Development of Novel Indole-Based Bifunctional Aldose Reductase Inhibitors/Antioxidants as Promising Drugs for the Treatment of Diabetic Complications

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Abstract: Aldose reductase (AR, ALR2), the first enzyme of the polyol pathway, is implicated in the pathophysiology of diabetic complications. Aldose reductase inhibitors (ARIs) thus present a promising therapeutic approach to treat a wide array of diabetic complications. Moreover, a therapeutic potential of ARIs in the treatment of chronic inflammation-related pathologies and several genetic metabolic disorders has been recently indicated. Substituted indoles are an interesting group of compounds with a plethora of biological activities. This article reviews a series of indole-based bifunctional aldose reductase inhibitors/antioxidants (ARIs/AOs) developed during recent years. Experimental results obtained in in vitro, ex vivo, and in vivo models of diabetic complications are presented. Structure–activity relationships with respect to carboxymethyl pharmacophore region somerization and core scaffold modification are discussed along with the criteria of “drug-likeness”. Novel promising structures of putative multifunctional ARIs/AOs are designed.

Keywords: indole; pyridoindole; triazinoindole; aldose reductase; inhibitor; diabetic complications; polyol pathway; antioxidant

1. Introduction

Aldose reductase (AR, ALR2), the first enzyme of the polyol pathway, was initially connected with the onset of diabetic complications (DCs) [1–6]. More recent evidence suggests that AR also plays a key role in inflammatory pathologies and in several genetic metabolic disorders in non-diabetic subjects. In this context, aldose reductase inhibitors (ARIs) have received much attention as a promising therapeutic strategy in targeting the above-mentioned disorders [7–19].

Yet, the etiology of DCs is multifactorial since multiple mechanisms contribute to their development. Complex multifactorial etiology of DCs stems from activation of several metabolic pathways by hyperglycemia. The polyol pathway serves as a major link to other pathways responsible for glucose toxicity including the oxidative stress [20–25]. The multifactorial nature of diabetic complications represents a great challenge in the development of efficient therapy. A multi-target directed approach in prevention of diabetic complications is oriented on the rational design of chemical entities able to affect simultaneously multiple key mechanisms, since targeting just one particular mechanism may
have a limited effect. This approach increases the chance of successful therapeutic intervention, decreases the risk of side effects, and is economical.

Substituted indoles are an interesting group of compounds with a plethora of biological activities. The indole scaffold, found in numerous natural and synthetic substances, is considered a useful structural subunit for drug design and discovery [26–30]. In our pursuit of multifactorial drugs to treat diabetic complications we were inspired by both an efficient antioxidant and free radical scavenging agent, stobadine (STB, 1a) [25,31–35], a drug of hexahydropyridoindole nature, and by the highly efficient ARI lidorestat, derivative of indol-1-yl acetic acid [36]. Accordingly, both tetrahydropyridoindole scaffold evolved from stobadine, and indol-1-yl acetic acid moiety were used in our lab during the last 15 years as starting fragments in developing of several promising series of aldose reductase inhibitors/antioxidants.

This article reviews several groups of indole-based bifunctional aldose reductase inhibitors/antioxidants designed and tested as potential drug candidates for the treatment of diabetic complications (Figure 1).

![Figure 1. The core scaffolds of the indole-based aldose reductase inhibitors/antioxidants.](image-url)

Experimental results obtained at the level of isolated enzymes, and in the models of free radical damage in vitro are presented. The enzyme inhibition data are complemented by molecular docking simulations into the ALR2 binding site followed by results from ex vivo and in vivo models of diabetic complications. Structure–activity relationships with respect to the core indole scaffold modifications are discussed along with the criteria of
“drug-likeness”. The findings thus obtained are extended to predictions of novel promising structures of multifunctional ARIs.

2. Studies at the Level of Isolated Enzymes and Free Radical Models in Vitro and in Silico: SAR

2.1. Inhibition of Aldo-Keto Reductases ALR2 and ALR1

Aldose reductase (AR, ALR2, EC 1.1.1.21) is a member of the aldo-keto reductase (AKR) superfamily, which is composed of monomeric proteins with approximately 315–330 residues, and with a molecular weight of 36 kD. It is the first enzyme of the polyol pathway and converts glucose to sorbitol in the presence of NADPH. The pathway is terminated by the second enzyme, sorbitol dehydrogenase, which converts sorbitol to fructose with NAD as a cofactor. In diabetic patients, the flux of glucose through the polyol pathway results in intracellular accumulation of sorbitol with consequent disruption of tissue osmotic homeostasis. The concurrent depletion of NADPH and the imbalance of the NADH/NAD+ ratio eventually lead to oxidative stress. As such, these metabolic rearrangements have been found implicated in the pathophysiology of diabetic complications [1–6]. Another member of the AKR superfamily is aldehyde reductase (ALR1, EC 1.1.1.2). ALR1 is present in all tissues and is responsible for the reduction of toxic aldehydes. ALR1 and ALR2 share a high degree of amino acid sequence (~65%) and structural homology and their co-inhibition may result in undesired side effects [12]. Thus, to test selectivity of novel ARIs, the closely related ALR1 is routinely employed.

Based on the premise that a bifunctional compound with joint antioxidant/aldehyde reductase inhibitory (AO/ARI) activities could be multifactorially beneficial, the first three series of novel derivatives (series 1 to 3), structurally based on the antioxidant drug stobadine [1a, (4aR,9bS)-2,8-dimethyl-2,3,4,4a,5,9b-hexahydro-1H-pyrido[4,3-b]indole] [31–35] were designed and synthesized.

The parent drug stobadine [1a, Table 1] as an efficient reactive oxygen species (ROS) scavenger was extensively studied in multiple models of diabetic complications with the aim to attenuate the oxidative component of glucose toxicity. Indeed, under conditions of an experimental glycation model in vitro, compound 1a was found to protect bovine serum albumin against glyco-oxidative damage [37–39]. Using a model of streptozotocin-diabetic rats in vivo, compound 1a was reported to attenuate pathological changes in diabetic cardiovascular system [40–44], kidneys [45–50], eye lens [51,52], and retina [53], vas deferens [54], peripheral nerves [55,56], and brain [57], to decrease matrix collagen cross-linking [46,58] and to reduce plasma cholesterol [41] and triglyceride levels [41,42] in diabetic animals. Stobadine (1a) treatment normalized calcium homeostasis in diabetic rat heart and liver [42], produced a beneficial effect on leukocyte function [59], inhibited doxorubicin-induced apoptosis [60], and ameliorated alloxan toxicity in mice [61,62].

The rationale for designing of the novel multi-target directed/bifunctional drugs was based on an idea of endowing the stobadine scaffold bearing the antioxidant activity with a carboxymethyl functional group, a key pharmacophore of aldose reductase inhibitors. Hexahydropyridoindole stobadine (1a) and its tetrahydro congener 2a, used as starting fragments of the drug design, were devoid of any ability to inhibit aldose reductase (Table 1).

Introduction of the carboxymethyl group into the hexahydropyridoindole 1a at the position 8 resulted in compound 1b with a very mild inhibition of ALR2 [1%,100 μM = 13%]. Linking the more lipophilic benzyl substituent into position 2 (compound 1c) did not affect the inhibition efficacy significantly. Yet inhibition activities in micromolar range were recorded for the unsaturated tetrahydropyridoindole congeners 2b–d. Visualization of low energy conformations of compounds 1c and 2c showed almost planar tricyclic moiety of the tetrahydropyridoindole 2c, contrasting with severe space distortion of the lipo-
philic heterocyclic backbone of the hexahydropyridoindole 1c (Figure 2) [63]. The presence of an extended aromatic planar region in the majority of potent ARIs is well documented as a crucial pharmacophoric element [64–66].

