Modulation of Vascular Reactivity by Novel Synthetic Benzopyran Analogues in Rat Aortas

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A substantial body of research described ATP-dependent potassium (K_{ATP}) channels, sarcolemmal and mitochondrial, with different physiological functions and pharmacological behaviour in various tissues (pancreatic â-cells, brain, skeletal muscle and vascular smooth muscle). The present study was purported to assess the effects of three novel benzopyran derivatives, KL-1487, KL-1492 and KL-1507, analogues of BMS191095 (a selective opener of mitoK_{ATP} channels) on vascular function and reactive oxygen species (ROS) production. To this aim, we performed organ bath experiments on rat aortic rings in the presence vs. absence of each of the mentioned compounds (10 µmol/L). Incubation of vascular rings with each benzopyran analogue elicited a significant decrease of the contractile response to phenylephrine vs. control, thus suggesting a vasodilator action. However, only two of the benzopyran derivatives, KL-1492 and KL-1507, significantly improved the endothelial-dependent relaxation. Interestingly, these effects were present also in endothelium denuded vessels. None of these compounds modified vascular ROS production. In conclusion, in murine aortic segments, KL-1492 and KL-1507, two novel benzopyran analogues elicited an endothelial-independent modulation of vascular reactivity. Further studies will elucidate whether these compounds may be useful in restoring the vascular response in pathological conditions associated with endothelial dysfunction.

Keywords: benzopyran derivatives, endothelial function, organ bath, vascular murine rings

The ATP-sensitive potassium channels (K_{ATP}) are highly investigated due to their unique property which is the coupling of membrane excitability with cellular energetics in several metabolic active tissues, such as heart, brain, pancreas, smooth and skeletal muscle, respectively[1, 2, 3]. The channels are closed under normal metabolic conditions and become activated in the setting of various types of metabolic stress, including conditions associated with tissue ischemia[4]. In the heart, studies have demonstrated the existence of two subtypes of K_{ATP}channels, sarcolemmal (sarcK_{ATP}) and mitochondrial K_{ATP} channels (mitoK_{ATP}); the channels are activated during ischemia/hypoxia due to an decrease in ATP concentration and protect the ischemic myocardium by several mechanisms [5]. Subsequently, a theory has emerged that the selective pharmacological modulation of K_{ATP} channels in the heart can have beneficial effects in cardiovascular pathology. The inhibition of sarc K_{ATP} channels was shown to decrease the incidence of ischemia-related arrhythmias (malignant tachyarrhythmia and atrial fibrillation) by decreasing the action potential duration [6], whereas mitoK_{ATP} opening was associated with the preservation of mitochondrial structure [7], increased ATP production and preservation during ischemia, increased functional recovery at reperfusion [8] and infarct size reduction [9].

The major limitation regarding the available compounds is represented by the lack of selectivity. Thus, there are several unselective agents that open both types of K_{ATP} channels such as: pinacidil, cromakalim, minoxidil, levosimendan, aprikalim, P-1060, EMD 60480. More recently, a selective mito K_{ATP} channel opener with benzopyran structure: BMS 191095 (fig.1), has been synthesized; the compound elicited cytoprotection in cell lines via the inhibition of Ca^{2+} influx from extracellular environment [10], the reduction of basal ROS production, and the improvement in ATP homeostasis [11, 12].

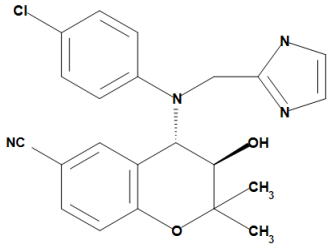

Fig. 1.The chemical structure of BMS 191095

The aim of the present study was to assess the effects of novel benzopyran derivatives, analogues of BMS191095 (KL-1487, KL-1492 and KL-1507) on vascular function and reactive oxygen species (ROS) production in a murine experimental model.

Experimental part

Material and methods

Sprague-Dawley female rats (weighing 250-350 g) were purchased from Cantacuzino Institute (Bucharest, Romania) and were acclimated for 2 weeks prior to the study. Animals were housed under standard conditions (constant temperature and humidity of 22.5 ± 2° C and 55 + 5%, 12-h light/dark cycle). Twenty-four hours prior to the

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experiment solid food was withdrawn with no limitation in water supply.

All experimental procedures used in this study were conducted in accordance with the Directive 2010/63/EU and the Romanian Law no. 43/May 2014 concerning the protection of animals used for scientific purposes. The experimental protocol was approved by the Committee for Research Ethics of Victor Babes University for Medicine and Pharmacy of Timisoara, Romania. Vascular segments were obtained after animal anaesthesia with an intraperitoneal injection of ketamine (30 mg/kg body mass) and xylazine (5 mg/kg body mass).

