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Zoniporide: a potent and highly selective inhibitor of human Na^+/H^+ exchanger-1

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Abstract

We evaluated the in vitro pharmacological profile of a novel, potent and highly selective Na^+/H^+ exchanger-1 (NHE-1) inhibitor, [1-(Quinolin-5-yl)-5-cyclopropyl-1*H*-pyrazole-4-carbonyl]guanidine hydrochloride monohydrate (zoniporide or CP-597,396). The potency and selectivity of zoniporide were determined via inhibition of $^{22}Na^+$ uptake by PS-120 fibroblast cell lines overexpressing human NHE-1, -2 or rat NHE-3. Additionally, potency for endogenous NHE-1 was confirmed via ex vivo human platelet swelling assay (PSA), in which platelet swelling was induced by exposure to sodium propionate. The pharmacological profile of zoniporide was compared with that of eniporide and cariporide. Zoniporide inhibited $^{22}Na^+$ uptake in fibroblasts expressing human NHE-1 in a concentration-dependent manner ($IC_{50} = 14$ nM) and was highly selective (157-fold and 15,700-fold vs. human NHE-2 and rat NHE-3, respectively). Zoniporide was 1.64- to 2.6-fold more potent at human NHE-1 than either eniporide or cariporide ($IC_{50} = 23$ and 36 nM, respectively). Zoniporide was also more selective at inhibiting human NHE-1 vs. human NHE-2 than either eniporide or cariporide (157-fold selective compared with 27- and 49-fold, respectively). All three compounds inhibited human platelet swelling with IC_{50} values in low nanomolar range. From these results, we conclude that zoniporide represents a novel, potent and highly selective NHE-1 inhibitor.

Keywords: Na^+/H^+ exchanger; Platelet swelling assay

1. Introduction

The Na^+/H^+ exchanger (NHE) plays crucial role in the regulation of intracellular pH by extruding protons in exchange for extracellular Na^+ in a 1:1 stoichiometry (Wakabayashi et al., 1997). At least six isoforms of NHE are known to exist, each with a distinct pharmacological profile (Orlowski, 1999). NHE-1, although ubiquitously distributed, is the predominant isoform expressed in the heart and is believed to play a major role in mediating Ca^{2+} overload and cellular necrosis following myocardial ischemia (Wakabayashi et al., 1997; Karmazyn, 1996). The exchanger is quiescent under physiological conditions and is activated by intracellular acidosis (Wallert and Frohlich, 1989).

During the past several years, amiloride and its derivatives have been extensively utilized to study NHE and its role during myocardial ischemia and reperfusion (Karmazyn et al., 1993; Tani and Neely, 1989). Although efficacious as cardioprotective agents, amiloride and its derivatives lack the potency and selectivity to be fully utilized as therapeutic agents (Pierce et al., 1993; Garcia et al., 1990). Recently, however, cariporide (Aventis) and eniporide (Merck KGaA) have been shown to be potent and selective inhibitors of NHE-1 (Weichert et al., 1997; Baumgarth et al., 1997) with potential for treatment of acute myocardial infarction. Here we report characterization of a structurally novel, potent and highly selective NHE-1 inhibitor, [1-(Quinolin-5-yl)-5-cyclopropyl-1*H*-pyrazole-4-carbonyl]guanidine hydrochloride monohydrate (zoniporide or CP-597,396) (Guzman-Perez et al., 2001).

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The cardioprotective efficacy of zoniporide was recently demonstrated both in vitro and in vivo using rabbit models of myocardial ischemia–reperfusion injury (Knight et al., 2001).

The in vitro pharmacological profile of zoniporide is presented and compared with that of eniporide and cariporide, two other NHE-1 inhibitors currently under clinical development. Zoniporide represents a novel class of potent NHE-1 inhibitors with the potential to prevent myocardial ischemic injury in a variety of cardiovascular diseases.

2. Materials and methods

2.1. Materials

Zoniporide (CP-597,396), cariporide and eniporide were synthesized at Pfizer Global Research and Development (Groton, CT). All drug stock solutions were made in dimethyl sulfoxide (DMSO) and diluted in buffer. Final DMSO concentration in assays was <1%. Carrier free $^{22}Na^+$ was purchased from Amersham (Piscataway, NJ). All molecular biological reagents were purchased from New England Biolabs (Beverly, MA) unless otherwise noted. All other reagents were of analytical grade and obtained commercially.