Table 1. Inhibition of rat lens ALR2 and rat kidney ALR1.

| Compound | Substituent | ALR2 IC₅₀ (μM)/I (%) | ALR1 IC₅₀ (μM) | SF a | Reference |
|----------|-------------|----------------------|----------------|------|-----------|
| 1a (STB) | -CH₃ | -H | -CH₃ | >100/1 | n.d. | - | [63] |
| 1b | -CH₃ | -H | -CH₂COOH | >100/13 | n.d. | - | [63] |
| 1c | -CH₃Ph | -H | -CH₂COOH | >100/19 | >3.000 | - | [63] |
| 1d | -COOEt | -H | -OCH₃ | >100/1 | n.d. | - | [67] |
| 1e | -CH₃ | -COCH₃ | -CH₃ | >100/1 | n.d. | - | [67] |
| 2a | -CH₃ | -H | -CH₃ | >100/1 | n.d. | - | [63] |
| 2b | -CH₃ | -H | -CH₂COOH | 54/n.d. | 3.081 | 57 | [63] |
| 2c | -CH₃Ph | -H | -CH₂COOH | 18/n.d. | 328 | 18 | [63] |
| 2d | -CH₂CH₂Ph | -H | -CH₂COOH | 16/n.d. | 603 | 38 | [63] |
| 2e | -COOEt | -H | -CH₂COOH | 4/n.d. | n.d. | - | [68] |
| 2f | -CH₂COOH | -H | -H | >100/38 | n.d. | - | [67] |
| 2g | -CH₂COOH | -H | -CH₃ | >100/22 | n.d. | - | [67] |
| 2h | -CH₂COOH | -H | -OCH₃ | >100/14 | n.d. | - | [67] |

a SF means a selectivity factor defined as IC₅₀(ALR1)/IC₅₀(ALR2). The values of SF may differ slightly from those shown in the original publication due to rounding of the IC₅₀ values.

Figure 2. Three-dimensional (3D) structures of the low energy conformations of the hexahydropyridoindole 1c (left) vs. tetrahydropyridoindole 2c (right). Reprinted with permission from [63]. Copyright (2008) Elsevier.
The computed stereoelectronic properties of compounds 1c and 2c along with a docking study into the ALR2-binding site (PDB: 1PWM) suggested an explanation for the higher inhibitory efficacies of the tetrahydropyridoindoles (2) in comparison to the hexahydropyridoindoles (1) shown in Figure 3. Planar conformation of the tricyclic tetrahydropyridoindole moiety of 2c enabled closer contacts between the carboxymethyl pharmacophore and the key interaction partners His110 and NADP+, in comparison with sterically distorted hexahydropyridoindole structure of compound 1c [63]. Cation–π interaction between protonated N2 nitrogen and benzene ring of Phe122 contributed to the stabilization of 2c-ALR2 complex, but also prevented 2c to adopt more advantageous position. Eliminating the basicity center at N2 by introducing an acyl substituent in derivative 2e resulted in significant improvement of the inhibition efficacy [68]. The selectivity of the tetrahydropyridoindoles 2b–2d in relation to ALR1 was characterized by selectivity factors (SFs) in the range of 18 to 57. It is noteworthy that compound 2c retained ALR2 inhibitory activity even for the enzyme isolated from diabetic rats, with an IC₅₀ of 16.6 μM. The selectivity index of compound 2c even slightly increased from 18 to 21 in the enzyme preparations from diabetic rats [69]. Compound 2c was reported [70] to be selective in relation to the enzymes of the glycolytic pathway of glucose elimination.

![Figure 3. Geometry of the active site for 1c (gray) and 2c (mustard yellow) derivatives optimized with the whole enzyme (PDB: 1PWM). Dashed yellow lines denote the hydrogen bonds; green line outlines the hydrophobic interaction of 2c with NADP+. Ionic and cation–π interactions are emphasized with magenta arrows. For transparency, only residues and NADP⁺ of 1c-ALR2 complex are visible, with exception of His110 [63].](image)

Shifting the carboxymethyl pharmacophore from position 8 to position 2, yielded derivatives 2f–h with markedly decreased inhibition efficacy (IC₅₀ >100 μM); therefore, this route of drug designing was not further followed [67].

Transferring the carboxymethyl pharmacophore from position 8 to position 5, yielded derivatives (series 3) with markedly enhanced aldotose reductase inhibition efficacy and selectivity (Table 2) [71]. Mild inhibition characterized by IC₅₀ in micromolar range was recorded for compound 3a with the isopropyl substituent in position 2. This alkylated tertiary nitrogen is characterized by a rather high basicity (pKa ~ 10, MarvinSketch Online 2016/ChemAxon), which ensures its complete protonation at physiological pH. The presence of a positive charge on the tertiary nitrogen, which predisposes these compounds to form double-charged zwitterion species, has apparently a detrimental effect on AR inhibition efficacy. Similarly, only modest AR inhibition was recorded for structurally related zwitterionic 8-carboxymethylated pyridoindoles [63,72].
Table 2. Inhibition of rat lens ALR2 and rat kidney ALR1.

| Compound | Substituent | ALR2 (IC₅₀ (nM)) | ALR1 (IC₅₀ (μM)) | SF * | Reference |
|----------|-------------|------------------|------------------|------|-----------|
| 3a       | -IPr        | 34,250           | >100             | >3   | [71]      |
| 3b       | -F          | 141              | 44               | 312  | [71]      |
| 3c       | -H          | 57               | 8                | 140  | [71]      |
| 3d       | -COCH₃      | 21               | 22               | 1048 | [71]      |
| 3e       | -COOEt      | 13               | 5                | 381  | [71]      |
| 3f       | -OCH₃       | 13               | 10               | 792  | [71]      |
| epalrestat |            | 227             | -                | -    | [71]      |
| valproic acid |        | -               | 56              | -    | [71]      |

* SF means a selectivity factor defined as IC₅₀(ALR1)/IC₅₀(ALR2). The values of SF may differ slightly from those shown in the original publication due to rounding of the IC₅₀ values.

On the other hand, AR inhibition activity of the low basicity derivatives possessing an acyl or ethoxycarbonyl substituent on N2 compounds 3b–f is characterized with IC₅₀ values in low and medium nanomolar range (Table 2). Based on SAR in this set of compounds, the flexible carbamate moiety of compounds 3e and 3f appears to fit better the enzyme-binding site contrary to alkyl– and aryl-acyl–substructures of compounds 3b–d, respectively. In the latter series, the inhibition efficacy decreased with increasing bulkiness of the N-substituent. The replacement of the methoxy group in position 8 of compound 3f by the more polar carboxylic group in compound 3e did not affect the resulting inhibition activity. Compounds 3b–f revealed higher inhibition efficacy in comparison with the clinically used epalrestat. Compound 3b is presently undergoing clinical evaluation under the name of setipiprant for treatment of androgenic alopecia [73]. On balance, these results establish the tetrahydropyrindolines carboxymethylated at position 5 as a prospective scaffold for designing efficient AR inhibitors.

