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Bioorganic & Medicinal Chemistry 16 (2008) 4908–4920
Bioorganic & Medicinal Chemistry

Carboxymethylated pyridoindole antioxidants as aldose reductase inhibitors: Synthesis, activity, partitioning, and molecular modeling

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Received 28 January 2008; revised 5 March 2008; accepted 14 March 2008
Available online 17 March 2008

Abstract—Starting from the efficient hexahydropyridoindole antioxidant stobadine, a series of carboxymethylated tetrahydro- and hexahydropyridoindole derivatives was synthesized and tested for the inhibition of aldose reductase, an enzyme involved in the etiology of diabetic complications. In vitro inhibiton of rat lens aldose reductase was determined by a conventional method. Kinetic analysis of (2-benzyl-2,3,4,5-tetrahydro-1H-pyrido[4,3-*b*]indole-8-yl)-acetic acid (**5b**) and (2-phenethyl-2,3,4,5-tetrahydro-1H-pyrido[4,3-*b*]indole-8-yl)-acetic acid (**5c**), the most potent compounds in this series with activities in micromolar range, showed uncompetitive inhibition. In addition to the importance of the acidic function, the inhibition efficacy was highly influenced by the steric conformation of the lipophilic aromatic backbone when comparing tetrahydro- and hexahydropyridoindole congeners. Selectivity with respect to the closely related aldehyde reductase was determined by measuring the corresponding inhibitory activities. Antioxidant action of the novel compounds was documented in a DPPH test and in a liposomal membrane model, oxidatively stressed by peroxyl radicals. The presence of a basicity center at the tertiary nitrogen, in addition to the acidic carboxylic function, predisposes these compounds to form double charged zwitterionic species, a characteristic which may remarkably affect their pH-lipophilicity profile. For compounds **5b** and **5c**, a maximal distribution ratio in a system comprised of 1-octanol/phosphate buffer was recorded near the neutral physiological pH, the region where the isoelectric point lies. Molecular docking simulations into the ALR2 active site performed for the zwitterionic species provided an explanation for the observed structure–activity relationships and the calculated parameters were in agreement with characteristic differences in the stereoelectronic profiles of the tetrahydro- versus hexahydropyridoindoles. 'Drug-likeness' of the novel aldose reductase inhibitors was assessed by applying the criteria of Lipinski's 'rule of five'.

1. Introduction

Although multiple biochemical pathways are likely to be responsible for the pathogenesis of diabetic complications, substantial evidence suggests a key role for the polyol pathway.[1–4] In tissues that do not require insulin for glucose uptake, the high systemic level of glucose developing during diabetes readily translates into high tissue levels of glucose. Aldose reductase (ALR2, E.C.1.1.1.21), the first enzyme of the polyol pathway, reduces some of this excess glucose to the organic osmolyte sorbitol in an NADPH-dependent manner. Due to its poor membrane penetration and slow metabolism by sorbitol dehydrogenase, sorbitol accumulates

Abbreviation: Ph, Phenyl.
Keywords: Aldose reductase inhibition; Pyridoindole; Antioxidant; Zwitterion; Diabetic complications.
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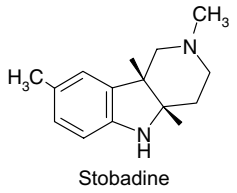
doi:10.1016/j.bmc.2008.03.039



Stobadine

Chart 1.

intracellularly, resulting in disruption and eventually death of the cells. Depletion of NADPH due to aldose reductase activity reduces intracellular glutathione (GSH), an endogenous antioxidant, thereby inducing oxidative stress.[5] In this way, the polyol pathway in cells is believed to contribute to the etiology of long-term diabetic complications such as cataract, retinopathy, nephropathy, neuropathy, micro-, and macroangiopathy. Under physiological conditions, ALR2 serves as an extrahepatic detoxifying enzyme against endogenous and xenobiotic aldehydes.[1]

Implicated in diabetes complications, aldose reductase is a potential target of drug action,[6] and the search for aldose reductase inhibitors (ARIs) offers an attractive strategy for preventing the onset or the progression of the complications.[7–10] A large variety of structurally diverse compounds have been identified as potent in vitro aldose reductase inhibitors. However, in humans, with few exceptions, these compounds have produced little evidence of clinical benefit, and some even produced deleterious side effects, prompting efforts to search more potent and specific inhibitors. Owing to ALR-2 pharmacophore requirements[11,12] for an acidic proton, most ARIs contain an acetic acid moiety or *N*-unsubstituted cyclic imides. Carboxylic acids are ionized at physiological pH resulting in their poor biological availability. Substitution of the acetic acid chain with the hydantoin or succinimide moiety partially improved the yield in the target tissues.[7,8] The bioisosteric principle may offer another way how to overcome unfavorable partitioning of acidic ARIs.[13,14]

The implications of oxidative stress and the polyol pathway in the etiology of diabetic complications are widely accepted. Since the inhibition of both the processes is desirable, a bifunctional compound with joint antioxidant/aldose reductase inhibitory (AO/ARI) activities could be multifactorially beneficial. In this study, we present carboxymethylated hexahydropyridoindoles and tetrahydropyridoindoles, structurally based on the hexahydropyridoindole antioxidant drug stobadine[15] (Chart 1) as aldose reductase inhibitors endowed with antioxidant activity.

2. Results and discussion

2.1. Design

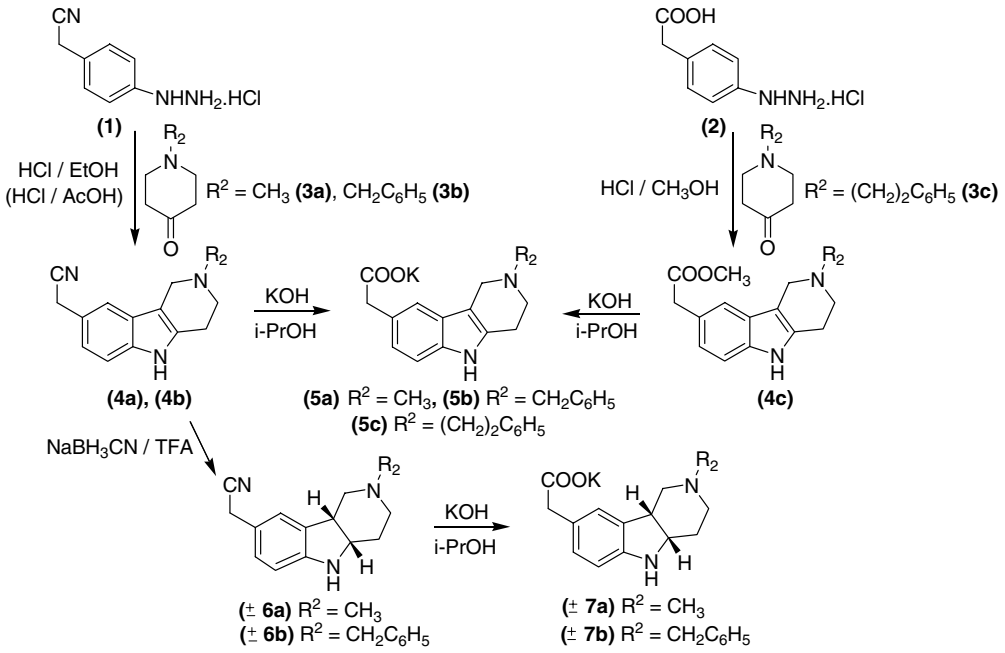
Oxidative stress and the involvement of the polyol pathway in the etiology of diabetic complications have been widely reported.[1,5,21] With the aim of inhibiting these processes, compounds such as pyridazines,[22] benzopyranes,[23] and pyridopyrimidines[24] were synthesized and were shown to display antioxidant as well as aldose reductase inhibitory activities under in vitro conditions. Based on the premise that a bifunctional compound with joint antioxidant/aldose reductase inhibitory (AO/ARI) activities could be multifactorially beneficial, in the current study we synthesized and tested new carboxymethylated pyridoindoles, structurally based on the antioxidant drug stobadine[15] (Chart 1). Under conditions of an experimental glycation model in vitro, the parent drug stobadine was found to protect bovine serum albumin against glyco-oxidative damage.[25] Using a model of streptozotocin-diabetic rats in vivo, stobadine was found to attenuate pathological changes in diabetic myocardium,[26,27] kidneys,[27,28] eye lens[29] and retina,[30] and vas deferens,[31] to decrease matrix collagen cross-linking,[28] and to reduce plasma cholesterol[26] and triglyceride[26,32] levels in diabetic animals. Stobadine treatment normalized calcium homeostasis in diabetic rat heart and liver[32] and produced a beneficial effect on leukocyte function.[33] The newly synthesized (Scheme 1) and investigated carboxymethylated pyridoindoles were two hexahydropyridoindoles (**7a**, **b**) and three tetrahydropyridoindoles (**5a**–**c**), with the R_2-substituents being -methyl (**5a**, **7a**), -benzyl (**5b**, **7b**), or -phenethyl (**5c**).

