Cardioprotective effects of novel tetrahydroisoquinoline analogs of nitrobenzylmercaptopurine riboside in an isolated perfused rat heart model of acute myocardial infarction

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Zhu Z, Hofmann PA, Buolamwini JK. Cardioprotective effects of novel tetrahydroisoquinoline analogs of nitrobenzylmercaptopurine riboside in an isolated perfused rat heart model of acute myocardial infarction. Am J Physiol Heart Circ Physiol 292: H2921–H2926, 2007. First published February 9, 2007; doi:10.1152/ajpheart.01191.2005.—We have investigated the cardioprotective effects of novel tetrahydroisoquinoline nitrobenzylmercaptopurine riboside (NBMPR) analog nucleoside transport (NT) inhibitors, compounds 2 and 4, in isolated perfused rat hearts. Langendorff-perfused heart preparations were subjected to 10 min of treatment with compound 2, compound 4, or vehicle (control) followed by 30 min of global ischemia and 120 min of reperfusion. For determination of infarct size, reperfusion time was 180 min. At 1 μM, compounds 2 and 4 provided excellent cardioprotection, with left ventricular developed pressure (LVDP) recovery and end-diastolic pressure (EDP) increase of 82.9 ± 4.0% (P < 0.001) and 14.1 ± 2.0 mmHg (P < 0.03) for compound 2-treated hearts and 79.2 ± 5.9% (P < 0.002) and 7.5 ± 2.7 mmHg (P < 0.01) for compound 4-treated hearts compared with 41.6 ± 5.2% and 42.5 ± 6.5 mmHg for control hearts. LVDP recovery and EDP increase were 64.1 ± 4.2% and 29.1 ± 2.5 mmHg for hearts treated with 1 μM NBMPR. Compound 4 was the best cardioprotective agent, affording significant cardioprotection, even at 0.1 μM, with LVDP recovery and EDP increase of 76.0 ± 4.9% (P < 0.003) and 14.1 ± 1.0 mmHg (P < 0.03). At 1 μM, compound 4 and NBMPR reduced infarct size, with infarct area-to-total risk area ratios of 29.13 ± 3.17 (P < 0.001) for compound 4 and 37.5 ± 3.42 (P < 0.01) for NBMPR vs. 51.08 ± 5.06% for control hearts. Infarct size was more effectively reduced by compound 4 than by NBMPR (P < 0.02). These new tetrahydroisoquinoline NBMPR analogs are not only potent cardioprotective agents but are, also, more effective than NBMPR in this model.

cardioprotection; nucleoside transport inhibitors; left ventricular developed pressure; end-diastolic pressure; ischemia; reperfusion; adenosine potentiation; infarct size reduction

IN THE MAMMALIAN HEART, the nucleoside adenosine is released in response to an ischemic insult, largely through ATP breakdown, to exert broad-spectrum cardioprotective actions through the activation of adenosine receptors (ARs), as well as AR-independent mechanisms (12, 16, 24, 35, 42, 43). Adenosine has also been implicated in ischemic preconditioning and postconditioning cardioprotective mechanisms (20). The cardioprotective actions of adenosine, however, are short-lived because of rapid reuptake through nucleoside transporters (NTs) into surrounding cells and metabolism. Thus approaches to augment extracellular adenosine concentrations and prolong its cardioprotective actions are being explored (16, 20, 35). One of these approaches is the inhibition of adenosine transport (47–50). The use of NT inhibitors to potentiate adenosine’s cardioprotective effects is attractive, because, in contrast to AR agonists, which activate ARs throughout the body and, thus, are prone to unwanted side effects (17, 26), NT inhibitors provide an event- and site-specific approach to cardioprotection with few side effects (20). NT inhibitors have been shown to elevate extracellular adenosine concentrations and, thereby, potentiate its cardioprotective effects (20, 31, 34). For example, enhancement of cardiac functional recovery after global ischemia by NT inhibition has been shown in cat (30), dog (33), and rabbit (34) isolated hearts.