With the aim of analyzing possible interaction modes, human recombinant enzyme AKR1B1 in complex with structurally related lidorestat (PDB: 1z3n) was used for in silico docking followed by optimization of the resulting complexes in a water environment. The trial revealed several common features for the set of the most efficient inhibitors 3c–f. The carboxylate group of these compounds and the one of lidorestat were found to align well. To avoid redundancy, details only for the derivative 3f were reported. As shown in Figure 4A, the carboxylate group is directed to the main residual trio of the “anion binding pocket”, Tyr48, His110, and Trp111.

The methylene group of the carboxymethyl substituent creates hydrophobic interactions with NADP⁺ with the exception of the least efficient inhibitor 3a, the structure of which was, similarly as for 2c, captured in cation–π interaction with Phe122 keeping it too far from NADP⁺. By their overall positions, compound 3f and lidorestat take up approximately the same space in the binding site with several specific differences. The trifluorobenzothiazole part of lidorestat creates strong π–π interactions with Trp111 and Phe122,
leaving no space available for water molecules in the binding pocket. However, compound 3f binds firmly to Leu300, leaving enough space in the cavity for two water molecules and creating H-bond with one of them. Water environment plays an important role in thermodynamic balance of ligand-enzyme interactions [74]. No relevant interactions were observable for the 8-methoxy moiety, which is in agreement with the experimental findings showing no difference between the inhibition efficacies of compounds 3e and 3f. Compound 3f and lidorestat induced different water arrangement near the cavity access. As shown in the fitted molecular surface (Figure 4B), lidorestat induced a narrower access to the cavity, keeping amino acids Trp219 and Leu300 closer in comparison with the complex of 3f. The amino group of Gln49 (colored in green in Figure 4B) is perpendicular to the surface of the protein in the complex with lidorestat (Figure 4B left), while for 3f this group is parallel to the surface (Figure 4B right) [71].

![Figure 4](image_url)

**Figure 4.** (A) Superposition of the binding modes of the compound 3f (magenta) and lidorestat (orange) complexed with human AKR1B1 (PDB code 1z3n) and NADP⁺ (enzyme and NADP⁺ is colored by element in complex with 3f and by yellow in complex with lidorestat). Green lines denote hydrophobic interactions, yellow dashed lines are hydrogen bonds and the red one is for π–π interaction. (B) Fitted molecular surface of the enzyme with superposed lidorestat (left) and 3f (right). The surfaces are colored per atom by element type with the exception of Gln49, Trp219, and Leu300 (green). Reprinted with permission from [71]. Copyright (2017) Elsevier.

To test selectivity, we used the comparison to the closely related aldehyde reductase (ALR1). All compounds 3a–f were found to be less active inhibitors of ALR1 compared to ALR2 (Table 2). The corresponding selectivity factors calculated for the most efficient compounds 3e and 3f were found to be 381 and 792, respectively. Methoxy substituent in position 8 of compound 3f thus ensured higher selectivity than the corresponding carboxyl group in compound 3e. This effect may be obviously assigned to the higher affinity to ALR1 of compound 3e bearing the carboxyl group, since the alterations of the 8-methoxy vs. 8-carboxyl substituents did not affect ALR2 inhibition. In silico study revealed a strong interaction of compound 3f with Phe122, Trp219, Leu300, and Tyr309 from the “specificity
mild pocket”, set of residues in ALR2 which are not conserved in ALRI [71]. The highest selectivity was recorded for compound 3d with the corresponding selectivity factor exceeding 1000.

One of the most efficient inhibitors, compound 3f, was tested for inhibition of human recombinant AKR1B1 and AKR1B10, yielding IC50 values 84 nM and 9434 nM, respectively, pointing to high selectivity relative to AKR1B10. Zopolrestat used as reference gave for AKR1B1 the IC50 value of 25 nM, while for AKR1B10 it was inactive. According to the docking study, selectivity of compound 3f towards AKR1B10 is related to the fact that in Cys298 is replaced by hydroxylated cysteine Cso299, which creates H-bond with the acetoxy group of compound 3f and keeps it too far from NADP+ to form the hydrophobic interaction with nicotinamide ring [71].

In our persistent search for highly efficient and selective ARIs, we were in the next stage of drug design inspired by lidorestat (Figure 5), one of the most efficient inhibitors of aldose reductase with reported IC50 value in low nanomolar region [36]. Chemically, lidorestat is a derivative of indol-1-yl acetic acid. Despite the failure of lidorestat in clinical trials and based on encouraging highly efficient derivatives 3c-f, we still had believed that indol-1-yl acetic acid moiety was a promising starting fragment for drug design. Indeed, in our preliminary study [75], indol-1-yl acetic acid 4a was identified as an ARI with a mild efficacy. We decided to employ this compound as a starting structural moiety (hit) in a fragment based drug design. By applying this approach to the virtual screening of ChemSpider database, three series (4 to 6) of indol-1-yl acetic acid derivatives, as summarized in Tables 3–5, were identified as promising ARIs and subjected to experimental SAR study for AR inhibition [76].

![Figure 5. Highly efficient ARI lidorestat, a derivative of indol-1-yl acetic acid [36].](image)

For comparison, two indol-1-yl propionic acid congeners (compounds 4b and 4i) were included in the experimental sample set. In addition, two structural variants lacking the acetic moiety on indolyl skeleton were incorporated (compounds 5b and 5c).

As shown in Table 3, unsubstituted indol-1-yl acetic acid (4a) inhibited ALR2 in low micromolar range. Insertion of a substituent at position 3 in compounds 4e-j, resulted in reduced inhibitory power towards ALR2. Introduction of additional methyl substituent in position 2, did not affect the inhibition significantly, as shown in the case of compound 4f [76]. On the other hand, combination of –CHO and –COCH3 substituents in position 3 with methyl in position 2 for compounds 4k and 4l, respectively, resulted in marked increase in inhibition efficacy as documented by substantial decrease of IC50 values (23- to 35-fold, respectively) in comparison with unsubstituted indol-1-yl acetic acid (4a) [71]. In compound 4m, the presence of benzyloxy group in position 5 resulted in about 10-fold improvement of inhibition when compared with the unsubstituted indol-1-yl acetic acid (4a), based on the experimental IC50 values [77].
Table 3. Inhibition of rat lens ALR2 and rat kidney ALR1.

![Molecule structure](image)

| Compound | Substituent | ALR2 | ALR1 | SF | Reference |
|----------|-------------|------|------|----|-----------|
| Z1       | R2          | R3   | R5   | IC₅₀(μM)/I (% ,100 μM) | IC₅₀(μM)/I (% ,100 μM) |
| 4a       | -CH₃COOH    | -H   | -H   | 7/n.d. | 80/n.d. | 11 [76] |
| 4b       | -CH₂CH₂COOH | -H   | -H   | 100/54 | >100/12 | >1 [76] |
| 4c       | -H          | -H   | -CH₃COOH | >100/38 | n.d./n.d. | - [75] |
| 4d       | -H          | -H   | -CH₃COOH | >100/11 | n.d./n.d. | - [75] |
| 4e       | -CH₃COOH    | -H   | -H   | 46/n.d. | >100/20 | >2 [76] |
| 4f       | -CH₃COOH    | -CH₃ | -CH₃N(CH₃)₂ | 42/n.d. | >100/22 | >2 [76] |
| 4g       | -CH₃COOH    | -H   | -H   | 35/n.d. | >100/32 | >2 [76] |
| 4h       | -CH₃COOH    | -H   | -CH₃N(Et)₂ | 30/n.d. | >100/38 | >3 [76] |
| 4i       | -CH₃COOH    | -H   | -CH₃N(Et)₂ | n.d./20 | >100/7 | - [76] |
| 4j       | -CH₃COOH    | -H   | -COBu | 14/n.d. | 68/n.d. | 5 [76] |
| 4k       | -CH₃COOH    | -CH₃ | -CHO  | 0.3/n.d. | 12/n.d. | 40 [71] |
| 4l       | -CH₃COOH    | -CH₃ | -COCH₃ | 0.2/n.d. | 11/n.d. | 55 [71] |
| 4m       | -CH₃COOH    | -H   | -H   | 0.7/n.d. | 5/n.d.  | 7 [77] |

*a SF means a selectivity factor defined as IC₅₀(ALR1)/IC₅₀(ALR2). b Rabbit lens ALR2. The values of SF may differ slightly from those shown in the original publication due to rounding of the IC₅₀ values.

