosterone. EO is a highly specific ligand for arterial smooth muscle Na^+ pumps with $\alpha 2$ subunits. The Na^+ pumps with $\alpha 2$ subunits are functionally coupled to the arterial myocyte NCX1. EO inhibits these Na^+ pumps, thereby presumably elevating sub–plasma membrane [Na^+] and promoting Ca^{2+} entry by NCX1. The rise in myocyte [Ca^{2+}] increases myogenic tone and contractility, and the ultimate result is an elevation in BP. In contrast, there is evidence that EO also activates the renal tubular α isoform ($\alpha 1$) of the Na^+ pump, with resulting Na^+ retention, volume expansion, and a rise in BP. The volume expansion may, in fact, promote the secretion of EO and thereby trigger the elevation of BP. Indeed, ouabain infused into normal rats elevates BP. Moreover, approximately 50% of patients with essential hypertension have plasma EO levels greater than 2 SD above the normal mean.

A novel digitoxigenin derivative, rostafuroxin (PST 2238) is a oubain antagonist that displaces ouabain from its binding site on the Na^+ pump. Rostafuroxin lowers BP in several salt-dependent rat hypertension models, in rats with ouabain-induced hypertension, and in about 40% of patients with essential hypertension. Several NCX inhibitors that have in common a benzyloxyphenyl structure are under investigation. They inhibit reverse-mode internal Na^+-dependent Ca^{2+} influx via the NCX and have specific efficacy in Na^+-dependent models of hypertension. They also have potential in other clinical conditions to protect against intracellular Ca^{2+} overload, such as ischemia/reperfusion injury and digitalis toxicity.

The evidence is compelling that the mechanism underlying the elevation of BP in a high proportion of hypertensive patients involves the interaction of EO, an adrenal steroid with a high affinity for the $\alpha 2$ isoform of the sodium pump. Inhibition of the pump by EO promotes a rise in intracellular [Ca^{2+}] via the NCX, and this stimulates a rise in smooth muscle arterial tone and thus BP. The several steps in this molecular pathway offer several possibilities in the design of new antihypertensive therapeutic agents.

REFERENCES

1. Kaplan NM. Kaplan's clinical hypertension. Philadelphia: Lippincott Williams & Wilkins; 2002.
2. Chobanian AV, Bakris GL, Black HR, et al, and the National High Blood Pressure Education Program Coordinating Committee. Seventh report of the Joint National Committee on