2.2. Chemistry

We describe (Scheme 1) the synthesis of putative pharmacologically active tetrahydropyridoindoles, 2-methyl- **(5a)**, 2-benzyl- **(5b)** and 2-phenethyl- (**5c**) 2,3,4,5-tetrahydro-1*H*-pyrido[4,3-*b*]indole-8-yl acetates, and hexahydropyridoindoles, (±)-2-methyl- (**7a**) and (±)-2-benzyl- (**7b**) (4a,9b)-*cis*-1,2,3,4,4a,9b-hexahydro-1*H*-pyrido[4,3-*b*]indole-8-yl acetates. The synthesis of the target compounds started from the previously reported[16,17] 4-cyanomethylphenylhydrazine hydrochloride (**1**) or 4-carboxymethylphenylhydrazine hydrochloride (**2**) and commercially available *N*-alkylsubstituted-4-piperidones **3a**–**c**. The initial step was carried out under Fischer's indole synthesis conditions[18] (Scheme 1).

Specifically, acetonitrile **4a** was synthesized by the treatment of **1** with **3a** in a refluxing 10% ethanolic HCl solution to give the product in a fair yield. A similar procedure was used to synthesize the methyl ester **4c** started from **2** and **3c**. However, in this case, best results were obtained by utilizing a 10% methanolic HCl solution. The condensation of **1** with **3b** in the above-mentioned acidified alcohols did not result in cyclization to **4b.** Alternatively, the corresponding hydrazone was first formed and then cyclized to the desired product **4b** with the action of acetic acid containing 6% HCl under control heating conditions. This two-step procedure was found to result consistently in high yields of **4b**.

The reduction of **4a** and **4b** with $NaBH_3CN$ in trifluoroacetic acid (TFA) gave hexahydropyridoindole compounds **6a** and **6b**. The reaction was found to be stereoselective, yielding the *cis*-configuration of the hydrogens in the positions 4a and 9b of the correspond-



Scheme 1.

ing hexahydropyridoindoles in more than 98% rate,[19] this finding being in agreement with relevant literary data.[20] The products were isolated as racemic mixtures and no attempt was made to separate the enantiomers. Finally, the hydrolysis of the intermediate products **4a**–**4c** and **6a**, **6b** in refluxing KOH/*i*-PrOH yielded the target products **5a**–**5c** and **7a**, **b**, which were isolated as potassium salts. Compound **7b** was characterized in the form of its sodium salt.

2.3. Enzyme inhibition

The compounds **5a**–**5c** and **7a**, **7b** were evaluated for their ability to inhibit the in vitro reduction of D,L-glyceraldehyde by partially purified ALR2 from rat lens, using zopolrestat as a reference (Table 1). In the primary screening studies, the three following questions were addressed: (i) the effect of introducing the carboxymethyl group into the molecule of stobadine, (ii) the effect of switching between hexahydropyridoindole and tetrahydropyridoindole type structure, and (iii) the effect of lipophilicity of the substituent $-R_2$. As shown, neither stobadine nor its unsaturated tetrahydropyridoindole form, dehydrostobadine, were active as inhibitors of ALR2. However, the introduction of the carboxymethyl group into the molecule of stobadine resulted in hexahydropyridoindole compounds **7a**, **7b** with a mild inhibition of ALR2. Activities in micromolar range were recorded for the unsaturated tetrahydropyridoindole congeners **5a**–**5c**.

Table 1. Inhibition of rat lens ALR2

Compound	IC_{50} (μM)
5a	54.0 ± 12.1
5b	18.2 ± 1.2
5c	16.7 ± 1.2
7a	380.7
7b	266.0 ± 4.1
Stobadine	>1000
Dehydrostobadine	>1000
Zopolrestat	0.005 ± 0.001

Experimental results are mean values from two or mean values ± SD from at least three experiments.

In the next step, we analyzed the enzyme kinetics for compounds **5b** and **5c**. Uncompetitive inhibition was observed in relation to the substrate D,L-glyceraldehyde and the cofactor NADPH (for **5b** see Figs. 1 and 2, respectively). Table 2 shows values of the corresponding kinetic parameters. The uncompetitive inhibition of ALR2 indicates that the glucose substrate would not compete with the inhibitor for the enzyme. Thus, under hyperglycemia conditions in diabetics, excessive glucose would not presumably decrease the inhibitory efficiency of the drug. The experimentally obtained K_m values for

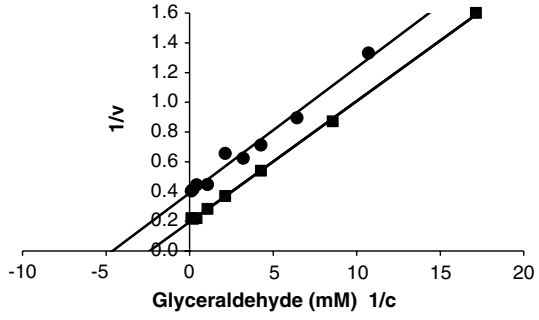


Figure 1. Inhibitory effect of compound **5b** on rat lens aldose reductase. Typical double reciprocal plot of the initial enzyme velocity versus the concentration of substrate (D,L-glyceraldehyde) in the presence or absence of **5b**: (■) no inhibitor; (●) 20 μM **5b** (uncompetitive type of inhibition).

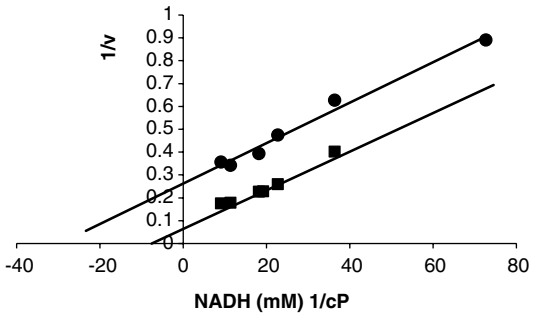


Figure 2. Inhibitory effect of compound **5b** on rat lens aldose reductase. Typical double reciprocal plot of the initial enzyme velocity versus the concentration of cofactor (NADPH) in the presence or absence of **5b**: (■) no inhibitor; (●) 20 μM **5b** (uncompetitive type of inhibition).

Table 2. Kinetic parameters of rat lens ALR2 and the inhibitory effect of compounds **5b** and **5c**

Varied substrate/ cofactor	K_m (μM)	V_{max} (10,000 OD/s/mg)	K_i[a] (μM)
D,L-Glyceraldehyde	585.0 ± 90.0	4.3 ± 1.3	17.9 ± 3.0 (**5b**) 17.7 ± 2.1 (**5c**)
NADPH	54.0 ± 12.0	6.8 ± 1.4	11.0 ± 1.9 (**5b**) 10.5 ± 1.9 (**5c**)

[a] Uncompetitive type of inhibition. Results are mean values ± SD from at least three experiments.

the aldose reductase, $(K_m)^{Glyceraldehyde} = 0.585$ mM and $(K_m)^{NADPH} = 0.054$ mM, were in the range of those determined by other authors for partially purified rat lens ALR2.[34–36]

An important feature of pharmacologically applicable ARIs is their selectivity of action. The co-inhibition of structurally related physiological oxidoreductases might have unwanted side effects. In testing for selectivity we used the comparison to an enzyme with the highest homology, aldehyde reductase (ALR1).[37,38] The IC_{50} values of compounds **5a**–**5c** and **7b**, for their inhibition of the reduction of glucuronide substrate by partially purified ALR1 from rat kidney, in comparison with the standard valproate, are shown in Table 3. The corresponding selectivity factors calculated for the tetrahydropyridoindole congeners **5a**–**5c** ranged from approx. 20 for **5b** through 40 for **5c** to about 60 for **5a**. These compounds are expected to have even less affinity for other enzymes that are not as closely related to ALR2.

Table 3. Inhibition of rat kidney ALR1

Compound	IC_{50} (μM)
5a	3081.3
5b	328.5 ± 21.9
5c	603.0
7b	>3000
Valproic acid	56.1 ± 2.7

Results are mean values from two or mean values ± SD from at least three experiments.