Table 4. Inhibition of rat lens ALR2 and rat kidney ALR1.

![Molecule structure](image)

| Compound | Substituent | ALR2 | ALR1 | SF | Reference |
|----------|-------------|------|------|----|-----------|
| Z1       | Z2          | R8   | IC₅₀(nM)/I (% ,100 μM) | IC₅₀(μM)/I (% ,100 μM) |
| 5a (CMTI) | -CH₃COOH    | -H   | -H   | 97/n.d. | 41/n.d. | 422 [76] |
| 5b       | -H          | -H   | -H   | >100/4 | n.d./n.d. | - [76] |
| 5c       | -H          | -CH₃COOH | >100/3 | n.d./n.d. | - [76] |
| 5d       | -CH₃COOH    | -CH₂CONH | -H   | 4060/n.d. | n.d./24 | - [76] |
| 5e       | -CH₃COOH    | -H   | -H   | 2240/n.d. | n.d./1.2 | - [76] |
| 5f       | -CH₃COOH    | -CH(CH₃)₂ | -H   | 2050/n.d. | 14/n.d. | 7 [76] |
| 5g       | -CH₃COOH    | -CH₃N(CH₃)₂CH₃ | -CH₃ | 330/n.d. | 20/n.d. | 61 [76] |

*a SF means a selectivity factor defined as IC₅₀(ALR1)/IC₅₀(ALR2). The values of SF may differ slightly from those shown in the original publication due to rounding of the IC₅₀ values.
Table 5. Inhibition of rat lens ALR2 and rat kidney ALR1.

| Compound | ALR2 IC₅₀ (µM) | ALR1 IC₅₀ (µM) | IC₅₀ (µM)/f (%) | SF | Reference |
|----------|---------------|----------------|----------------|----|-----------|
| 6a       | 12            | 100/49         | 8              |    | [76]      |
| 6b       | 10            | n.d./n.d.      | -              |    | [76]      |
| 6c       | 3             | 5/n.d.         | 2              |    | [76]      |
| 6d       | 3             | 25/n.d.        | 8              |    | [76]      |

*SF means a selectivity factor defined as IC₅₀(ALK)/IC₅₀(ALR2). The values of SF may differ slightly from those shown in the original publication due to rounding of the IC₅₀ values.

Profoundly increased inhibition was recorded for the thixo triazine derivatives 5a and 5g, with IC₅₀ values in submicromolar range (Table 4). Values of IC₅₀ in low micromolar range were recorded for the remaining thioxotriazine derivatives 5d-f [76]. The strongest inhibition of ALR2 was found for 5a (centirestat, CMTI) with IC₅₀ = 97 nM. The structure–activity relationship in the series of 5 and 6 points to the necessity of a concurrent presence of both, the carboxymethyl group in position 5 and the terminal sulfur to achieve best affinity. Shifting of the carboxymethyl group from indole nitrogen to sulfur in compound 5c or its absence in compound 5b resulted in a loss of activity.

High resolution X-ray crystallography of the human recombinant AKR1B1 enzyme complexed with centirestat (5a, PDB: 4QX4) demonstrated a unique pattern of centirestat binding, with the specificity pocket closed, contrary to the interaction of the structurally related lidorestat [76]. As shown in greater detail in Figure 6a, two molecules of the inhibitor 5a were visible in the determined difference electron density, one in the entrance of the binding pocket and the second one as expected inside the binding pocket. The oxygen atom of the carboxylate group of the first inhibitor molecule 5a and N2 of the second one form an H-bond (2.8 Å, Figure 6b). The proton is most likely contributed by the nitrogen of the triazine ring of the second molecule of compound 5a, as we assume that the carboxylate was deprotonated under the applied conditions. The other carboxylate oxygen atom of the first molecule of compound 5a is in H-bond distance to the backbone nitrogen of Ser302 (2.8 Å). Furthermore, the aromatic core skeleton makes a face-to-face π-stacking to Trp219 (3.4 Å, Figure 6c). Figure 6d depicts the superposition of face-to-face π-stacking to Trp219 (3.4 Å, Figure 6c). Figure 6d depicts the superposition of face-to-face π-stacking to Trp219 (3.4 Å, Figure 6c). Molecular dynamics simulations corroborated [78] the above results of the X-ray crystallographic assay.
The other polycyclic derivatives 6a–d revealed moderate inhibition of ALR2 characterized by IC50 values in low micromolar range (Table 5).

All tested compounds of the series 4 to 6 were found to be less active on ALR1 compared to ALR2. It should be noted that the most selective compound 5a, with a selectivity factor above 400, is also the most potent ARI in the series 4 to 6 (Tables 3–5).

Considering excellent “lead-likeness” of centirestat (5a) [76], we proceeded with optimization of its thioxotriazinoidole scaffold by replacing sulfur with oxygen, with the
aim to improve the inhibitory efficacy and selectivity. Based on preliminary molecular modeling and docking calculations, a series of 2-(3-oxo-2H-[1,2,4]triazino[5,6-b]indol-5(3H)-yl)acetic acid derivatives 7a-d was proposed, synthesized and their AR inhibitory efficacy and selectivity determined [79].

The preliminary molecular modeling and docking study on protein conformer from PDB: 4QX4 pointed to preferable fitting of the O-derivative into the inhibitor-binding site of AR. Indeed, the O-variant of centristeat (5a), oxotriazinoindole, compound 7a (OTI), demonstrated significantly increased inhibition efficacy characterized by IC50 values 2-3 times lower compared to compound 5a, depending on the solvent used. SAR evaluation in the series of novel OTI derivatives revealed that the presence of simple substituents at N2 position of compound 7a decreased their ALR2 inhibition efficacy. The most remarkable decrease was recorded for compound 7c with the lipophilic benzyl substituent.