Prevention, Detection, Evaluation and Treatment of High Blood Pressure. Hypertension 2003;42:1206–52.
3. Swales JD. Dietary sodium restriction in hypertension. In: Laragh JH, Brenner BM, editors. Hypertension: pathophysiology, diagnosis and management. 2nd ed. Vol 1. New York: Raven Press; 1995. p. 283–98.
4. Guyton AC. Long term arterial control: an analysis from animal experiments and computer and graphic models. Am J Physiol Regul Integr Comp Physiol 1990;259:R865–77.
5. Borst JGG, Borst-deGeus A. Hypertension explained by Starling's theory of circulatory homeostasis. Lancet 1963;1:677–82.
6. Cowley AW Jr. Long term control of arterial blood pressure. Physiol Rev 1992;72:231–300.
7. Oparil S, Zaman MA, Calhoun DA. Pathogenesis of hypertension. Ann Intern Med 2003;139:761–76.
8. Hamlyn JM, Blaustein MP, Bova S, et al. Identification and characterization of a ouabain-like compound from human plasma. Proc Natl Acad Sci U S A 1991;88:6259–63.
9. Ludens JH, Clark MA, DuCharme DW, et al. Purification of an endogenous digitalis-like factor from human plasma for structural studies. Hypertension 1991;17:923–9.
10. Shaikh IM, Lau BWC, Siegfried BA, Valdes R. Isolation of digoxin-like immunoreactive factors from mammalian adrenal cortex. J Biol Chem 1991;226:13672–8.
11. Bagrov AY, Fedorova OV, Dmitrieva RI, et al. Characterization of a urinary bufodienolide Na^+ K^+-ATPase inhibitor in patients after acute myocardial infarction. Hypertension 1993;31:1097–103.
12. Mathews WR, DuCharme DW, Hamlyn JM, et al. Mass spectral characterization of an endogenous digitalis-like factor from human plasma. Hypertension 1991;17:930–5.
13. Kawamura A, Guo J, Itagaki Y, et al. On the structure of endogenous ouabain. Proc Natl Acad Sci U S A 1999;96:6654–9.
14. Boulanger BR, Lilly MP, Hamlyn JM, et al. Ouabain is secreted by the adrenal gland in awake dogs. Am J Physiol 1993;264:E413–9.
15. Cayosa LB, Hamilton BP, Manunta P, et al. Effect of ACTH and angiotensin II on plasma ouabain [abstract]. J Hypertens 1994;12(Suppl 3):164.
16. Laredo J, Hamilton BP, Hamlyn JM. Secretion of endogenous ouabain from bovine adrenocortical cells: role of the zona glomerulosa and zona fasciculata. Biochem Biophys Res Commun 1995;212:487–93.
17. Hamlyn JM, Laredo J, Shah JR, et al. 11-Hydroxylation in the biosynthesis of endogenous ouabain: multiple implications. Ann N Y Acad Sci 2003;986:685–93.
18. Gottlieb SS, Rogowski AC, Weinberg M, et al. Elevated concentrations of endogenous ouabain in patients with congestive heart failure. Circulation 1992;86:420–5.
19. Manunta P, Stella P, Rivera R, et al. Left ventricular mass, stroke volume and ouabain-like factor in essential hypertension. Hypertension 1999;34:450–6.
20. Pierdomenico SD, Bucci A, Manunta P, et al. Endogenous ouabain and hemodynamic and left ventricular geometric patterns in essential hypertension. Am J Hypertens 2001;14:44–50.
21. Blaustein MP. Sodium ions, calcium ions, blood pressure regulation and hypertension; a reassessment and a hypothesis. Am J Physiol Cell Physiol 1977;232:C165–73.
22. Iwamoto T, Kita S, Zhang J, et al. Salt-sensitive hypertension is triggered by Ca^{2+} entry via Na^+/Ca^{2+} exchanger type-1 in vascular smooth muscle. Nat Med 2004;10:1193–9.
23. Zhang J, Lee MY, Cavalli M, et al. Sodium pump $\alpha 2$ subunits control myogenic tone and blood pressure in mice. 2005. [DOI 10.1113/jphysiol.2005.091801]
24. James PF, Grupp IL, Grupp G, et al. Identification of a specific role for the Na,K-ATPase alpha 2 isoform as a regulator of calcium in the heart. Mol Cell 1999;3:555–63.
25. Manunta P, Rogowski AC, Hamilton BP, Hamlyn JM. Ouabain-induced hypertension in the rat: relationships among plasma and tissue ouabain and blood pressure. J Hypertens 1994;12:549–60.
26. Juhaszova M, Blaustein MP. Distinct distribution of different Na^+ pump alpha subunit isoforms in plasmalemma. Physiological implications. Ann N Y Acad Sci 1997;834:524–36.
27. Dostanic I, Paul RJ, Lorenz JN, et al. The α_2 isoform of Na,K-ATPase mediates ouabain-induced hypertension in mice and increased vascular contractility in vitro. Am J Physiol Heart Circ Physiol 2005;288:H477–85.
28. Golovina VA, Song H, James PF, et al. Na^+ pump α_2-subunit expression modulates Ca^{2+} signaling. Am J Physiol Cell Physiol 2003;284:C475–86.
29. Pamnani MB, Whithehorn WV, Clough DL, Haddy FG. Effects of canrenone on blood pressure in rat with reduced renal mass. Am J Hypertens 1993;3:188–95.
30. Ferrari P, Bianchi G. Lessons from experimental genetic hypertension. In: Laragh JH, Brenner BM, editors. Hypertension: pathophysiology, diagnosis and management. New York: Raven Press; 1995. p. 126.
31. Toka HR, Luft FC. Monogenic forms of human hypertension. Semin Nephrol 2002;22:81–8.
32. Cusi D, Barlassina C, Azzani T, et al. Polymorphism of α adducin and salt sensitivity in patients with essential hypertension. Lancet 1997;349:1353–7.
33. Bianchi G, Tripodi G, Casari G, et al. Two point mutations within the adducin genes are involved in blood pressure variation. Proc Natl Acad Sci U S A 1994;91:3999–4003.
34. Bianchi G, Ferrari P, Staessen JA. Adducin polymorphism detection and impact on hypertension and related disorders. Hypertension 2005;45:331–40.
35. Kato N, Sugiyama T, Nabika T, et al. Lack of association between the alpha-adducin locus and essential hypertension in the Japanese population. Hypertension 1998;31:730–3.
36. Ishikawa K, Katsuya T, Sato N, et al. No association between alpha-adducin 460 polymorphism and essential hypertension in a Japanese population. Am J Hypertens 1998;11:502–6.
37. Delva P, Devynck MA, Degan M, et al. Plasma levels of an endogenous Na–K pump inhibitor in relation to hemodynamic data in cardiopathic patients. Clin Sci 1991;81,part 1:23–9.
38. Ferrandi M, Molinari I, Barassi P, et al. Organ hypertrophic signaling within caveolae membrane subdomains triggered by ouabain and antagonized by PST 2238. J Biol Chem 2004;279:33306–14.
39. Haas M, Askari A, Xie Z, Involvement of Src and epidermal growth factor receptor in the signal-transducing function of Na^+/K^+-ATPase. J Biol Chem 2000;275:27832–7.
40. Manunta P, Hamilton BP, Hamlyn JM. Salt intake and depletion increase circulating levels of endogenous ouabain in normal men. Am J Physiol Regul Integr Comp Physiol 2005. R-00648-2005.R1
41. Ferrari P, Torielli L, Ferrandi M, et al. PST 2238: a new antihypertensive compound that antagonizes the long-term pressor effect of ouabain. J Pharmacol Exp Ther 1998;285:83–94.