2.2. NHE-expressing cells

PS120 fibroblast cells expressing human NHE-1 and rat NHE-3 isoforms were obtained from Professor J. Pouyssegur (Nice, France) and were cultured as previously described (Counillon et al., 1993). Human NHE-2-expressing cells were generated at Pfizer Global Research and Development. Full-length human NHE-2 cDNA was cloned by 5′ and 3′ rapid amplification of cDNA ends (RACE) procedures utilizing a published partial sequence (Dudeja et al., 1998). The sequence confirmed full-length human NHE-2 sequence (sequence will be submitted to GeneBank) and is 90% identical to the rat NHE-2 sequence (Collins et al., 1993). The pcDNA3.1/HuNHE-2 plasmid was transfected into PS120 cells utilizing Fugene6 (Boehringer Mannheim, Indianapolis, IN) transfection agent according to manufacturer's protocol. Individual clones were selected based on their functional NHE-2 activity.

2.3. $^{22}Na^+$-uptake assay

Cells expressing three different NHE isoforms (human NHE-1, -2, or rat NHE-3) were seeded in 24-well collagen coated plates and grown to confluency in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, penicillin (50 μg/ml) and streptomycin (50 μg/ml). Initial rates of $^{22}Na^+$ uptake by these NHE expressing cells were determined by first subjecting them to intracellular acidification by NH_4^+ prepulse followed by incubation in $^{22}Na^+$-uptake medium as described by Counillon et al. (1993) with minor modifications. Briefly, the culture medium was removed and cells were incubated for 60 min at 37 °C in acidification medium (50 mM ammonium chloride, 70 mM choline chloride, 5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5 mM glucose, 15 mM HEPES, pH 7.5). Cells were washed rapidly twice with wash buffer (120 mM choline chloride, 5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5 mM glucose, 15 mM morpholinopropanesulfonic acid, pH 7.0). The cells were then incubated for 6 min at 37 °C in an uptake medium (0.2 μCi/ml carrier free $^{22}Na^+$ in wash buffer containing 1 mM ouabain) in the presence or absence of increasing concentrations of inhibitors. At the end of the incubation, cells were washed three times with ice-cold 0.1 M $MgCl_2$ followed by solubilization with 0.5 ml of 0.1 N NaOH for 30 min. Aliquots (150 μl) were counted in a liquid scintillation counter (Beckman, Fullerton, CA) for incorporated radioactivity. 5-(*N*,*N*-hexamethylene)amiloride (100 μM) was added in one set of control wells in each plate. NHE-dependent $^{22}Na^+$ uptake was defined as the difference between the uptake of $^{22}Na^+$ in the presence and absence of 100 μM 5-(*N*,*N*-hexamethylene) amiloride.

2.4. Platelet swelling assay (PSA)

Platelet swelling assay was performed as described earlier by Rosskopf et al. (1991) with minor modifications. Briefly, blood (~ 10 ml) was drawn by venipuncture from normal healthy human volunteers into tubes containing anticoagulant, EDTA. Platelet-rich plasma was obtained by centrifugation of whole blood at 170 × *g* for 10 min at room temperature. The upper two thirds of the supernatant was removed and used for platelet swelling assay. All measurements were performed at room temperature and were completed within 4 h of venipuncture. PSA was performed in a Shimadzu Spectrophotometer (2401PC) (Baltimore, MD) fitted with a 12-cell changer. Assays were routinely performed in triplicates. Propionate medium (400 μl; sodium propionate 140 mM, HEPES 20 mM, glucose 10 mM, KCl 5 mM, $MgCl_2$ 1 mM, $CaCl_2$ 1 mM, pH 6.7) was placed in plastic disposal cuvettes (1-cm path length) in the spectrophotometer and 100-μl sample of platelet-rich plasma was directly added. NHE-1 inhibitors were added to propionate medium where indicated. Final volume and concentrations of propionate medium were adjusted when inhibitors were added to keep them consistent with controls without inhibitors. Increasing concentrations of amiloride (1–100 μM) were added as reference agent where indicated. Changes in optical density (OD) at 680 nm were recorded for 5 min at 6-s intervals. Rate constants were calculated from slopes generated during the first 42 s as described by Rosskopf et al. (1991).

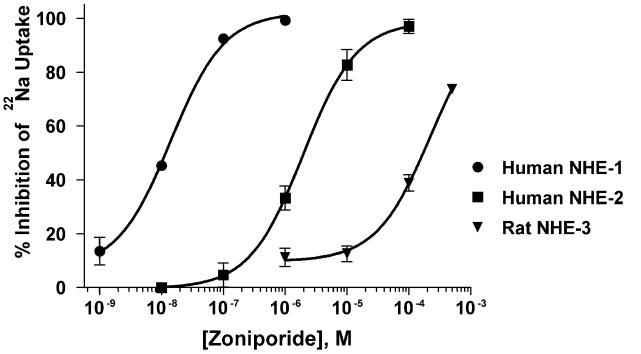

Fig. 1. Concentration-dependent inhibition of human NHE-1, -2 and rat NHE-3-mediated, 5-(*N*,*N*-hexamethylene)amiloride-sensitive, $^{22}Na^+$-uptake by zoniporide. All values are means ± S.E. from at least three experiments.