2.4. Antioxidant activity

According to the published data, stobadine has been postulated as a chain-breaking antioxidant characterized by its ability to scavenge chain-propagating peroxyl radicals.[39,40] As shown in Table 4, in agreement with our previously published results,[41] stobadine and its carboxymethylated hexahydropyridoindole derivatives **7a** and **7b** rapidly reacted with α,α′-diphenyl-β-picrylhydrazyl (DPPH), their antiradical activity being from 4 to 8 times higher than that of their tetrahydropyridoindole analogues **5a**–**5c**. An absorbance decrease at 518 nm at time intervals of 5 min was used as a measure of the antiradical activity of the compounds tested. DPPH, as a weak hydrogen atom abstractor, is considered a good kinetic model for peroxyl $ROO^{\cdot}$ radicals.[42] In a homogeneous solution system of DPPH in ethanol, antioxidant activity stems from an intrinsic chemical reactivity toward radicals. In membranes, however, the relative reactivity may be different since it is determined also by additional factors, such as location of the antioxidant and radicals, ruled predominantly by their partition ratios between water and lipophilic compartments.

In our further experiments, unilamellar dioleoyl L-α-phosphatidylcholine (DOPC) liposomes were used as model membranes.[41,43] Peroxidation of liposomes was induced by a water-soluble radical generator, 2,2′-azobis(2-amidinopropane)hydrochloride (AAPH), which simulates an attack by free radicals from the aqueous region. The monoene DOPC was used in order to minimize adventitious heat-induced autooxidation of liposomes. Indeed, no measurable accumulation of hydroperoxides was observed in the reaction system of liposomes heated to 50 °C when the initiator AAPH was omitted. In a complete reaction system, DOPC liposomes/AAPH/buffer, lipid peroxidation proceeded at a constant rate, with a near linear time-dependent increase of lipid hydroperoxides observed, without an induction period (Fig. 3). The carboxymethylated tetrahydropyridoindole **5b** effectively suppressed oxidation and gave a distinct lag phase in the lipid peroxide accumulation curve, indicating a much higher reaction rate of the compound with peroxyl radicals compared to the rate of chain propagation of phosphatidylcholine peroxidation. However, the presence of polar acidic function in

Table 4. Antiradical activities of substituted pyridoindoles in a DPPH test[a]

Compound	Absorbance decrease ($-\Delta A$/5 min)
5a	0.031 ± 0.007
5b	0.030 ± 0.006
5c	0.031 ± 0.009
7a	0.132 ± 0.014
7b	0.187 ± 0.020
Stobadine	0.239 ± 0.019
Dehydrostobadine	0.033 ± 0.008

Results are mean values ± SD from at least three measurements.
[a] The ethanolic solution of DPPH radical (50 μM) was incubated in the presence of the compound tested (50 μM). Absorbance decrease at 518 nm during the first 5 min interval was determined.

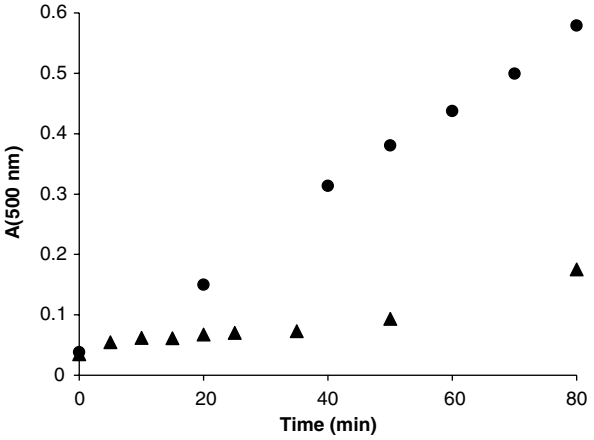


Figure 3. Typical kinetic curve of AAPH-induced peroxidation of DOPC liposomes (●) and the appearance of inhibition period in the presence of compound **5b** (250 μM, ▲). DOPC liposomes (0.8 mM) were incubated in the presence of AAPH (10 mM) in phosphate buffer (pH 7.4, 20 mM) at 50 °C.

Table 5. Antioxidant activity of compounds **5b** and **7b** in comparison with stobadine, dehydrostobadine, and standard trolox

Compound	IC_{50}[a] (μM)
5b	168.1 ± 14.5
7b	75.6 ± 2.5
Stobadine	25.3 ± 14.6
Dehydrostobadine	72.7 ± 19.8
Trolox	93.5 ± 8.5

Results are mean values ± SD from at least three independent incubations.

[a] Values of IC_{50} determined for the inhibition of AAPH-induced peroxidation of DOPC liposomes at the time interval of 80 min. DOPC liposomes (0.8 mM) were incubated in the presence of AAPH (10 mM) in phosphate buffer (20 mM; pH 7.4) at 50 °C.

the molecule of **5b** is expected to hinder its passage into the lipid compartment, reflected by correspondingly lower distribution ratio of **5b** compared with its more lipophilic congener dehydrostobadine (Table 6). This lipophilicity drop eventually may account for lower overall antioxidant efficiency of **5b** and **7b**, based on IC_{50} values (see Table 5), observed in the liposomes in spite of the fact that the intrinsic DPPH antiradical activity of **5b** and **7b** are similar to those of parent dehydrostobadine and stobadine, respectively (see Table 4). Still, the antioxidant activity of hexahydropyridoindole **7b** is comparable to that of the standard trolox.

2.5. Partitioning

Distribution ratios (*D*), defined by the total concentration of a solute in organic phase divided by that in aqueous phase, were determined for the compounds studied by a standard shake-flask technique. Experimental *D* values obtained at the physiological pH are shown in Table 6. The pH-lipophilicity profile experimentally determined for compounds **5b** and **5c** in the system of 1-octanol/buffer was characterized by a bell-shaped curve, with a maximal distribution ratio near neutral pH (Fig. 4). This behavior is in contrast with that of

Table 6. Distribution ratios of the substituted pyridoindoles in the system 1-octanol/phosphate buffer

Compound	Distribution ratio (D)[a]
5a	0.13 ± 0.05
5b	0.35 ± 0.02
5c	0.51 ± 0.03
7a	<0.10
7b	0.20 ± 0.04
Stobadine	2.50 ± 0.08
Dehydrostobadine	6.00 ± 0.42

Results are mean values ± SD from at least three independent experiments.

[a] Distribution ratio in the system of 1-octanol/0.1 M phosphate buffer (pH 7.4) determined by a shake-flask method.

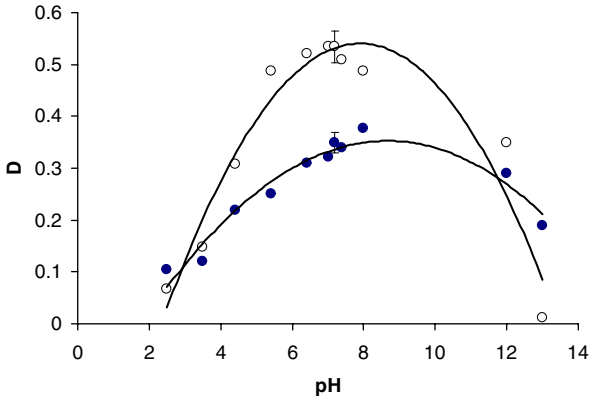


Figure 4. pH-distribution profile of compounds **5b** (●) and **5c** (○) in 1-octanol/buffer system. Experimental points represent mean values from two measurements or a mean value ± SD from four measurements.

acidic ARIs whose carboxylic acid function is ionized at neutral pH resulting in a sharp drop in distribution ratios and poor biological availability under physiological conditions. Owing to the presence of a basicity center at the tertiary nitrogen, the compounds are typical ampholytes.[44] Dissociation constants were determined for compound **5b** as follows: $pK_a^{Acidic} = 4.34 \pm 0.07$ and $pK_a^{Basic} = 8.65 \pm 0.07$. Since $pK_a^{Acidic} < pK_a^{Basic}$, the acidic and basic functionalities can be ionized at the same time resulting in double charged zwitterionic species.[44] Zwitterionic ampholytes often exhibit incompletely understood effects of intramolecular neutralization that may render the zwitterionic species markedly more lipophilic than the single charged cations and anions, giving typically a lipophilicity profile of a characteristic bell-shape with the maximum around the isoelectric point.[44] The parabolic distribution profiles shown for **5b** and **5c** are those of typical zwitterionic ampholytes.

Switch from the methyl substituent in compound **5a** to more lipophilic benzyl- or phenethyl-substituents in compounds **5b** and **5c**, respectively, resulted in significant increase in the distribution ratios (Table 6) with expected consequences in their potentially increased biological availability to lipid compartments under in vivo conditions. On the other hand, the lipophilicity of the $-R_2$ substituent affected the aldose reductase inhibition just marginally compared with the effect of reduc-

ing the tetrahydropyridoindole moiety to that of hexahydropyridoindole, as discussed below.