Several chemical classes of compounds have been shown to inhibit mammalian NT processes (for review see Ref. 14), but only a few, such as nitrobenzylmercaptopurine riboside (NBMPR), dilazep, diprydamole, and drafzaine, have demonstrated effective inhibitory activity (8, 27, 29, 31, 32). Diprydamole has a high affinity for binding to plasma proteins (21), which compromises its bioavailability, and it is nonspecific as well, inhibiting phosphodiesterase and stimulating prostacyclin release (17, 23). The lidoflazine class of compounds, which includes drafzaine, also causes nonspecific cell membrane stabilization (16). Thus, because the present repertoire of NT inhibitors lacks suitable drug development leads, novel compounds are needed.

Among the known NTs (11, 20), the equilibrative NT type 1 (ENT1) is by far the most important in mammalian tissues, especially the human myocardium (4) and, therefore, the NT that might be the most promising for targeting in cardioprotective therapeutics. The most selective ENT1 inhibitors are nucleoside analogs represented by NBMPR (Fig. 1) (34, 35). NBMPR alone or in combination with erythyrho-9-(2-hydroxy-3-nonyl)-adenine (EHNA), a potent adenosine deaminase inhibitor, has been shown to attenuate myocardial stunning (1, 2, 5, 7). However, NBMPR is not a suitable clinical candidate because of its susceptibility to metabolic loss of the 6-nitrobenzyl group, causing a 1,000-fold loss in potency and yielding a 6-mercaptopurine riboside metabolite, which may be mutagenic (13) and immunosuppressive (15).

We recently reported the synthesis and potent ENT1 transporter inhibitory activity of a series of novel 1,2,3,4-tetrahydroisoquinoline analogs of NBMPR (60). The ENT1 inhib-
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Fig. 1. Nucleoside transport inhibitors. NBMPR, nitrobenzylmercaptopurine riboside.

Inhibitory constants ($K_i$) are provided in Table 1. Compound 4 was the most potent, with a $K_i$ of 0.45 nM, which was lower than that of the standard NBMPR ($K_i = 0.70$ nM) (60). Compound 2, which was also tested in the present study, exhibited a $K_i$ of 250 nM. Compound 4 and its counterparts are attractive, because, in contrast to NBMPR, they will not readily lose a nitrobenzyl group as a result of metabolism to produce inactive metabolites (see above). Thus we are interested in exploring the cardioprotection potential of these novel tetrahydroisoquinoline derivative NT inhibitors. We report a comparative study of the cardioprotective effects of two tetrahydroisoquinoline analogs, compounds 4 and 2, and NBMPR in isolated rat hearts. The results indicate that compounds 4 and 2 are better cardioprotective agents than the standard compound NBMPR.

MATERIALS AND METHODS

Compounds. Compounds 2 and 4 were synthesized according to methods reported previously (60). NBMPR was purchased from Aldrich (Milwaukee, WI). The structures of the compounds are shown in Fig. 1.

Animals. Female Wistar rats (225–249 g body wt; Harlan Sprague Dawley, Indianapolis, IN) were maintained in the animal care facility of the University of Tennessee Health Science Center and used in accordance with the guidelines of the Animal Care and Use Committee of the University of Tennessee Health Science Center and used in accordance with the guidelines of the Animal Care and Use Committee of the University of Tennessee Health Science Center and used in accordance with the guidelines of the Animal Care and Use Committee of the University of Tennessee Health Science Center and used in accordance with the guidelines of the Animal Care and Use Committee of the University of Tennessee Health Science Center and used in accordance with the guidelines of the Animal Care and Use Committee of the University of Tennessee Health Science Center and used in accordance with the guidelines of the Animal Care and Use Committee of the University of Tennessee Health Science Center.