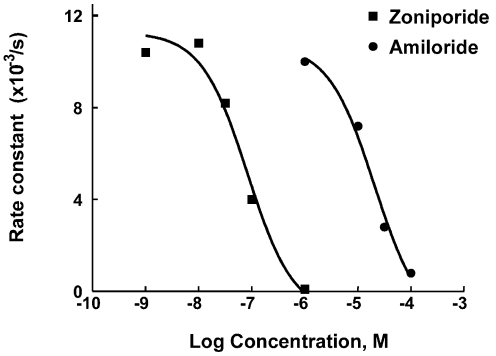

Fig. 2. Zoniporide inhibition of endogenous NHE-1-mediated human platelet swelling induced by ex vivo intracellular acidification of platelet-rich plasma. The control rate constant was 11×10^{-3}/s. The NHE inhibitor, amiloride, was used for comparison. Values represent data from a typical experiment. Experiments were done in triplicate and repeated at least three times.

2.5. Statistical analyses

Data are expressed as means ± S.E. The statistical significance was analyzed by Student's *t*-test. A *P* value less than 0.05 was considered statistically significant.

3. Results

3.1. Inhibition of NHE-mediated $^{22}Na^+$ uptake in cells expressing NHE-1, -2 or -3

Zoniporide inhibited $^{22}Na^+$ uptake in fibroblasts expressing human NHE-1 in a concentration-dependent manner (Fig. 1). Zoniporide also inhibited $^{22}Na^+$ uptake in fibroblasts expressing human NHE-2 or rat NHE-3 isoforms, but with significantly lower potencies when compared with human NHE-1 (Fig. 1). The IC_{50} values (concentration of zoniporide required to inhibit maximal $^{22}Na^+$ uptake by 50%) for the inhibition of human NHE-1, -2 and rat NHE-3 were 0.014 ± 0.002, 2.2 ± 0.37 and 220 μM, respectively (Fig. 1 and Table 1). Additionally, zoniporide demonstrated a significant ($P < 0.05$), although modest, improvement in potency over eniporide and cariporide (1.64- and 2.6-fold, respectively) at inhibiting $^{22}Na^+$ uptake by cells expressing human NHE-1 (Table 1). Zoniporide exhibited greater selectivity at inhibiting human NHE-1 vs. human NHE-2 than either eniporide or cariporide (157-fold selective compared with 27-fold and 49-fold, respectively) (Table 1). Zoniporide was evaluated against a panel of ion transporters/channels using specific ligand binding assays. No significant interactions ($IC_{50} > 10$ μM) of zoniporide were observed at Na^+–K^+ ATPase, Na^+/Ca^{2+} antiporter, Ca^{2+} channel (L type) and Na^+ channel (site 2) (data not shown).

3.2. Inhibition of NHE-1-mediated platelet swelling

Zoniporide inhibited NHE-1-mediated platelet swelling induced by ex vivo intracellular acidification of human platelet rich plasma in a concentration-dependent manner (Fig. 2). Amiloride, a nonselective and weak NHE inhibitor (IC_{50} at human NHE-1 was 4.77 ± 0.33 μM in $^{22}Na^+$-uptake assay), was used as a reference agent (Fig. 2). The IC_{50} (concentration of zoniporide required to decrease the rate of human platelet swelling by 50%) was 0.059 ± 0.014 μM (Table 2). Zoniporide, eniporide and cariporide and were all highly potent at inhibiting human platelet swelling (Table 2).

Table 1
Selectivity and potency of zoniporide compared with eniporide and cariporide

Compound	^{22}Na uptake IC_{50} (μM) (fold selectivity vs. NHE-1)		
	Human NHE-1	Human NHE-2	Rat NHE-3
Zoniporide	0.014 ± 0.002	2.2 ± 0.37 (157)	220 (15,700)
Eniporide	0.023 ± 0.001[a]	0.62 ± 0.29 (27)	~ 500 (~ 21,740)
Cariporide	0.036 ± 0.013[a]	1.75 ± 0.56 (49)	~ 1000 (~ 27,777)

Data are the means ± S.E. for each group; $n = 3–8$.
[a] Significantly different ($P < 0.05$) from zoniporide. IC_{50} values of all three compounds at human NHE-2 and rat NHE-3 were significantly ($P < 0.05$) different compared with their respective IC_{50} values at human NHE-1.

Table 2
Inhibition of ex vivo human platelet swelling by zoniporide, eniporide and cariporide

Compound	PSA IC_{50} (μM)
Zoniporide	0.059 ± 0.014
Eniporide	0.039 ± 0.001
Cariporide	0.023 ± 0.005[a]

Data are the means ± S.E. for each group; $n = 2–8$.
Values between zoniporide and eniporide were not significantly different ($P > 0.05$).
[a] Significantly different ($P < 0.05$) from zoniporide.