2.6. Molecular modeling

Based on the comparison of the appropriate IC_{50} values listed in Table 1, the unsaturated tetrahydropyridoindole type compound **5b** was found to be an over one order more efficient inhibitor of ALR2 than the saturated hexahydropyridoindole congener **7b**. To relate the inhibitory efficiencies of compounds **5b** and **7b** to their structural features, we performed several computational studies. The lower inhibitory activity of **7b** in relation to **5b** may be due to the specific distinction of the stereoelectronic profiles of these compounds. Visualization of low energy conformations showed almost planar tricyclic moiety of the tetrahydropyridoindole **5b**, contrasting with severe space distortion of the lipophilic heterocyclic backbone of hexahydropyridoindole **7b** (see Fig. 5). The presence of an extended aromatic planar region in the majority of potent ARIs is well documented as a crucial pharmacophoric element.[11,12,45–48]

The values of total interaction energies E_{int} between ALR2 residues and the individual compounds studied were calculated by docking followed by total optimization of the inhibitor-ALR2-$NADP^+$ ternary complex. The calculated results of E_{int} obtained for double-charged zwitterionic species, −90.7 kcal/mol for **5b**, −77.3 and −74.5 kcal/mol for (−)-**7b** and (+)-**7b**, respectively, and that of −107.0 kcal/mol for standard zopolrestat agreed well with the order of the inhibitory activities. For structure **5b** a much lower value of E_{int}, −59.3 kcal/mol, was calculated for the uncharged molecular species. The neutral molecule is practically the only form expected to be in equilibrium with corresponding zwitterions around the physiological pH, being in this case close to $pH_{iso} = 6.5$, calculated on the basis of the macroscopic dissociation constants.

Individual interaction energies between the inhibitor molecules and the specific amino acid residues of the active site of ALR2 (Tyr48, His110, Trp111)[49] and $NADP^+$, calculated for **5b** and for the theoretically more efficient (−)-enantiomer of **7b**, are summarized in Table 7. Interaction with Tyr48 seems to be crucial for the inhibitory activity of the compounds since comparison of the E_{int} with Tyr48 gives values of −19.4 and −9.4 kcal/mol for **5b** and (−)-**7b**, respectively. This effect is reflected also by the geometric parameters of the minimized complexes: the mutual distance between the carboxylic oxygen of the inhibitor and the hydrogen of Tyr48 was found to be 1.6 Å for **5b**, but 2.7 Å for (−)-**7b** (Fig. 6). Molecular electrostatic potential of ALR2 complexed with (−)-**7b** and **5b** derivatives after total optimization is shown in Figure 7.

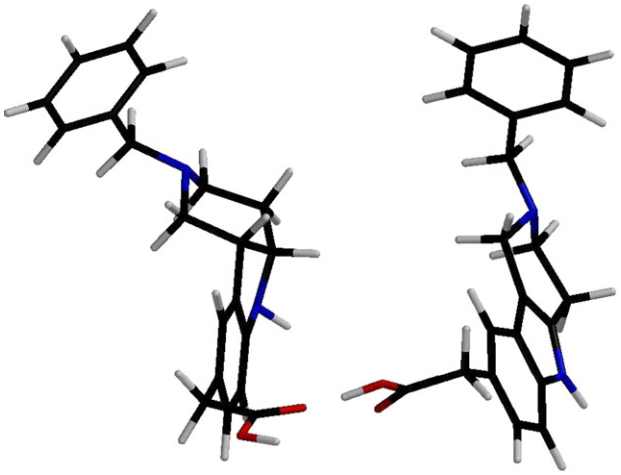


Figure 5. Stereoview of hexahydropyridoindole (−)-**7b** (left) versus tetrahydropyridoindole **5b** (right).

Table 7. Individual interaction energies between the inhibitors and the specific amino acid residues of ALR2

Compound	E_{int} ($I - R$) (kcal/mol)			
	$NADP^+$	Tyr48	His110	Trp111
5b	−12.5	−19.4	−3.4	−2.6
(−)-**7b**	−9.2	−9.4	−3.8	−3.8
Zopolrestat	0.0	−19.3	−12.3	−14.1

2.7. 'Drug-likeness'

The 'drug-likeness' of the novel aldose reductase inhibitors was assessed on the basis of their structural properties by applying the Lipinski's 'rule of five', and moreover the polar surface area and the number of rotatable bonds as two additional molecular descriptors[50–52] (Table 8). For all the drug candidates studied, none of the criteria was violated, thus predicting their good oral bioavailability.

3. Conclusions

The carboxymethylated hexahydropyridoindoles **7a**, **7b** and tetrahydropyridoindoles **5a**–**5c** structurally based on the antioxidant drug stobadine are presented as novel aldose reductase inhibitors endowed with antioxidant activity. The computed stereoelectronic properties of compounds **7b** and **5b** along with a modeling study of the ALR2-binding site could provide an explanation for the higher inhibitory efficiencies of the tetrahydropyridoindoles in comparison to the hexahydropyridoindoles. The distribution profile of the representative inhibitors **5b** and **5c** showed maximal extraction yield around pH 7 in a 1-octanol/water system. This behavior can obviously be accounted for by the zwitterionic ampholyte nature of the compounds and by higher lipophilicity of the doubly charged zwitterionic form when compared to a singly charged species. Properties implicit to 'drug-likeness', inferred from simple molecular descriptors, rendered novel aldose reductase inhibitors potential drugs with expected good bioavailability and with a prospect of further optimization that could lead to compounds of even higher potency. The zwitterionic principle thus offers an interesting way how to improve potentially the bioavailability of aldose reductase inhibitors bearing an acidic function.

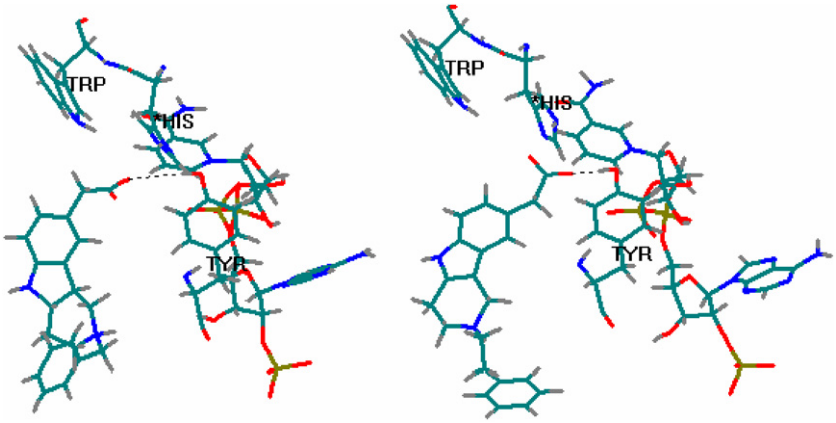


Figure 6. Geometry of the active site for (−)-**7b** (left) and **5b** (right) derivative optimized with the whole enzyme. Dashed line denotes the hydrogen bonds between the carboxylic oxygen and the hydrogen of Tyr48.