Organ Bath Studies

Organ bath experiments were performed on rat aortic rings in the presence of diclofenac (10 μmol/L) in order to inhibit the synthesis of prostaglandins. The concentration of phenylephrine, used for preconstriction, was adjusted to obtain a preconstriction level of 80% of the contraction elicited by KCl(80 mmol/L). Endothelium-dependent relaxation to cumulative concentrations of acetylcholine (Ach) was recorded in the presence vs. the absence of benzopyran derivatives, analogues of BMS 191095 (KL-1487, KL-1492 and KL-1507; 10μmol/L) and compared with the effect of HMR 1098 (the cardioselective inhibitor of sarcK_{ATP} channels, 10 μmol/L) according to a protocol standardized in ref [13]. In a separate group of experiments, endothelium was removed by a treatment with CHAPS (5 mg/mL dissolved in glucose solution 50g/L, exposure for 40 s).

ROS Measurements

Hydrogen peroxide production was assessed in rat aortic samples, in the presence *vs.* the absence of benzopyran analogues (30 min preincubation with KL1478, KL1492, KL1507, 10 μM) by Ferrous iron xylenol orange OXidation (FOX) assay, as previously described [14,15] using the PeroxiDetect Kit (Sigma Aldrich). The principle of the assay is that peroxides oxidize Fe^{2+} to Fe^{3+} ions at acidic *p*H. The Fe^{3+} ion will form a colored adduct with xylenol orange (XO,3,32 -bis[N,N-*bis*(carboxymethyl) aminomethyl]-o-cresolsulfonephthalein, sodium salt), which is measured spectrofotometrically at 560 nm.

Statistics

All values are presented as mean ± SEM. Relaxations were calculated from individual dose-response curves. The values were analysed using the GraphPad Prism 5.To determine the global vascular reactivity the following variables of the Hill equation for a sigmoidal relationship: maximal response, concentration required to achieve 50% of the maximal response – EC50 (-log[M]) and the Hill slope, were computed. Values of $p<0.05$ were considered statistically significant.

Reagents

All reagents used were of the highest quality available and were purchased from Sigma-Aldrich, Invitrogen, Applichem and Abcam.The novel compounds were synthesized and kindly provided by Dr. L.Kiss from the Department of Pharmaceutical Chemistry, University of Szeged, Hungary.

Results and discussions

Ex Vivo Stimulation With The Novel Benzopyran DerivativesReduced Contractility And Improved Relaxation in Rat Aortic Rings

All three benzopyran derivatives determined a significant decrease of the contraction to PHE vs. control(fig. 2A), as well as an important improvement of the endothelial-dependent relaxation (fig.2B), observations that are relevant to a vasodilator action of these novel compounds.

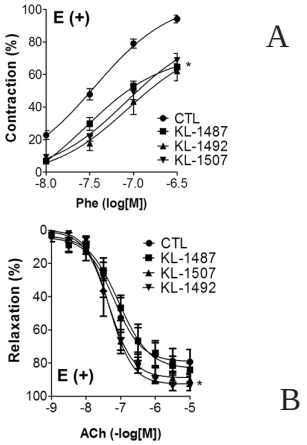

Fig. 2. Phenylephrine-induced contraction(A) and acetylcholine-induced relaxation(B) in rat aortic rings with normal endothelium (E+) in the presence of the investigated compoundsvs. the non-treated rings (n=5-6/group, *<0.05)

The Presence Of An Intact Endothelium Is Not Necessary ForThe Vascular Effects Of The Benzopyran Derivatives

In order to investigate whether the above mentioned vascular effects are dependent on a functional endothelium, we repeated the experiments in endothelium -denuded aortic rings. Interestingly, both the decrease in contractility (fig. 3A) and the improvement in relaxation (fig. 3B), were still present, strongly suggesting that the vascular effects of these compounds are endothelial-independent.

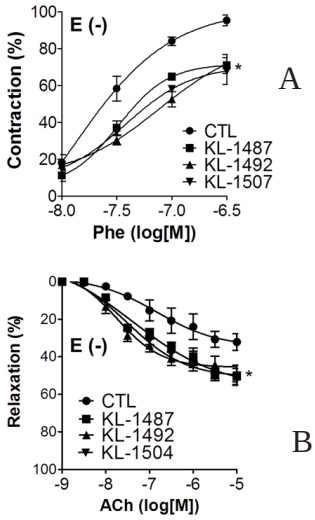

Fig. 3. Phenylephrine-induced contraction(A) and acetylcholine-induced relaxation (B) in rat aortic rings with denuded endothelium (E-) in the presence of the investigated compoundsvs. the non-treated rings (n=4-6/group, *<0.05).