All compounds evaluated were less efficient inhibitors of ALR1 compared to ALR2. With exception of compound 7b, the ALR1 IC50 values of the OTI derivatives (7a, 7c, and 7d) were found to be over 100 μM. For these derivatives, the percentage inhibition I(%) at 100 μM concentration of the inhibitor was determined. Estimates of the particular selectivity factors calculated for the most efficient inhibitors 7a and 7d were found to be >2381 and >1177, respectively. It is striking to observe the enormous increase of the selectivity factor in the couple centristeat (5a) vs. its O isostere 7a (from 422 to >2381). Molecular docking into the binding site of ALR1 offered a feasible explanation: cation–π interaction of protonated Arg312 in ALR1 with the aromatic ring of O-derivative 7a supported by two H-bonds was found to keep this molecule out of reach of NADP+ cofactor in contrast to more tightly attached centristeat 5a (Figure S52 in [79]). In addition, a hydrophobic interaction between the methylene residue of an acetate group of compound 7a and 5a, respectively, and the nicotinamide ring of NADP+ should also be taken into consideration [74]. Based on the IC50 values shown in Table 6, compound 7a inhibits ALR2 more efficiently (IC50 = 42 nM) than the reference inhibitor epalrestat (IC50 = 227 nM, Table 2).

Table 6. Inhibition of rat lens ALR2 and rat kidney ALR1.

| Compound | Substituent | ALR2 IC50 (nM) | ALR1 IC50 (μM) | SF * | Reference |
|----------|-------------|----------------|----------------|------|-----------|
| 7a (OTI) | -H          | 42             | >100/24        | >2381| [79]      |
| 7b       | -CH3COOH    | 120            | 20/n.d.        | 166  | [79]      |
| 7c       | -CH2Ph      | 434            | >100/38        | >230 | [79]      |
| 7d       | -CH3OCH3    | 85             | >100/13        | >1177| [79]      |

*SF means a selectivity factor defined as IC50(ALR1)/IC50(ALR2). The values of SF may differ slightly from those shown in the original publication due to rounding of the IC50 values.

Figure 7 shows superposition of centristeat (5a) and its O-analogue 7a in the inhibitor-binding site of AKR1B1. Polar carboxymethyl groups of both compounds were found to align well. Slight distortion of the fused planar aromatic system was apparently caused by higher rotational flexibility of the carboxymethyl moiety of compound 7a (in cyan) owing to less bulky oxygen compared to the sulfur of the original thioctriazinoindole 5a (in...
red, van der Waals radius of O is 1.4 Å, while for S it is 1.9 Å. As a result, compound 7a was able to create stronger H-bond with Leu300 (contact distance 2.8 Å vs. 3.2 Å) and gave more favorable hydrophobic interaction (2.0 kJ/mol for compound 7a vs. 0.6 kJ/mol for cemtirestat 5a) with NADP⁺ (contact distance 3.6 Å vs. 3.8 Å). Compound 7a interacts with the residues of the specificity pocket Phe122 and Leu300 while letting the specificity pocket closed, which is in line with the previously published crystal structure of AKR1B1 complexed with cemtirestat (5a, PDB: 4QX4). These in silico findings provided an explanation of better fitting of the O-derivative 7a into the binding site of AR when compared with its parent molecule of cemtirestat (5a).

![Superposition of compound 7a and cemtirestat (5a) in the binding site of AKR1B1](image)

**Figure 7.** Superposition of compound 7a and cemtirestat (5a) in the binding site of AKR1B1 (PDB: 4QX4). Cemtirestat (5a) is colored in red and the corresponding amino acid residues in orange. Compound 7a is shown in cyan and the complementary amino acid residues in dark blue. H-bonds are indicated as yellow and hydrophobic interactions as green lines. Reprinted with permission from [79]. Copyright (2020) American Chemical Society.

In the next stage, we focused on the development of novel oxotriazinoindole inhibitors of aldose reductase designed to fit an unoccupied ALR2 pocket shown in PDB: 4QX4 surrounded by the amino acid residues Trp219, Ala299, and Leu301. In order to utilize this pocket for additional ligand–enzyme interactions, novel N-benzyl(oxotriazinoindole) derivatives 8a–d have been designed and developed (Table 7) [80].

Molecules 8a–d were tested for their ability to inhibit the reduction of D,L-glyceraldehyde using ALR2 isolated from the rat eye lenses. Unsubstituted benzyl analogue 7c was used as a reference inhibitor. To assess selectivity, we used a structurally related detoxification enzyme (an antitarget), aldehyde reductase (ALR1), isolated from the rat kidneys. We found out that all substituted N-(benzyl) derivatives 8a–d exhibited from 2- to 6-fold better inhibitory efficacy than unsubstituted analogue 7c. In addition, they also revealed low inhibition of ALR1, which resulted in good (compounds 8b–d) and excellent (compound 8a) enzyme selectivity (Table 7).
Table 7. Inhibition of rat lens ALR2 and rat kidney ALR1.

![Chemical structure](image)

| Compound | Substituent | ALR2 IC₅₀ (nM) | ALR1 IC₅₀ (µM)/I (%) at 100 µM | SF a | Reference |
|----------|-------------|----------------|--------------------------------|-------|-----------|
| 7c       | -H          | 434            | >100/38                        | >230  | [79]      |
| 8a       | -CN         | 76             | 100/51                         | 1316  | [80]      |
| 8b       | -CONH₂      | 236            | 100/50                         | 424   | [80]      |
| 8c       | -COOH       | 139            | 59/62                          | 424   | [80]      |
| 8d       | -CH₂OH      | 244            | >100/33                        | >410  | [80]      |

*SF means a selectivity factor defined as IC₅₀(ALR1)/IC₅₀(ALR2). The values of SF may differ slightly from those shown in the original publication due to rounding of the IC₅₀ values.

The results confirmed the proposed additional interactions of substituted N-(benzyl) derivatives 8a–d within the interactive pocket of ALR2. The best ALR2 inhibition (IC₅₀ = 76 nM) and selectivity relative to ALR1 (SF = 1316) was obtained for compound 8a containing a N-(2-cyanobenzyl) group. Predicted binding position of compound 8a in an active site of ALR2 shows an H-bond of a cyano group with Ala299 from backbone (2.4 Å). Moreover, the aromatic ring of a benzyl moiety forms two π–π interactions with Trp219 (3.9 and 4.6 Å) (Figure 8).

![Predicted binding pose](image)

Figure 8. Predicted binding pose and interaction analysis of 8a in the binding site of an ALR2 (PDB: 4QX4). Reprinted with permission from [80]. Copyright (2021) Elsevier.

In spite of the predicted similar positions and interactions of compounds 8d and 8a, the derivative 8d exhibited about 3-times lower inhibitory activity characterized with IC₅₀ = 244 nM. The additional reason for the lower activity of compound 8d could be the desolvation and the rotation penalty caused by a –CH₂OH group. Desolvation penalty effect is a subject of recent publication [81].

Derivatives 8b,c (–CONH₂, –COOH, respectively) revealed 2- to 3-fold lower inhibitory activity in comparison to compound 8a (–CN). Besides desolvation penalty, the lower
The activity of a carbamoyl derivative 8b could be also caused by conformational penalty of –CONH$_2$ group. The symmetry of the delocalized carboxylate group in 8c (–COO$^-$) exhibited better inhibition activity than –CONH$_2$ in compound 8b and –CH$_2$OH in compound 8d. In addition, the outer part of the studied binding pocket and the benzyl group are well water accessible (Figure 9) and, as a consequence, the benzyl group could provide two conformers.

**Figure 9.** Solvents accessible surface of the unsubstituted benzyl derivative 7c. Blue color indicates water accessible parts. Reprinted with permission from [80]. Copyright (2021) Elsevier.