42. Ferrari P, Ferrandi M, Tripodi G, et al. PST 2238: a new antihypertensive compound that modulates Na, K-ATPase in genetic hypertension. J Pharmacol Exp Ther 1999;288:1074–83.
43. Ferrari P, Ferrandi M, Torielli L, et al. Antihypertensive compounds that modulate the Na–K pump. Ann N Y Acad Sci 2003;986:694–701.
44. Blaustein MP, Lederer WJ. Sodium/calcium exchange: its physiological implications. Physiol Rev 1999;79:763–854.
45. Wakimoto K, Kobayashi K, Kuro OM, et al. Targeted disruption of Na^+/Ca^{2+}exchanger gene leads to cardiomyocyte apoptosis and defects in heartbeat. J Biol Chem 2005;275:36991–8.
46. Iwamoto T. Forefront of Na^+/Ca^{2+} exchanger studies: molecular pharmacology of Na^+/Ca^{2+} exchange inhibitors. J Pharmacol Sci 2004;96:27–32.
47. Matsuda T, Arakawa N, Takuma K, et al. SEA0400, a novel and selective inhibitor of the Na^+-Ca^{2+} exchanger attenuates reperfusion injury in the in vitro and in vivo cerebral ischemic models. J Pharmacol Exp Ther 2001;298:249–56.
48. Kuramochi T, Kakefuda A, Yamada H, et al. Synthesis and structure-activity relationships of benzyloxyphenyl derivatives as a novel class of NCX inhibitors: effects on heart failure. Bioorg Med Chem 2005;13:725–34.
49. Iwamoto T, Inoue Y, Ito K, et al. The exchanger inhibitory peptide region-dependent inhibition of Na^+/Ca^{2+} exchange by SN-6. Mol Pharmacol 2004;66:45–55.
50. Iwamoto T, Kita S, Uehara A, Imanaga I, et al. Molecular determinants of Na^+/Ca^{2+} exchange (NCX1) inhibition by SEA0400. J Biol Chem 2004;279:7544–53.
51. Lee C, Visen NS, Dhalla NS, et al. Inhibitory profile of SEA0400 [2-[4-[(2,5-difluorophenyl)methoxy]phenoxy]-5-ethoxyaniline] assessed on the cardiac Na+-Ca2+ exchanger, NCX1.1. J Pharmacol Exp Ther 2004;311:748–57.