Langendorff-perfused heart preparations and measurement of ventricular function. Rats were anesthetized with pentobarbital sodium (30 mg/kg ip), and their hearts were quickly removed and placed in ice-cold, oxygenated modified Krebs-Henseleit buffer [in mM: 4.7 KCl, 118 NaCl, 1.2 MgSO$_4$, 1.2 KH$_2$PO$_4$, 25 NaHCO$_3$, 11 glucose, and 1.3 CaCl$_2$ (pH 7.4)]. After cannulation via the aorta, the hearts were mounted on a Langendorff apparatus, perfused with 37°C oxygenated (95% O$_2$-5% CO$_2$) modified Krebs-Henseleit buffer, and placed in a 100-ml organ bath that was filled with oxygenated modified Krebs-Henseleit buffer and maintained at 37°C. A left atriotomy was performed, and a water-filled cellophane balloon fixed to a pressure transducer (BLPR, World Precision Instruments, Sarasota, FL) was inserted into the left ventricle for determination of left ventricular developed pressure (LVDP). Before initiation of the experiment, the volume in the intraventricular balloon was changed with a micrometer syringe to adjust left ventricular end-diastolic pressure (EDP) to 5–15 mmHg. Hearts were paced at 300 beats/min, and pacing voltage was set at twice the threshold value. External pacing was stopped during global normothermic ischemia and resumed after 5 min of reperfusion. For all determinations of ventricular performance, the pressures from the transducer were digitized using an analog-to-digital conversion board (model NB-MIO-16XL-18 μs, National Instruments, Austin, TX) and saved in a Macintosh computer. Data acquisition and analysis were carried out using LABVIEW software (National Instruments).

Experimental protocol. After a 20-min baseline equilibration period, the hearts were randomly assigned for treatment with modified Krebs-Henseleit buffer containing 0.01% DMSO (vehicle control) or individual test compounds at the appropriate concentration for 10 min. Then the hearts were subjected to 30 min of global normothermic ischemia followed by 120 min of reperfusion. The time line of the experimental protocol is shown in Fig. 2.

Preischemic LVDP and EDP were determined by averaging LVDP and EDP values, respectively, for the final 5 min of the 20-min baseline perfusion. Similarly, postischemic LVDP and EDP were taken as the average LVDP and EDP, respectively, for the last 5 min of the 120-min reperfusion. Postischemic LVDP recovery was calculated as postischemic LVDP divided by preischemic LVDP. Postischemic EDP rise was determined as the difference between postischemic and preischemic EDP. Hearts were included in data analysis only if their preischemic LVDP values were stable at 80–130 mmHg during preischemic equilibration.

Determination of infarct size. Hearts used for infarct size experiments were subjected to 20 min of equilibration, 10 min of treatment with control or test compound, 30 min of global ischemia, and 180 min of reperfusion. Before global ischemia, each heart was subjected to one of three treatments: 0.01% DMSO (vehicle control), 1 μM NBMPR, or 1 μM compound 4.

After 3 h of reperfusion, hearts were removed from the apparatus, and the ventricular myocardium was frozen at −20°C for 2 h and subsequently cut into 2-mm-thick transverse sections. The heart slices were incubated in 1% triphenyltetrazolium chloride in 0.1 M phosphate buffer (pH 7.4) at 37°C for 10 min. After incubation in triphenyltetrazolium chloride, which stained surviving tissue red, the slices were fixed in 10% formalin solution for 20 min. Both sides of each slice were then scanned into a computer for planimetric analysis using Photoshop version 8.0 (Adobe, Mountain View, CA) and ImageJ 1.31v (National Institutes of Health). Since hearts were subjected to global ischemia, the total cross-sectional areas were defined as the total risk areas. The infarcted area-to-total risk area ratio of the two sides of each slice was calculated. The final infarct size of each heart was the average of infarcted area-to-total risk area ratio of all slices.

Table 1. $K_i$ values of NBMPR, compound 2, and compound 4

<table>
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<tr>
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<th>$K_i$, nM</th>
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<tr>
<td>NBMPR</td>
<td>0.70</td>
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<tr>
<td>Compound 2</td>
<td>250</td>
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<tr>
<td>Compound 4</td>
<td>0.45</td>
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Inhibitory constants ($K_i$) were determined by flow cytometry. NBMPR, nitrobenzylmercaptopurine riboside. Data are from Ref. 60.
Statistical analysis. Data were analyzed by ANOVA and Student’s t-test and Kruskal-Wallis post hoc test; \( P < 0.05 \) was taken to indicate statistically significant differences between treatment groups. Values are means ± SE.