The first conformer allows the formation of the predicted H-bond with an orientation of a polar group inside the pocket and the second conformer prefers an orientation towards the solvent. Consequently, very polar groups would not be oriented inside the pocket, where the predicted H-bonds could be formed, but owing to solvation, they would remain oriented towards a water environment out of the pocket. Therefore, significant H-bond with Ala299 or Leu301 would not be formed as predicted and thus the least solvated derivative 8a (–CN) revealed the highest inhibitory activity.

Table 8 summarizes the human recombinant enzyme AKR1B1 inhibition data of the most promising ARIs representing the four leading scaffolds. The inhibition efficacies and selectivities of AKR1B1 are compared with those of rat lens ALR2.

**Table 8.** Inhibition of human recombinant enzymes AKR1B1 and AKR1B10 by the most promising ARIs in comparison with the rat ALR2.

| Compound | IC$_{50}$ (nM)/SF | rat-ALR2 | Human-AKR1B1 | Reference |
|----------|--------------------|----------|---------------|-----------|
| 3f       | 13/792 $^a$        |          | 84/112 $^b$     | [71]      |
| 4m       | 700/7 $^a$         |          | 5400/n.d. $^b$ | [77]      |
| 5a (CMTI)| 100/422 $^a$       |          | 57/375 $^b$    | [76]      |
| 7a (OTI) | 42/$>$2 381 $^a$   |          | 66/852 $^b$    | [79]      |

$^a$ SF means a selectivity factor defined as IC$_{50}$(ALR1)/IC$_{50}$(ALR2); $^b$ SF defined as IC$_{50}$(AKR1B10)/IC$_{50}$(AKR1B1).
2.2. Antioxidant Activity

As mentioned above, the hexahydropyridoindole scaffold of stobadine (1a) and its tetrahydro-congener 2a had been used as core structures in designing bifunctional agents combining antioxidant activity with aldose reductase inhibitory action. The antioxidant and free radical scavenging ability of stobadine stems from ability of the indole to form a resonance-stabilized nitrogen-centered radical, which is formed after one-electron removal followed by deprotonation (Figure 10) [82]. It has been well documented that structural alterations in the close proximity of the indole nitrogen, affecting its hydrogen donating ability, are crucial for the free radical scavenging efficiency [83,84].

![Figure 10. One-electron oxidation of protonated stobadine (1a) in a water solution followed by deprotonation of the indole nitrogen of its oxidized form to give a resonance-stabilized, nitrogen-centered radical. Modified according to [82].](image)

Aromatization of the hexahydropyridoindole skeleton of stobadine (1a) into its tetrahydro-congener 2a significantly lowered the antioxidant activity, while acetylation of the indole nitrogen in compound 1e completely abolished the ability to scavenge free radicals [83]. The results are fully in agreement with the notion that the antioxidant activity of stobadine and the related pyridoindoles is mediated via the indole nitrogen center [82,84,85].

As shown in Table 9, in agreement with our previously published results [83,84], stobadine (1a) and its carboxymethylated hexahydropyridoindole derivatives 1b and 1c rapidly reacted with DPPH, their antiradical activities being from 4 to 8 times higher than those of their tetrahydropyridoindole analogues 2a–d in which nitrogen lone pair is involved in the indole aromatic system. In the series of hexahydropyridoindoles 1a–c, the presence of the carboxymethyl group in position 8, decreased the DPPH scavenging ability markedly. On the other hand, analogous carboxymethyl substitution in the tetrahydrocongeners 2a–d did not affect the antioxidant activity significantly. Notably, substitution of the methyl group in position 8 by the more electron-donating methoxy substituent yielding compound 1d, with augmented electron density on the aromatic ring, markedly increased the free radical scavenging activity compared to the parent stobadine (1a). The initial rate of DPPH decolorization by compound 1d was comparable with that of equimolar trolox. The presence of the carbamate moiety in position 2 decreased the basicity of the nitrogen, without affecting the intrinsic chemical reactivity with free radicals [86]. However, it had profound consequences for the bioavailability of the drug as discussed below (Section 2.3). Acetylation of the indole nitrogen (position 5) of the parent stobadine (1a) yielded a completely non-active derivative 1e (Table 9). This finding is in line with the above-mentioned mechanism of free radical scavenging action of the hexahydropyridoindoles according to which the presence of a free hydrogen on the indole nitrogen (position 5) is a prerequisite for the antiradical activity.

Introducing the carboxymethyl pharmacophore into indole nitrogen (position N5), yielded derivatives 3a–f with appreciable aldose reductase inhibition efficacy as shown in Table 2, yet with abolished antioxidant activity as reported in [67,71].
Table 9. Antiradical activities in a DPPH test a.

| Compound | Absorbance Decrease (-ΔA/5 min) | Reference |
|----------|---------------------------------|-----------|
| 1a (STB) | 0.239                           | [63,83,86]|
| 1b       | 0.132                           | [63]      |
| 1c       | 0.187                           | [63]      |
| 1d       | 0.514                           | [86]      |
| 1e       | <0.016                          | [83]      |
| 2a       | 0.033                           | [63,83]   |
| 2b       | 0.031                           | [63]      |
| 2c       | 0.030                           | [63]      |
| 2d       | 0.031                           | [63]      |
| trolox   | 0.503                           | [83,86]   |

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.

Similarly, in the series 4, indol-1-yl acetic acid (4a) was devoid of any antioxidant activity as shown in Table 10. On the other hand, in the case of the indol-3-yl acetic acid derivatives with unsubstituted nitrogen 4c, 4d, the antiradical activity higher than that of the structurally related reference antioxidant melatonin was recorded. The electron donor substituent -OCH₃ in the more efficient derivative 4d facilitates the delocalization of the unpaired electron of the intermediate indolyl radical originating after free radical encounter [75].

In the series 5, the antiradical activity of the most efficient AR inhibitor cemtirestat (5a) markedly exceeded that of equimolar melatonin (Table 10). On placing an isopropyl group on the S atom resulting in compound 5f, thus preventing thione–thiol tautomerism and deprotonation of the –SH group, the antiradical activity of compound 5f was completely lost [87].

Table 10. Antiradical activities in a DPPH test a

| Compound | Absorbance Decrease (-ΔA/30 min) | Reference |
|----------|---------------------------------|-----------|
| 4a       | 0.001                           | [75]      |
| 4c       | 0.069                           | [75]      |
| 4d       | 0.089                           | [75]      |
| 5a (CMTI) | 0.219                        | [87]      |
| 5f       | 0.009                           | [87]      |
| 7a (OTI) | 0.057                           | [79]      |
| 7d       | 0.042                           | [79]      |
| melatonin | 0.022                         | [79]      |

a The ethanolic solution of DPPH radical (50 μM) was incubated in the presence of the compound tested (200 μM). Absorbance decrease at 518 nm during the first 30 min interval was determined.

Taking into account the above findings and spin density calculations, an S-centered charge localization in one electron-oxidized radical of cemtirestat (5a) was suggested as shown in Figure 11 [88]. The total stoichiometry of DPPH scavenging by cemtirestat (5a) was determined. Under the experimental conditions used, one molecule of compound 5a was found to quench about 1.5 DPPH radicals [87].
As shown in Table 10, the oxygen isosteric congeners 7a and 7d of cemtirestat (5a) affected the kinetics of DPPH decolorization only marginally, pointing to their decreased free radical scavenging ability compared to that of sulfur-containing cemtirestat (5a). Yet the free radical scavenging efficacy of compounds 7a and 7d still exceeded that of the equimolar melatonin used as a standard.