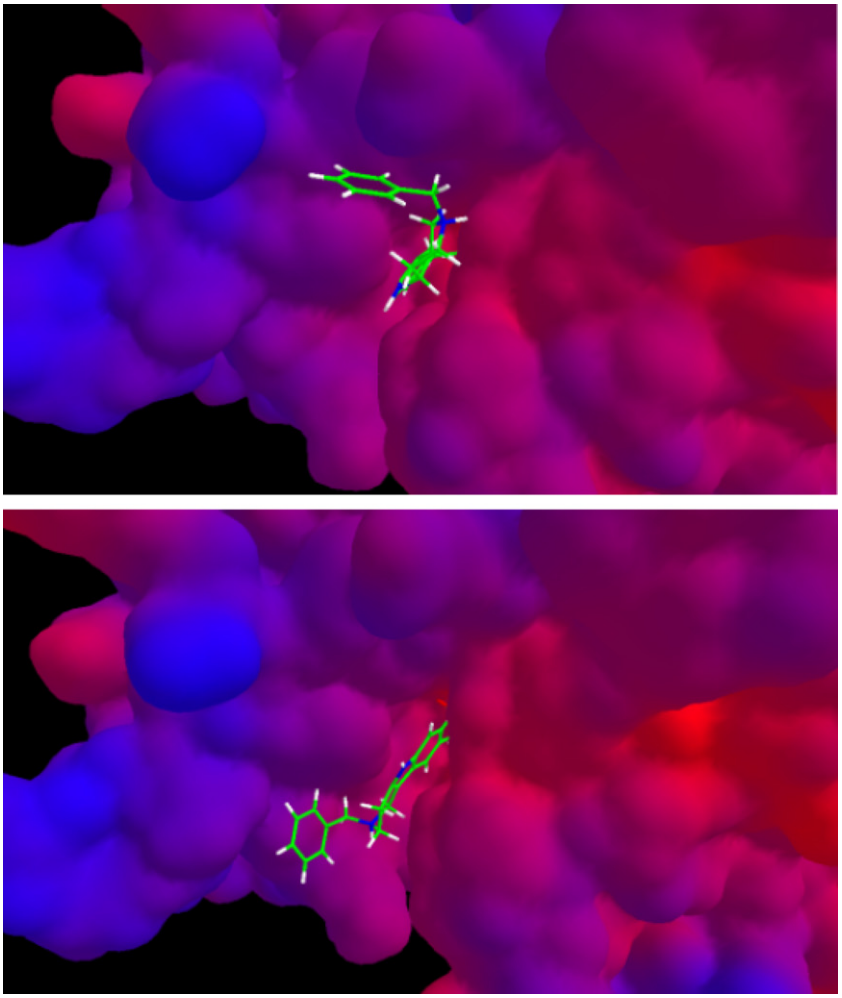


Figure 7. Molecular electrostatic potential of ALR2 complexed with (−)-**7b** (up) and **5b** (below) derivatives after total optimization.[73] Due to sterical restrictions, the saturated hexahydroderivative (−)-**7b** is not able to insert into the cavity of the active site sufficiently and thus to contact the specific amino acid residues in proper interaction distances as the unsaturated tetrahydropyridoindole derivative **5b**.

4. Experimental

4.1. Chemistry

4.1.1. General. NMR spectra were recorded on Bruker Avance DPX 300 spectrometer operating at 300.13 MHz for ^{1}H and 75.46 MHz for ^{13}C. ^{1}H and ^{13}C NMR spectra were acquired using internal acetone (^{1}H $\delta = 2.225$; ^{13}C $\delta = 31.07$) as a reference standard in $CDCl_3$ or D_2O used as solvents. To assign the signals, the COSY, HSQC, TOCSY, NOESY, and DEPT techniques were used. Elemental analyses were performed

Table 8. 'Drug-likeness' of the carboxymethylated pyridoindoles

Compound	MW (<500)	NH/OH (<5)	N/O (<10)	*C*log *P* (<5)	PSA < 120 Å^2	nROTB (<8)
5a	244.32	2	4	1.57	56.33	3
5b	320.42	2	4	2.97	56.33	4
5c	334.45	2	4	3.38	56.33	5
7a	246.34	2	4	1.13	52.56	3
7b	322.44	2	4	2.60	52.56	4

MW, molecular weight; OH/NH, number of H-bond donors; O/N, number of H-bond acceptors; *C*log *P*, calculated decimal logarithm of octanol/water partition coefficient[71]; PSA, polar surface area[72]; nROTB, number of rotatable bonds.

on EA 1108 Carlo Erba (for **5b** also by Galbraith Laboratories, Knoxville, TN, USA) and were within ±0.4% of the theoretical values. Melting points were determined on a Kofler melting point apparatus with digital thermometer (DT012C) and are uncorrected. The purity of the intermediate products (**4a**–**4c**) and (**6a**, **6b**) was determined by GC–MS on Hewlett–Packard GC HP 5980 using the column HP-5 (12 m × 0.22 mm × 0.33 μm) and mass detector Hewlett–Packard MSD HP 5970B. Molecuar masses of the final potassium salts (**5a**–**5c**) and (**7a**, **7b**) were determined on MALDI TOF IV (Shimadzu, Kratos Analytical, Manchester, UK) instrument. 2,5-Dihydroxy benzoic acid was used as a matrix. Samples were irradiated by 337 nm photons from the nitrogen laser. Typically, 100 shots were summed into a single mass spectrum. Stobadine, (−)-*cis*-2,8-dimethyl-2,3,4,4a,5,9b-hexahydro-1H-pyrido[4,3-*b*]-indole, and its unsaturated analogue dehydrostobadine, 2,8-dimethyl-2,3,4,5-tetrahydro-1H-pyrido[4,3-*b*]-indole were supplied by the Institute of Experimental Pharmacology, Slovak Academy of Sciences as hydrochloride salts.[53,54] The starting materials 4-cyanomethylphenylhydrazine hydrochloride (**1**) and 4-carboxymethylphenylhydrazine hydrochloride (**2**) were synthesized by published procedures in yields comparable with literary data.[16,17] All solvents and reactants were purified before use according to standard procedures.[55] Commercially available 1-methyl-4-piperidone (**3a**) and 1-benzyl-4-piperidone (**3b**) were purified by vacuum distillation and 1-(2-phenethyl)-4-piperidone (**3c**) was crystalized from *n*-hexane. Column chromatography was done by using the flash chromatography technique and was carried out on silica gel 60 (230–400 mesh, Merck). L-α-Phosphatidylcholine dioleoyl (C18:1, [*cis*]-9) (DOPC) (99% grade), 2,6-di-*tert*-butyl-*p*-cresol (BHT), and α,α′-diphenyl-β-picrylhydrazyl (DPPH) radical were obtained from Sigma Chemical Co. (St. Louis, MO, USA). 2,2′-azobis (2-amidinopropane)hydrochloride (AAPH) was obtained from Fluka Chemie GmbH (Buchs, Switzerland). Other chemicals were purchased from local commercial sources and were of analytical grade quality. All solvents used for lipid peroxidation studies were deaereated under nitrogen.

4.1.2. 2-Methyl-2,3,4,5-tetrahydro-1*H*-pyrido[4,3-*b*]indole-8-yl acetonitrile (4a). The mixture of **1** (18.36 g, 0.1 mol), **3a** (11.31 g, 0.1 mol), and 500 ml of anhydrous 10% HCl ethanolic solution was stirred and refluxed for 5 h under argon atmosphere. After evaporation of the solvent to dryness, water (300 mL) was added, stirred for a few minutes, then the solution was carefully made basic to pH 10 with 40% aqueous NaOH, cooled to 5–8 °C and stirred vigorously for 30 min. The solid material formed was filtered, washed with water and dried. The crude product was dissolved in a hot mixture of 3% triethanolamine in toluene (600 mL), cooled and filtered through a short (5 cm high and 3 cm diameter) column of silica gel. The column was washed several times with the same solvent as used for dissolving the product, and the filtrates were concentrated under reduced pressure to afford crude **4a**, which was crystalized at −5 °C from methanol, filtered and washed with a small volume of cold methanol to yield pure **4a** (12.97 g, 57.6%): mp: 164–166 °C; ^{1}**H NMR** ($CDCl_3$, 25°): 2.54 (s, 3H, CH_3N), 2,78 (t, 2H, H-4), 2,84 (t, 2H, H-3), 3,68 (s, 2H, H-1), 3.80 (s, 2H, CH_2CN), 6.93 (d, 1H, H-6), 7.10 (d, 1H, H-7), 7.31 (s, 1H, H-9), 8.81 (s, 1H NH); ^{13}**C NMR** ($CDCl_3$, 25 °C): 23.68 (C-4), 23.74 (CH_2CN), 45.79 (CH_3N), 51.63 (C-3), 52.41 (C-1), 108.40 (C-9b), 111.24 (C-6), 116.84 (C-9), 119.04 (CN), 120.42 (C-8), 120.82 (C-7), 126.46 (C-9a), 133.30 (C-5a), 135.68 (C-4a); **MS** *m*/*z* (%) = 225 (M^+, 46), 182 (100), 154 (14), 140 (7), 128 (7) 99 (11).

4.1.3. 2-Benzyl-2,3,4,5-tetrahydro-1*H*-pyrido[4,3-*b*]indole-8-yl acetonitrile (4b). The mixture of **1** (18.36 g, 0.1 mol), **3b** (8.93 g, 0.1 mol) and methanol (500 mL) was stirred and refluxed for 1.5 h in argon atmosphere and then the solution was evaporated under reduced pressure to dryness. The solution of 6% HCl in acetic acid (600 mL) was added to the residue and the reaction mixture was stirred and heated up to reflux under argon atmosphere for 10 s, and then was stirred without heating for 15 min. The reaction mixture was heated again to a short reflux, and the further procedure was as in the previous step. This procedure was repeated six times, then the mixture was concentrated under reduced pressure, diluted with water (300 mL), alkalized with 40% aqueous NaOH to pH 10 and extracted several times with CH_2Cl_2. The combined organic phases were washed with brine, dried (Na_2SO_4), and concentrated under reduced pressure. The crude product was dissolved in a hot mixture of 3% triethanolamine in toluene and treated as previously described for **4a**, crystalized from methanol at −5 °C, filtered and washed with a small volume of cold methanol to give pure **4b** (25.47 g, 84.5%): mp: 120–122 °C; ^{1}**H NMR** ($CDCl_3$, 25 °C): 2.78 (t, 2H, H-4), 2.84 (t, 2H, H-3), 3.72 (s, 2H, H-1), 3.79 (s, 2H, CH_2CN), 6.93 (d, 1H, H-6), 7.14 (d, 1H, H-7), 7.20–7.42 (m, 6H, H-9, Ph-H), 8.23 (s, 1H, NH); ^{13}**C NMR** ($CDCl_3$, 25 °C): 23.7 (C-4, **C**H_2CN), 49.59 (C-3), 50.14 (C-1), 62.41 (**C**H_2Ph), 108.43 (C-9b), 111.24 (C-6), 116.97 (C-9), 119.05 (CN),

120.51 (C-8), 120.92 (C-7), 126.55 (C-9a), 127.43, 128.5, 129.35, 138.00 (C-Ph), 133.48 (C-5a), 135.58 (C-4a); **MS** *m/z* (%) = 301 (M^+, 18), 182 (100), 154 (4), 91 (27).