RESULTS

Functional response to global normothermic ischemia and reperfusion after treatment with NT inhibitors. Preischemic LVDP and EDP were not significantly different between all groups before drug/vehicle treatment. Representative data showing the effects of vehicle and compound 4 on LVDP are shown in Fig. 3. After 10 min of treatment with vehicle (0.01% DMSO), 30 min of global normothermic ischemia, and 120 min of reperfusion, the average postischemic LVDP of control hearts was 41.6 ± 5.2% of preischemic LVDP (Fig. 4). Treatment with 1 \( \mu \)M compound 4 caused a statistically significant increase in cardiac functional recovery compared with control hearts, improving the postischemic LVDP to 79.2 ± 5.9% relative to preischemic LVDP (\( P < 0.002 \)). Treatment with the prototype es (ENT1) nucleoside transporter inhibitor NBMPR at 1 \( \mu \)M also produced a significant postischemic LVDP recovery compared with control hearts, with an average postischemic LVDP recovery of 64.1 ± 4.2% of preischemic LVDP (\( P < 0.03 \)). Interestingly, treatment with the weak es (ENT1) transporter inhibitor compound 2 (60) at 1 \( \mu \)M also resulted in a significantly higher postischemic LVDP recovery (82.9 ± 4.0%, \( P < 0.03 \)) than in NBMPR-treated hearts. Thus compounds 4 and 2 at 1 \( \mu \)M demonstrated equivalent excellent improvement of functional recovery in ischemic-reperfused hearts, despite their very different NT inhibitory potencies (60). Nevertheless, 1 \( \mu \)M is four times the \( K_i \) of compound 2. We were surprised to find that NBMPR, which is similar in potency to compound 4 as an NT inhibitor (60), caused significantly less functional recovery than both of the tetrahydroisoquinoline compounds at the same dose level of 1.0 \( \mu \)M (Fig. 4). Treatment of hearts with 1 \( \mu \)M compound 4 or compound 2 also resulted in a significant improvement in EDP, as evidenced by the reduction in the average postischemic rise in EDP (Fig. 5): 42.5 ± 6.5 mmHg for control vs. 7.5 ± 2.7 mmHg (\( P < 0.01 \)) and 14.1 ± 5.0 mmHg (\( P < 0.05 \)) with compounds 4 and 2, respectively. At 1 \( \mu \)M, NBMPR, in contrast to either tetrahydroisoquinoline analog, did not significantly reduce the rise in postischemic EDP (29.1 ± 2.5 mmHg, \( P < 0.25 \)) compared with control. The significant reductions in the postischemic rise in EDP induced by com-
this protective effect translated to protection of the myocardium against ischemia-reperfusion tissue injury. We used compound 4, which showed excellent improvement in functional recovery, and compared it with the standard agent NBMPR in infarct size determination experiments. The results, which are presented in Fig. 6, show that compound 4 or NBMPR reduced infarct size relative to vehicle treatment. The average infarct size in vehicle-treated (control) rat hearts was 51.08 ± 5.06% of the total risk area, whereas those for compound 4- and NBMPR-treated hearts were 29.13 ± 3.17% and 37.5 ± 3.42%, respectively. This translates to infarct size reductions of 40% and 20% by compound 4 (P < 0.001) and NBMPR (P < 0.01), respectively. The ability of compound 4 to reduce infarct size was significantly greater than that of NBMPR (P < 0.02), which reflects the superiority of compound 4 to NBMPR with regard to improvement in left ventricular functional recovery and cardiac contracture (Figs. 4 and 5).