Unilamellar 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) liposomes were used as model membranes to study an overall antioxidant activity. Peroxidation of liposomes was induced by the water-soluble radical generator 2,2’-azobis(2-methylpropionamidine) dihydrochloride (AAPH) which simulates an attack by free radicals from the aqueous region. The carboxymethylated tetrahydropyridoindole 2c effectively suppressed oxidation and gave a distinct lag phase in the lipid peroxide accumulation curve (Figure 12). As shown in Table 11, the overall antioxidant efficiency, based on IC50 values, of the carboxymethylated congener 2c, is significantly lower in comparison with the parent compound 2a in spite of the fact that the intrinsic antiradical activity determined in the DPPH test is similar for both compounds (Table 9). The lipophilicity drop caused by the presence of the polar carboxymethyl group in 2c is obviously responsible for the marked decrease in the overall antioxidant efficacy in liposomes. A similar situation was recorded for the hexahydropyridoindole congeners 1a vs. 1c.
(0.8 mM) were incubated in the presence of AAPH (10 mM) in phosphate buffer (pH 7.4, 20 mM) at 50 °C. Reprinted with permission from [63]. Copyright (2008) Elsevier.

Table 11. Inhibition of AAPH-induced peroxidation of DOPC liposomes by compounds 1a,c; 2a,c; and 5a,f in comparison with the standard trolox and melatonin.

| Compound   | IC50 (μM) | Reference |
|------------|-----------|-----------|
| 1a (STB)   | 25.3      | [63]      |
| 1c         | 75.6      | [63]      |
| 2a         | 72.7      | [63]      |
| 2c         | 168.1     | [63]      |
| 5a (CMTI)  | 121.9     | [89]      |
| 5f         | >250      | [68]      |
| trolox     | 86.0      | [68]      |
| melatonin  | 131.1     | [89]      |

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 for 80 min.

In the homogeneous model system of DPPH in ethanol, the antioxidant activity of a compound is determined by its intrinsic chemical reactivity with the radicals. In membranes, however, the apparent reactivity may be different since it is affected by the distribution ratio of the antioxidant between water and lipid compartments [63].

2.3. Physicochemical Properties and Bioavailability

The bioavailability of the majority of drugs depends only on their lipophilicity. The more polar the drug, the less efficiently it can penetrate the lipid membrane. Increasing the lipophilicity improves the absorption. Extremely lipophilic compounds have disadvantage of poor solubility in water and very slow absorption.

Obviously, when considering biological availability of a drug in general, the molecule has not only to be lipophilic enough, as characterized by corresponding distribution coefficient (logP), but also neutral at the actual pH, the property that is affected by acid-base behavior. For acidic and basic drugs, pKa constants markedly affect distribution between water and lipid compartments. In the case of highly lipophilic drugs, it is advantageous if their pKa is not too far away from the pH neutral point. In their ionized form, they are water soluble, while in their neutral form, with which they are in equilibrium, they are lipophilic and membrane penetrable. In the context of the compounds reviewed here, this notion can be illustrated by the two following examples.

2.3.1. Example 1. Hexahydropyridoindole Antioxidants

The substituted hexahydropyridoindoles 1a and 1d (Figure 13) have been postulated as chain-breaking antioxidants [62,82–86,90,91]. The center of the antioxidant activity was identified to reside at the indole nitrogen. Structural alterations in the close proximity of the indole nitrogen, especially aromatic substitution in positions o- and p-, were found to influence the antioxidant efficacy. On the other hand, alteration in the synthetically accessible position N2 provides an opportunity to vary basicity and lipophilicity of the compounds, thus modifying bioavailability, without affecting the intrinsic reactivity with free radicals [83,84].
The most efficient antioxidant of the hexahydropyridoindole series 1d was projected with the aim to increase the free radical scavenging activity compared to the parent stobadine (1a) by substituting the aromatic methyl group with the more electron-donating methoxy group. At the same time, for the sake of bioavailability improvement, the basicity decrease of the molecule was achieved by replacement of the methyl substituent in position N2 by an appropriate acyl substituent, designed so that the lipophilicity of the parent molecule 1a would not change significantly.

Based on the DPPH test, the free radical scavenging activity of compound 1d was more than twice higher than that of equimolar stobadine (1a) and comparable with that of the standard trolox (Table 9). In this homogeneous cell-free system, antioxidant activity reflects the intrinsic chemical reactivity towards radicals. In membranes, however, the relative reactivity of antioxidants may be different since it is determined also by additional factors such as mutual location of the antioxidant and radicals at the membrane, ruled predominantly by their actual distribution ratios between water and lipid compartments.

Based on partition coefficients, compounds 1d and 1a have very similar lipophilicity, characterized by the respective calculated partition coefficients (ClogP) values of 1.95 and 1.79 [86]. At pH 7.4, however, with regard to the variance of basicity of the proton-binding center represented by the piperidine (N2) nitrogen, their actual calculated distribution ratios (ClogD) differ markedly. The indole nitrogen (N5) with very low pKa (for compound 1a pKa = 3), remains unprotonated at pH 7.4. For compound 1a, as a high basicity N-methyl derivative with pKa= 8.5 [86], the acidobasic equilibrium with respect to the piperidine nitrogen (N2) is strongly shifted to its protonation at neutral pH, which is reflected by the low distribution ratio ClogD regardless of the high partition coefficient ClogP. In contrast, in compound 1d, the acyl substituent at N2 lowers the basicity of this site profoundly (pKaq = -3.7). Therefore, the protonation of this nitrogen is negligible at physiological pH, which is reflected by high actual distribution ratio ClogD at pH 7.4, reaching almost the value of the partition coefficient ClogP [86].

Table 12 compares the antioxidant effects of the hexahydropyridoindoles 1a and 1d in the cellular system of isolated rat erythrocytes oxidatively stressed by AAPH- and t-BuOOH-derived peroxyl radicals, respectively. In the first case, when the red blood cells were exposed to the peroxyl radicals generated in the medium outside the cells by thermal decomposition of hydrophilic AAPH initiator, compound 1d was found to be less protective than equimolar stobadine (1a) at 10 μM concentration. On the other hand, when peroxyl radicals were generated inside the erythrocytes, by degradation of the lipophilic t-BuOOH, compound 1d protected the cells more efficiently than equimolar stobadine (1a) at 10 μM concentration. To account for the apparent discrepancy, the variance of basicity of compound 1a vs. compound 1d should be taken into consideration as indicated above.
Table 12. Effects of compound 1a (stobadine) and compound 1d on AAPH- or t-BuOOH-induced hemolysis of isolated rat erythrocytes.

| Compound        | AAPH Lag Time (min) | t-BuOOH Lag Time (min) |
|-----------------|---------------------|------------------------|
| Control         | 80                  | 84                     |
| 1a (STB), 10 μM | 126                 | 157                    |
| 1d, 10 μM       | 90                  | 257                    |

*Erythrocyte suspensions (1.5%) were incubated with 30 mM AAPH or 250 μM t-BuOOH in the absence (Control) or the presence of the indicated compounds [86].

2.3.2. Example 2: ARIs of Zwitterionic Nature

The presence of a basicity center at the tertiary nitrogen in compounds 2b–d, in addition to the acidic carboxylic group, predisposes these compounds to form double charged zwitterionic species. The pH-lipophilicity profile experimentally determined for compounds 2c and 2d in the system of 1-octanol/buffer was characterized by a bell-shaped curve, with a maximal distribution ratio near neutral pH (Figure 14) [63]. The presence of zwitterions was experimentally proved [72].