4.1.4. Methyl ester of 2-phenethyl-2,3,4,5-tetrahydro-1H-pyrido[4,3-*b*]indole-8-yl acetic acid (4c). The mixture of **2** (17.06 g, 0.1 mol) and **3c** (20.33 g, 0.1 mol) in a solution of 10% HCl in anhydrous CH_3OH (500 mL) was stirred and refluxed for 6 h under argon atmosphere. After evaporation of the solvent, water (300 mL) was added, and carefully made basic to pH 10 under cooling to 5–8 °C and vigorous stirring for 30 min by addition of 40% aqueous NaOH solution. The solid material was extracted by the addition of several portions of CH_2Cl_2. The combined organic phases were washed with brine, dried (Na_2SO_4), and concentrated under reduced pressure. The crude product was subjected to flash chromatography procedure as described for **4a** followed by crystalization from ether. Recrystalization from methanol/ether yielded **4c** (15.82 g, 45.4%): mp: 127–129 °C; **^{1}H NMR** ($CDCl_3$, 25 °C): 2.80–3.08 (m, 8H, H-3, H-4, CH_2CH_2Ph), 3.58 (s, 2H, H-1), 3.68 (s, 3H, CH_3O), 3.95 (s, 2H, $CH_2CO_2CH_3$), 6.96–7.32 (m, 8H, H-6, H-7, H-9, Ph-H), 8.48 (br s, 1H, NH); **^{13}C NMR** ($CDCl_3$, 25 °C): 22.72 (C-4), 33.14 ($CH_2\mathbf{C}H_2Ph$), 41.32 (CH_3O), 45.76 ($\mathbf{C}H_2CO_2CH_3$), 49.29 (C-3), 50.55 (C-1), 59.13 ($N\mathbf{C}H_2CH_2Ph$), 110.94 (C-6, C-9b), 117.91 (C-9), 122.81 (C-7), 124.97 (C-4a), 125.95, 126.47, 128.61, 128.74 (C-Ph), 131.75 (C-9a), 135.35 (C-5a), 138.94 (C-8), 172.96 (CO); **MS** *m/z* (%) = 348 (M^+, 1), 334(18), 215 (100), 156 (54), 91 (24).

4.1.5. General procedure for the reduction of tetrahydropyridoindoles (4a and 4b). To the magnetically stirred cold solution (−5 to 0 °C) of **4a** or **4b** (0.05 mol) in TFA (125 mL), 95% $NaBH_3CN$ (6.61 g, 0.1 mol, 2.0 equiv) was gradually added during a 40-min period. Then after removing the cooling bath, the reaction mixture was stirred at room temperature for 2.5 h. The reaction mixture was carefully poured into an ice-cold solution of NaOH (70 g) in water (105 mL), with the final pH being approx. 10. The target bases **6a** or **6b** were taken up in CH_2Cl_2 by repeated extraction. The combined organic phases were washed with brine, dried (anhydrous Na_2SO_4), and concentrated under reduced pressure. The residue was dissolved in dry methanol (15 mL) at 40–50 °C, acidified by the addition of a 15% methanolic HCl solution (15 mL), well mixed, and then crystalized at −5 °C overnight. The crystals of the dihydrochloride salts of **6a** and **6b** were filtered, washed with a small volume of cold methanol and acetone, and dried at 90 °C during a 1-h period to give pure compound **6a** or **6b**. For NMR analysis and the final hydrolysis step, free bases were isolated as follows: the dihydrochloride salts were dissolved in water and after the addition of 40% aqueous NaOH to reach pH 10, the mixture was extracted several times with CH_2Cl_2. The combined organic phases were washed with brine, dried with anhydrous Na_2SO_4, filtered and concentrated under reduced pressure to dryness to produce the bases of hexahydropyridoindole **6a** or **6b** for further applications.

4.1.5.1. (±)-2-Methyl-(4a,9b)-*cis*-1,2,3,4,4a,9b-hexahydro-1H-pyrido[4,3-*b*]indole-8-yl acetonitrile·2 HCl (6a). Isolated 10.3 g, (68.6% yield), mp: 172–175 °C; **^{1}H NMR** ($CDCl_3$, 25 °C): 1.78, 1.92 (m, m, 1H, 1H, H-4,4′), 2.27 (s, 3H, NCH_3), 2.3–2.39 (m, 3H, H-1, H-3,3′), 2.63 (dd, 1H, H-1′), 3.21 (dd, 1H, H-9b), 3.63 (s, 2H, CH_2CN), 3.72 (br s, 1H, NH), 3.81 (dd, 1H, H-4a), 6,63 (d, 1H, H-6), 6.95 (d, 1H, H-7), 7.05 (s, 1H, H-9); **^{13}C NMR** ($CDCl_3$, 25 °C): 22.96 ($\mathbf{C}H_2CN$), 28.62 (C-4), 40.88 (C-9b), 46.36 ($\mathbf{C}H_3N$), 51.04 (C-3), 56.58 (C-1), 57.04 (C-4a), 110.11 (C-6), 118.52 (CN), 123.03 (C-7), 119.72 (C-9), 127.21 (C-9a), 132.77 (C-5a), 150.32 (C-8); **MS** *m/z* (%) = 227 (M^+, 64), 184 (18), 155 (52), 71 (100), 58 (96).

4.1.5.2. (±)-2-Benzyl-(4a,9b)-*cis*-1,2,3,4,4a,9b-hexahydro-1H-pyrido[4,3-*b*]indole-8-yl acetonitrile·2 HCl (6b). Isolated 13.41 g (71.3% yield), mp: 197–201 °C; **^{1}H NMR** ($CDCl_3$, 25 °C) 1.72, 1.90 (m, m, 1H, 1H, H-4,4′), 2.23 (dd, 1H, H-1), 2.43 (m, 2H, H-3,3′), 2.62 (dd, 1H, H-1′), 3.13 (dd, 1H, H-9b), 3.47 (s, 2H, NCH_2Ph), 3.52 (s, 2H, CH_2CN), 3.76 (dd, 1H, H-4a), 4.11 (br s, 1H, NH), 6.57 (d, 1H, H-6), 6.85 (s, 1H, H-9), 6.89 (d, H-7), 7.31 (m, 5H, Ph-H); **^{13}C NMR** ($CDCl_3$, 25 °C): 22.79 ($\mathbf{C}H_2CN$), 28.35 (C-4), 40.69 (C-9b), 48.96 (C-3), 54.17 (C-1), 57.58 (C-4a), 62.82 ($\mathbf{C}H_2Ph$), 109.90 (C-6), 118.48 (CN), 119.42 (C-7), 123.15 (C-9), 126.86, 127.05, 128.03, 128.87 (C-**Ph**), 132.98 (C-9a), 138.01 (C-5a), 150.31 (C-8); **MS MALDI** *m/z* (%): 303 (M+H).

4.1.6. General procedure for hydrolysis of 4a–c and 6a–b. The mixture of KOH (0.15 mol, 3.0 equiv), isopropanol (100 mL) and water (4 mL) was flushed off the air by bubbling with argon for 10 min. Then, the appropriate compounds **4a**–**c** or **6a**, **b** (0.05 mol, 1.0 equiv) were added and the reaction mixture was heated and stirred under argon atmosphere on reflux. In the case of compound **4c**, a 1-day reaction time was used. Elimination of free ammonia indicated progress of the reaction in the case of the acetonitriles **4a**, **b** and **6a**, **b**. After 5 days of refluxing, the release of ammonia stopped, the reaction mixture was concentrated under reduced pressure to dryness and the residue was dissolved in air-free water (150 mL), warmed with active charcoal and filtered. Under intensive stirring in ice/NaCl bath, an ice-cold solution of 20% aq HCl was slowly added to the filtrate (temperature kept in the range 3–7 °C) in argon atmosphere to adjust pH to approx. 6.5, when the solid product started to crystallize. The intensive stirring continued for further 4 h at the above temperature, the mixture was filtered and the solid was washed with a small volume of cold air-free water to afford an amino acid product, which was dried over P_2O_5 and argon atmosphere to constant weight. Potassium salts of the appropriate amino acids were prepared by careful treatment of their suspensions in dry methanol (150 mL) with solid $KHCO_3$. Then the mixtures were filtered through a short (5 cm high and 3 cm diameter) column of silica gel. The column was washed with methanol, the filtrates were concentrated under reduced pressure to dryness and the crude products were recrystalized as potassium (sodium) salts at −5 °C from the solvents listed in Table 9.