4. Discussion

NHE-1, although ubiquitously distributed, is the predominant isoform expressed in the heart and has been implicated in the pathophysiology of myocardial ischemia, postischemic dysfunction and cell death. At the onset of myocardial ischemia, intracellular acidosis occurs as a consequence to anaerobic metabolism and ATP hydrolysis. This intracellular acidosis activates NHE-1 leading to the buildup of intracellular Na^+, which in turn activates Na^+-K^+ ATPase. Activation of Na^+-K^+ ATPase results in increased ATP consumption, depletion of high-energy stores and perhaps cell death (Hendrikx et al., 1994; Scholz et al., 1993). Additionally, buildup of intracellular Na^+ leads to the activation of Na^+/Ca^{2+} exchanger, resulting in an influx of Ca^{2+} ions. This accumulation of intracellular Ca^{2+} during ischemia–reperfusion contributes to pathophysiological changes leading to cardiac arrhythmias, myocardial stunning, cellular necrosis and ischemic contracture (Hendrikx et al., 1994; Scholz et al., 1993; Garcia-Dorado et al., 1997; Tani and Neely, 1989).

Since activation of myocardial NHE-1 plays a critical role in mediating ischemia–reperfusion injury, pharmacological inhibition of NHE-1 offers an attractive approach to reduce ischemia–reperfusion associated myocardial damage. Karmazyn (1988) demonstrated salutary effect of NHE-1 inhibition in postischemic ventricular recovery more than a decade ago. Since then, numerous studies using selective and potent inhibitors have confirmed the cardioprotective effects of NHE-1 inhibition (Scholz et al., 1995; Gumina et al., 1998; Humphreys et al., 1999). Selective NHE-1 inhibitors cariporide (Aventis) and eniporide (Merck KGaA) are currently being evaluated in the clinic, cariporide in high-risk cardiac patients, and eniporide for the treatment of acute myocardial infarction. The GUARDIAN trial evaluated efficacy of cariporide in patients with acute coronary syndromes. Although, cariporide failed to demonstrate overall benefit over placebo on the primary end points of death or MI, sub-group analysis indicated a significant benefit, with the highest cariporide dose of 120 mg limited to high-risk patients undergoing coronary artery bypass graft surgery (Theroux et al., 2000). The ESCAMI trial evaluated the effect of eniporide on infarct size and clinical outcomes in patients undergoing thrombolytic therapy or angioplasty for acute myocardial infarction. Administration of eniporide before reperfusion therapy failed to limit infarct size or improve clinical outcomes (Zeymer et al., 2001). One could conclude from these clinical trials that selective NHE-1 inhibitors need to be present at sufficient concentrations in the myocardium prior to the onset of an ischemic event as during coronary artery bypass graft surgery.

Here we have characterized [1-(Quinolin-5-yl)-5-cyclopropyl-1*H*-pyrazole-4-carbonyl]guanidine hydrochloride monohydrate (zoniporide) (Guzman-Perez et al., 2001), a structurally novel, potent and highly selective inhibitor of the human NHE-1 isoform. The potency and selectivity of zoniporide was determined via inhibition of $^{22}Na^+$ uptake by fibroblast cell-lines overexpressing human NHE-1, -2 and rat NHE-3. Additionally, zoniporide potently inhibited endogenous NHE-1-mediated ex vivo human platelet swelling with an IC_{50} value in low nanomolar range. All three NHE-1 inhibitors tested (zoniporide, cariporide and eniporide) were highly potent and selective at human NHE-1 in $^{22}Na^+$-uptake assay. Although zoniporide was significantly more potent than either cariporide or eniporide at inhibiting human NHE-1 in the $^{22}Na^+$-uptake assay, this was a modest improvement which was not evident in the ex vivo human platelet swelling assay. The reason(s) for this is not yet clear, but the platelet swelling assay may be less sensitive to these small differences in potency. More importantly, zoniporide possesses markedly improved selectivity over cariporide and eniporide for NHE-1 vs. NHE-2 and -3, which may translate into a reduced likelihood of clinical side-effects. Zoniporide has been shown to be cardioprotective in vitro and in vivo using rabbit models of ischemia–reperfusion injury in the absence of any untoward cardiac or hemodynamic changes (mean arterial pressure, heart rate, left ventricular developed pressure, coronary flow and rate pressure product) (Knight et al., 2001).

In conclusion, zoniporide is a novel NHE-1 inhibitor with similar potency, but with improved NHE-1 selectivity relative to other known inhibitors. Combined with the preclinical cardioprotective efficacy (Knight et al., 2001) of this compound, these data indicate zoniporide has therapeutic potential as a cardioprotective agent.

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