**DISCUSSION**

We have demonstrated the cardioprotective effects of novel tetrahydroisoquinoline NT inhibitors, which were recently discovered in our laboratory (60). Our findings that these new NT inhibitors enhance postischemic ventricular functional recovery, attenuate myocardial contracture, and reduce infarct size are consistent with previous work showing the cardioprotective effects of NT inhibitors (1, 2, 5, 7–10, 20, 24, 25, 29, 30, 33, 34). Site-specific inhibition of NT during ischemia and reperfusion has been reported to attenuate stunning of the myocardium (8) by preventing superoxide generation or activating ARs. Interestingly, bradycardia caused by the use of EHNA alone was prevented when NBMPR was combined with EHNA (29). NBMPR has also been shown to improve LVDP recovery and ATP repletion and enhance endogenous adenosine accumulation in rat hearts (25). Zughai et al. (61) showed that NBMPR and EHNA augment endogenous adenosine during ischemia and reperfusion in vivo to attenuate myocardial stunning in dogs. They also demonstrated that augmentation of endogenous adenosine levels is an effective therapeutic ap-
approach to protection against reversible postischemic dysfunction.

Cardioprotective mechanisms of adenosine involve vasodilatation, prevention of platelet aggregation, inhibition of catecholamine release, inhibition of neutrophil activation, induction of myocyte protein kinase (PKC) cytoprotective mechanisms, and opening of ATP-sensitive potassium channels, which result from activation of AR signaling cascades (24). It is generally believed that A1AR and A2aAR are associated with ischemic preconditioning (19, 24, 31, 32, 42, 44, 46), whereas A2bAR activation appears to mediate the tissue protective and anti-inflammatory actions of adenosine during reperfusion (24, 31, 39). A2bAR and A2bAR and A3AR have also been implicated in the recently discovered phenomenon of postconditioning (22, 59). NT inhibition not only causes extracellular accumulation of adenosine, but it can also cause retention/trapping of adenosine within cardiomyocytes (1, 54), which can help replenish essential adenine nucleotides, such as ATP. Specificity of NBMPR and congeners is known to be different from that of other NT inhibitors such as the lidoflazine analogs or dipyridamole (14). In the guinea pig atrium, NBMPR and dipyridamole increased interstitial fluid adenosine concentration (28). It is possible that NBMPR and compounds 2 and 4 produce their cardioprotective effects in the isolated rat heart by increasing interstitial adenosine concentrations and/or entrapping adenosine within cardiomyocytes (25). The use of AR antagonists will show whether an increase in interstitial adenosine concentrations is involved in the cardioprotective actions. Measurement of myocyte adenosine retention in treated hearts vs. untreated hearts will indicate whether adenosine entrapment contributes to the cardioprotection.

Our results establish that compounds 2 and 4 are cardioprotective agents. Compound 4 was significantly more effective than compound 2 at low doses, but not at the highest tested dose (1 μM), at which the two compounds were equipotent in enhancing postischemic cardiac functional recovery. Taken together, the results suggest that more than one mechanism may be operating in the cardioprotective effects. At lower doses, it appears that the NT inhibition mechanism may be the most important, since compound 4 is a much more effective NT inhibitor than compound 2 (60). This notion is further underscored by the superiority of compounds 2 and 4 to NBMPR, which has NT inhibitory activity similar to that of compound 4.

Ischemia-reperfusion injury is the primary cause of complications of cardiac surgery and the underlying cause of death in the majority of patients who die within 30 days after cardiac surgery (37, 58). Current myocardial protection approaches are limited and inadequate, especially in high-risk patients (38, 57), for whom more effective cardioprotective therapies are needed. In this context, it is significant that the novel tetrahydroisoquinoline compounds are superior to NBMPR. In these compounds, the nitrobenzyl moiety of NBMPR is locked into the nitro-1,2,3,4-tetrahydroisoquinoline system, and they are less likely to lose this moiety in vivo. This overcomes a major disadvantage of NBMPR relative to metabolic loss of the 6-nitrobenzyl moiety to produce the practically inactive 6-mercapto purine riboside metabolite, which may be mutagenic (13) and immunosuppressive (15), especially if there is a need for chronic use in very high-risk patients. For this reason, it is interesting to pursue these new compounds for the development of novel cardioprotective therapies.

GRANTS

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REFERENCES