4.1.6.1. Potassium 2-methyl-2,3,4,5-tetrahydro-1H-pyrido[4,3-*b*]indole-8-yl acetate (5a). **^{1}H NMR** (D_2O, 25 °C): 2.35 (s, 3H, CH_3N), 2,73 (br s, 4H, H-3, H-4), 3,50 (s, 4H, H-1, CH_2COOK), 7.00 (d, 1H, H-7), 7.25 (s, 1H, H-9), 7.25 (d, 1H, H-6); **^{13}C NMR** (D_2O, 25 °C): 23.30 (C-4), 44.86 (N–CH_3), 45.48 (CH_2COOK), 51.21 (C-1), 52.15 (C-3), 107.24 (C-9b), 112.00 (C-6), 118.31 (C-9), 123.30 (C-7), 126.42 (C-9a), 128.88 (C-4a), 133.91 (C-5a), 135.62 (C-8), 182.82 (COOK); **MS MALDI** *m*/*z* = 283 (M+H).

4.1.6.2. Potassium 2-benzyl-2,3,4,5-tetrahydro-1H-pyrido[4,3-*b*]indole-8-yl acetate (5b). **^{1}H NMR** (D_2O, 25 °C) 2.35 (br s, 2H, H-4), 2.44 (br s, 2H, H-3), 3.37 (s, 2H, NCH_2Ph), 3.43 (s, 2H, CH_2CO_2K), 3.48 (s, 2H, H-1), 6.93–7.23 (m, 8H, H-6, H-7, H-9, Ph-H); **^{13}C NMR** (D_2O, 25 °C) 23.09 (C-4), 45.61 (CH_2CO_2K), 49.13 (C-3), 49.71 (C-1), 61.74 (NCH_2Ph), 106.96 (C-9b), 111.97 (C-6), 118.42 (C-9), 123.31 (C-7), 126.55 (C-4a), 128.50, 128.71 129.30, 130.85, (C-Ph), 133.52 (C-9a), 135.63 (C-5a), 137.22 (C-8), 182.74 (COOK); **MS MALDI** *m*/*z* = 359 (M+H).

4.1.6.3. Potassium 2-phenethyl-2,3,4,5-tetrahydro-1H-pyrido[4,3-*b*]indole-8-yl acetate (5c). **^{1}H NMR** (D_2O, 25 °C): 2.45–2.71 (m, 8H, H-3, H-4, CH_2CH_2Ph), 3.44 (s, 2H, CH_2CO_2K), 3.51 (s, 2H, H-1), 6.94–7.29 (m, 8H, H-6, H-7, H-9, Ph-H); **^{13}C NMR** (D_2O, 25 °C): 23.19 (C-4), 33.46 (CH_2Ph), 45.59 (CH_2CO_2K), 49.30 (C-3), 50.31 (C-1), 59.46 (NCH_2CH_2Ph), 107.03 (C-9b), 111.99 (C-6), 118.37 (C-9), 123.32 (C-7), 126.57 (C-4a), 127.18, 128.79, 129.55, 129.65 (C-Ph), 133.88 (C-9a), 135.66 (C-5a), 141.10 (C-8), 182.72 (COOK); **MS MALDI** *m*/*z* = 373 (M+H).

4.1.6.4. Potassium salt of (±)-2-methyl-(4a,9b)-*cis*-1,2,3,4,4a,9b-hexahydro-1H-pyrido[4,3-*b*]indole-8-yl acetic acid (7a). **^{1}H NMR** (D_2O, 25 °C): 1.78, 1.83 (m, m, 1H, 1H, H-4,4′), 2.30 (s, 3H, CH_3-N), 2.32 (um, 1H, H-1), 2.52, 2.82 (um, m, 1H, 1H, H-3,3′), 2.95 (um, 1H, H-1), 3.30 (dd, 1H, H-9b), 3.44 (s, 2H, CH_2COOK), 3.80 (dd, 1H, H-4a), 6.81 (d, 1H, H-6), 7.02 (d, 1H, H-7), 7.12 (s, 1H, H-9); **^{13}C NMR** (D_2O, 25 °C): 27.73 (C-4), 41.14 (C-9b), 44.83 (**CH_2**COOK), 45.64 (CH_3N), 51.43 (C-3), 56.99 (C-1, C-4a), 113.02 (C-6), 125.61 (C-7), 129.36 (C-9), 133.45 (C-9a), 149.22 (C-5a), 164.62 (C-8), 172.01 (COOK); **MS MALDI** *m*/*z* = 285 (M+H).

Table 9. Chemical data for the carboxymethylated tetrahydropyridoindoles **5a**–**5c** and hexahydropyridoindoles **7a**, **7b**

Compound	Recrystalization solvent	Formula[a] (Molecular mass)	Yield[b] (%)
5a	*i*-PrOH/CH_3OH	$C_{14}H_{15}N_2O_2K$ (282.39)	50.5
5b	CH_3OH/acetone	$C_{20}H_{19}N_2O_2K$ (358.48)	67.3
5c	*i*-PrOH	$C_{21}H_{21}N_2O_2K$ (372.51)	48.6
7a	CH_3OH/acetone	$C_{14}H_{17}N_2O_2K$ (284.40)	38.7
7b	*i*-PrOH/acetone	$C_{20}H_{21}N_2O_2Na$ (344.38)	41.5

[a] Elemental analyses for C, H, N were within ±0.4% of the calculated value (for details see Supporting information).

[b] The yields are based on the corresponding acetonitriles **4a**, **4b** or **6a**, **b** and the methylester **4c**.

4.1.6.5. Sodium salt of (±)-2-benzyl-(4a,9b)-*cis*-1,2,3,4,4a,9b-hexahydro-1H-pyrido[4,3-*b*]indole-8-yl acetic acid (7b). **^{1}H NMR** (D_2O, 25 °C): 1.80, 1.94 (m, m, 1H, 1H, H-4,4′), 2.26 (dd, 1H, H-1), 2.51, 2.65 (m, m, 1H, 1H, H-3,3′), 2.78 (dd, 1H, H-1′), 3.20 (dd, 1H, H-9b), 3.39 (s, 2H, CH_2COOK), 3.59 (m, AB quartet, 2H, NCH_2Ph), 3.77 (dd, 1H, H-4a), 6.77 (d, 1H, H-6), 6.96 (s, 1H, H-9), 6.99 (d, 1H, H-7), 7.38 (m, 5H, Ph-H); **^{13}C NMR** (D_2O, 25 °C): 27.55 (C-4), 40.91 (C-9b), 44.98 (**CH_2**COONa), 49.50 (C-3), 53.97 (C-1), 57.57 (C-4a), 62.93 (N**CH_2**Ph), 112.67 (C-6), 125.74 (C-7), 129.48 (C-9), 128.80, 129.97, 131.15, 133.61 (C-Ph), 136.48 (C-9a), 149.40 (C-5a), 163.70 (C-8), 182.36 (COONa); **MS MALDI** *m*/*z* = 345 (M+H).

4.1.7. Partitioning. The distribution ratios *D* in 1-octanol/buffer systems, defined by total concentration of a solute in organic phase divided by that in aqueous phase, were measured using the shake-flask technique[56] at room temperature. The organic and aqueous phases were mutually saturated. Compounds were dissolved in aqueous buffer solutions (0.1 M phosphate or citrate buffers) with KCl (0.15 M) in final concentration of 100 μM; the solutions were shaken with 1-octanol for 3 h. Both aqueous and organic phase volumes were 3 mL. The phases were separated by centrifugation for 1 h. The organic layer was removed with a Pasteur pipette. The concentration of the solute was determined in both phases by UV spectrophotometry.

4.1.8. DPPH test. To investigate the antiradical activity of the pyridoindole derivatives, the ethanolic solution of DPPH (50 μM) was incubated in the presence of a compound tested (50 μM) at laboratory temperature. The absorbance decrease, recorded at λ_{ax} = 518 nm, during the first 5-min interval was taken as a marker of the antiradical activity.