Benzopyran Derivatives Do Not Interfere With Basal Production of Vascular Hydrogen Peroxide

Subsequently, we have assessed the effects of the three compounds(10 μM) on the basal H_2O_2 production in intact aortic rings. However, none of the investigated compounds (KL-1487; KL-1492, KL-1507) interferedwith ROS generation in the normal murine vessels (fig 4).

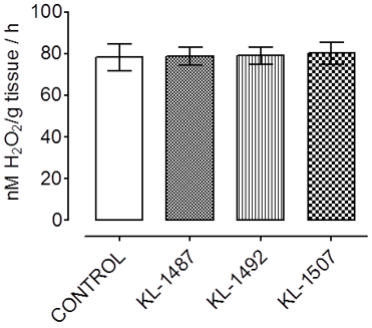

Fig. 4.Rat aortic H_2O_2 generation measured by FOX assay in the presence of KL-1478, KL-1492, KL-1507 *vs.* the non-treated rings (n=6/group)

In the present study,performed in rat aortic rings, we investigated the effects of three novel benzopyran derivatives (KL-1487, KL-1492 and KL-1507) on vascular reactivityand ROS production, respectively.We have previously described in isolated rat heart mitochondria that these compounds elicited an uncoupling effect and also,respiratory inhibition (in high doses) in a K^+-independent manner. Moreover, we found that, when applied in the highest concentrationtested (150 microM) all compounds were able to decrease the H_2O_2 release [16]. All these effects have been reported to be cardioprotective, in the settings of I/R injury.

Since their discovery in cardiac myocytes, K_{ATP} channels have been described in numerous other cell types, including vascular smooth muscle cells [17], where theiropening caused membrane hyperpolarization, a decrease in intracellular calcium concentration and also vasodilation [4].Numerous K_{ATP} openers like pinacidil, nicorandil and cromakalim have vasorelaxant effects on systemic and coronary blood vessels and also increase blood flow [4], thus being involved in reactive coronary hyperemia, metabolic coronary vasodilation and local control of coronary blood flow [18,19]. Conversely, glibenclamide (the classic K_{ATP} inhibitor) was reported to cause depolarization in vascular smooth muscle cells, a significant increase in vascular resistance and thus, a decrease in arterial diameter [20, 21].

However, controversial results are available in the literature with respect to the effects of the selective channel opener, BMS 191095. Accordingly, both no effects on the vascular activity [22] and a vasodilator action of the compound (at 50 and 100 microM) on cerebral arteries, have been reported[23]. Also, it has been reported that vasodilatation can be elicited by two different mechanisms, depending on the molecular structure of the compounds. Studies conducted on mesenteric arteries with pinacidil and diazoxide were able to demonstrate that vasodilation elicited by pinacidil required subunit SUR2B, whereas diazoxide-induced vasodilation occurred independently of SUR-containing KATP channels and involved inhibition of mitochondrial ETC [24]. In the case of BMS 191095, studies showed that, unlike diazoxide [23], the mitochondria-mediated vasodilation was independent of ROS production, but with a common pathway for both agents, namely the phosphoinositide-3 kinase/endothelial nitric oxide synthase pathway [25].

Several studies demonstrated that activation of K_{ATP} channels expressed in endothelial cells can trigger NO release [26] by increasing the transmembrane Ca2+ influx [27]; the production of other autacoids,such as prostaglandins, is also regulated via intracellular calcium [28, 29].Other studies have demonstrated that K_{ATP} opening is not involved in the endothelial-dependent relaxation mediated by acetylcholine (ACh) [30, 31].

Our study indicates that only two of the tested compounds, KL-1492 and KL-1507 (but not KL-1487), significantly improved relaxation in the presence of cumulative doses of ACh (fig. 2B). Interestingly, the effect was independent on the presence of a functional endothelium, an intriguing observation that clearly requires further investigation for the elucidation of the underlying signal transduction. Moreover, at variance from the results obtained in isolated mitocondria, no effect on ROS generation could be detected.

Conclusions

We have demonstrated here that *in vitro* application of two novel benzopyran analoguues, KL-1492 and KL-1507, modulate vascular reactivity in healthy murine aortic segments without interfering with ROS generation. Whether the beneficial effects can be recapitulated in conditions associated endothelial dysfunction, such as diabetes mellitus, are currently under investigation.

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