4.1.9. Liposome peroxidation. The methods were described previously.[41,43] L-α-phosphatidylcholine dioleoyl (DOPC, C18:1, [*cis*]-9, 15.7 mg) was dissolved in chloroform (5 mL) and placed into a round-bottom flask. The solvent was subsequently removed under nitrogen and the resulting thin film on the walls was dispersed in phosphate buffer (20 mL, 20 mM, pH 7.4) by vigorous stirring for 2 min followed by sonification for the same period of time. A suspension of unilamellar liposomes (1 mM DOPC) was thus obtained. The liposomes (final concentration of 0.8 mM DOPC) were incubated in the presence of different concentrations of the compounds tested and of the water-soluble initiator AAPH (final concentration 10 mM) at 50 °C for different periods of time. Aliquots (1 mL) of the incubation mixtures were extracted by 2 mL portions of ice-cold mixture $CHCl_3$/MeOH (2:1, v/v) containing BHT (0.05%). Lipid hydroperoxide content was determined by thiocyanate method according to Mihaljevic[57] by sequentially adding $CHCl_3$/MeOH (2:1, v/v) mixture (1.4 mL) and the thiocyanate reagent (0.1 mL) to 1-mL aliquots of the liposome extracts. The reagent was prepared by mixing equivalent volumes of methanolic solution of KSCN (3%) and ferrous-ammonium sulfate solution (45 mM in 0.2 mM HCl). After the mixture was left at the ambient

temperature for at least 5 min, the absorbance at 500 nm was recorded by Hewlett–Packard Diode Array Spectrophotometer 8452A.

4.1.10. Determination of the proton dissociation constant. The potentiometric determination[58–60] was performed with Precision Digital pH Meter OP-208/1 equipped with glass electrode OP-0718 P and saturated calomel electrode OP-0830 P (Radelkis, Budapest, Hu). Automatic byuret OP 930 (Radelkis, Budapest, Hu) was used for titrations. The compound **5b** was dissolved in distilled water free of carbon monoxide at a concentration of about 2 mM. The solution was acidified by 0.1 M HCl to pH of about 1.8 and titrated with fresh solution of NaOH (0.1 M) at 22 °C ± 1 °C and at a constant ionic strength $I = 0.1$ M set by KCl. Three parallel titrations were performed.

4.2. Enzyme section

4.2.1. General. NADPH, β-mercaptoethanol, D,L-glyceraldehyde, D-glucuronate, and sodium valproate were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Diethylaminoethyl cellulose DEAE DE 52 was from Whatman International Ltd (Maidstone, England). Zopolrestat (lot # 43668-12-7F) was supplied as gift samples by Pfizer (Groton, CT, USA). Other chemicals were purchased from local commercial sources and were of analytical grade quality. Total protein in enzyme preparations was determined according to Geiger and Bessman.[61]

4.2.2. Animals. Male Wistar rats 8–9 weeks old, weighing 200–230 g, were used as organ donors. The animals came from the Breeding Facility of the Institute of Experimental Pharmacology Dobra Voda (Slovak Republic). The study was approved by the Ethics Committee of the Institute and performed in accordance with the Principles of Laboratory Animal Care (NIH publication 83-25, revised 1985) and the Slovak law regulating animal experiments (Decree 289, Part 139, July 9th 2003).

4.2.3. Preparation of ALR2. ALR2 from rat lens was partially purified using a procedure adapted from Hayman and Kinoshita[62] as follows: lenses were quickly removed from rats following euthanasia and homogenized in a glass homogenizer with a teflon pestle in 5 vol of cold distilled water. The homogenate was centrifuged at 10,000*g* at 0–4 °C for 20 min. The supernatant was precipitated with saturated ammonium sulfate at 40%, 50%, and then at 75% salt saturation. The supernatant was retained after the first two precipitations. The pellet from the last step, possessing ALR2 activity, was dispersed in 75% ammonium sulfate and stored in smaller aliquots in liquid nitrogen container.

4.2.4. Preparation of ALR1. ALR1 from rat kidney was partially purified according to the reported procedure of Costantino et al.[23] as follows: kidneys were quickly removed from rats following euthanasia and homogenized in a knife homogenizer followed by processing in a glass homogenizer with a teflon pestle in 3 vol of 10 mM sodium phosphate buffer, pH 7.2, containing 0.25 M sucrose, 2.0 mM EDTA dipotassium salt, and 2.5 mM β-mercaptoethanol. The homogenate was centrifuged at 16,000*g* at 0–4 °C for 30 min and the supernatant was subjected to ammonium sulfate fractional precipitation at 40%, 50%, and 75% salt saturation. The pellet obtained from the last step, possessing ALR1 activity, was redissolved in 10 mM sodium phosphate buffer, pH 7.2, containing 2.0 mM EDTA dipotassium salt and 2.0 mM β-mercaptoethanol to achieve total protein concentration of approx. 20 mg/mL. DEAE DE 52 resin was added to the solution (33 mg/mL) and after gentle mixing for 15 min removed by centrifugation. The supernatant containing ALR1 was then stored in smaller aliquots in liquid nitrogen. No appreciable contamination by ALR2 in ALR1 preparations was detected since no activity in terms of NADPH consumption was observed in the presence of glucose substrate up to 150 mM.

4.2.5. Enzyme assays. ALR1 and ALR2 activities were assayed spectrophotometrically by determining NADPH consumption at 340 nm and were expressed as decrease of the optical density (OD)/s/mg protein. To determine ALR2 activity,[63] the reaction mixture contained 4.67 mM D,L-glyceraldehyde as a substrate, 0.11 mM NADPH, 0.067 M phosphate buffer, pH 6.2 and 0.05 mL of the enzyme preparation in a total volume of 1.5 mL. The reference blank contained all the above reagents except the substrate D,L-glyceraldehyde to correct for oxidation of NADPH not associated with reduction of the substrate. The enzyme reaction was initiated by the addition of D,L-glyceraldehyde and was monitored for 4 min after an initial period of 1 min at 30 °C. ALR1 activity[23] was assayed analogically using 20 mM D-glucuronate as a substrate in the presence of 0.12 mM NADPH in 0.1 M phosphate buffer pH 7.2 at 37 °C. Enzyme activities were adjusted by diluting the enzyme preparations with distilled water so that 0.05 mL of the preparation gave an average reaction rate for the control sample of 0.020 ± 0.005 absorbance units/min. The effect of inhibitors on the enzyme activity was determined by including in the reaction mixture each inhibitor at required concentrations. The inhibitor at the same concentration was included in the reference blank. IC_{50} values (the concentration of the inhibitor required to produce 50% inhibition of the enzyme reaction) were determined from the least-square analysis of the linear portion of the semilogarithmic inhibition curves. Each curve was generated using at least four concentrations of inhibitor causing an inhibition in the range from at least 25% to 75%.

4.3. Computational methods

Low energy conformations (starting geometries) of the compounds were obtained by semi-empirical (AM1) geometry optimization[64] followed by Monte Carlo (MMFF94) conformational search.[65] The compounds were docked into the active site of the enzyme (PDB structure 1PWM—human ALR2 complexed with Fidarestat)[66] by the program DOCK (version 3.5)[67] utilizing the flexible ligands option. Candidate configurations of

the molecules were visualized using the InsightII 2.1 package (Biosym Technologies Inc., San Diego, CA USA) and selected based on active site interactions with the C4 of the nicotinamide ring for the coenzyme as well as with the active site residues Tyr48, His110, and Trp111. Individual structures were assigned correct bonds, hybridization states, and charges prior to minimization. The ternary complex (enzyme, $NADP^+$ and the inhibitor) was fully energy minimized using the Discover 2.7 package (Biosym Technologies, San Diego, CA, USA) on an O2 (R12000) workstation (Silicon Graphics, Mountain View, CA, USA) according to procedures described previously.[68–70] Calculations were performed including a constant valence force field incorporating the simple harmonic function for bond stretching and excluding all nondiagonal terms (cut off distance between 26 and 33 Å) using the algorithms steepest descents and conjugate gradients (down to a maximum atomic root-mean-square derivative of 10.0 kcal/Å and 0.1 kcal/Å, respectively). Individual contributions of the interactions between the residues (Tyr48, His110, and Trp111) or the cofactor $NADP^+$ and the inhibitor were calculated by Discover. Predictions of partition coefficients ($C\log P$) and polar surface areas (PSA) were made by relevant fragment-based methods.[71,72]

Acknowledgments

This work was supported by VEGA Grants No. 2/5005/25, 2/7074/27, VEGA M.S. SR No. 4293/07, APVT Grant No. 20-020802, APVV No. 51-017905, COST-B35 and 6th FP-NoE (Dr. Durisova). We are thankful to Dr. Anna Tsantili-Kakoulidou, Ph.D. for the permission to use the Pallas program.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2008.03.039.

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