![Figure 14. pH-distribution profile of the zwitterionic compounds 2c (●) and 2d (○) in 1-octanol/buffer system. D, experimentally determined distribution ratios at a given pH. Reprinted with permission from [63]. Copyright (2008) Elsevier.](image)

The isoelectric pH lying closely to the physiologically relevant pH 7.4 predisposes these compounds for good bioavailability. This behavior is in contrast with that of the acidic ARIs (not possessing a basic group) whose carboxylic acid function is ionized at neutral pH resulting in a sharp drop in distribution ratios and poor biological availability under physiological conditions.

3. Biological Activity in the Rat Models of Oxidative Stress and Diabetic Complications

3.1. Antioxidant Activity of Centirestat (5a) in DOPC Liposomes Oxidatively Stressed by AAPH

A simple membrane model of unilamellar DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine) liposomes was employed to assess the antioxidant action of the aldose reductase inhibitor 5a (cemtirestat) in comparison with that of the standard melatonin and
trolox. The hydrophilic azo-initiator AAPH [2,2’-azobis(2-methylpropionamide) dihydrochloride] was used to generate peroxyl radicals in water phase. Compound 5a efficiently hindered the peroxidation and produced a distinct initial lag phase of about 40 min (Figure 15). Spectrophotometric assay revealed significant consumption of compound 5a during the induction period. After total depletion of compound 5a, lipid peroxidation resumed with the same rate as that in the absence of the drug [89]. Interestingly, compound 5a blocked the peroxidation even when supplemented to pre-peroxidized liposomes in the stage of the advanced peroxidation. This finding can be explained by a direct interference of compound 5a with the chain propagation within the liposomes.

![Figure 15](image)

Figure 15. Time-dependent peroxidation of DOPC liposomes induced by AAPH in the absence (●) and in the presence of compound 5a (250 μM, ○) and 5a degradation curve (○). 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. Reprinted with permission from [89]. Copyright (2020) Elsevier.

Based on the IC₅₀ values obtained, compound 5a inhibited liposome peroxidation slightly more efficiently than melatonin, yet less effectively than trolox (Table 11) [89].

3.2. Antioxidant Activity of Cemtirestat (5a) in the ex Vivo Model of t-BuOOH Induced Hemolysis

Red blood cells have been used as a model to investigate oxidative damage in biomembranes. Exposure of erythrocytes to free radicals may lead to a number of membrane changes resulting eventually in hemolysis. Lipid peroxidation and protein oxidation are likely to play a key role in the hemolytic process. In this model, oxidative damage of plasma membrane was induced by t-BuOOH. Owing to its high lipophilicity, t-BuOOH is rapidly taken up by red blood cells, which is followed by generation of peroxy radical [92]. Plasma membrane attack by reactive oxygen species from the intracellular region is simulated in this model. As shown in Figure 16, rat erythrocytes exposed to 250 μM t-BuOOH underwent progressive hemolysis followed by the release of hemoglobin. The onset of t-BuOOH-induced hemolysis was shifted from the starting zero point by the time...
interval designated as lag period. In the presence of compound 5a, the lag period increased significantly in a concentration-dependent manner. At 1 mM concentration of compound 5a, no hemolysis occurred during the 3-h incubation period [87]. Based on lag phase prolongation, it can be deduced that the erythrocytes were protected by compound 5a against t-BuOOH-induced hemolysis. Considering the fact that the damaging oxygen species are generated from t-BuOOH intracellularly, the above finding indicates that compound 5a is taken up readily by the cells.

![Figure 16. Hemolysis curves induced by t-BuOOH. Erythrocyte suspensions (1.5%) were incubated with 250 μM t-BuOOH in the presence of 100 μM (●) of 5a (cemtirestat). Control incubations (○). Modified according to [87].](image)

### 3.3. Inhibitory Effect of ARIs on Sorbitol Accumulation in Isolated Rat Eye Lenses ex Vivo

Table 13 summarizes the effects of the aldose reductase inhibitors 2c, 3d, 3f, 4m, 5a, and 7a on sorbitol accumulation in the isolated rat eye lenses incubated ex vivo with high glucose in comparison with standard epalrestat. There were recorded concentration-dependent inhibitory effects on the basis of which the IC₅₀ values were extrapolated.

| Compound | IC₅₀ (μM)/I (% @10 μM) | Reference |
|----------|-------------------------|-----------|
| 2c       | ~100/5                  | [93]      |
| 3d       | ~10/52                  | [71]      |
| 3f       | ~10/53                  | [71]      |
| 4m       | ~100/28                 | [77]      |
| 5a (CMTI) | ~100/38                | [76]      |
| 7a (OTI) | ~10/51                  | [79]      |
| epalrestat | >50/12                | [71,77]   |

Glucose, 50 mM; time of incubation, 3 h; 37 °C.

The highest efficacy with estimated IC₅₀ values around 10 μM was shown for the most efficient ARIs 3d, 3f, and 7a. The above findings indicate uptake of the compounds
studied by the eye lens tissue followed by their interference with the polyol pathway via inhibition of the cytosolic ALR2. Interestingly, the organ inhibition efficacy of 2c is comparable with that of the markedly more efficient ARIs 4m and 5a, which likely could be explained by higher bioavailability owing to the zwitterionic nature of compound 2c.

3.4. Inhibitory Effect of ARIs on Sorbitol Accumulation in the Sciatic Nerve of STZ Diabetic Rats In Vivo

In untreated diabetic rats, significant elevation of sorbitol concentration in the sciatic nerve was recorded. The compounds studied were administered intragastrically (50 mg/kg/day) for five consecutive days. The treatment resulted in about 20% inhibition of sorbitol accumulation in the sciatic nerve as summarized for compounds 2c, 3f, and 5a in Table 14.

Table 14. Accumulation of sorbitol in the sciatic nerve of the rats under conditions of STZ-induced experimental diabetes. Effect of the compounds 2c, 3f and 5a (IC50(ALR2): 18,000, 13, 97 nM, respectively) a.

| Sorbitol in the Sciatic Nerve, I (%) b | 2c [93] | 3f [71] | 5a (CMTD) [94] |
|--------------------------------------|-------|-------|-------------|
| Drug treated diabetic rats           | 25    | 19    | 20          |

a The drugs were administered intragastrically for five consecutive days according to the following dosage schedule: 25 mg/kg twice daily (8:30 and 15:30) for the first four days and 25 mg/kg on the fifth day three hours before sacrificing the animals. b Percentage of the inhibition relative to the control untreated rats.

This result points to a ready uptake of the compounds into the central compartment after their intragastrical administration followed by their supply to the peripheral nerves and inhibition of AR-mediated sorbitol accumulation. Yet, the sorbitol levels in the nerves were not normalized to control values. Optimization of the dosage regimen may improve the therapeutic outcome.

4. Conclusions and Future Perspectives

With regard to the multifactorial pathophysiological origin of diabetic complications, a therapeutic approach based on the use of multi-target directed drugs has been forwarded. Based on the premise that a bifunctional compound with joint antioxidant/aldose reductase inhibitory (AO/ARI) activities could be multifactorially beneficial, we were inspired by both an efficient antioxidant stobadine (1a), a drug of hexahydropyridoindole nature, and by the highly efficient ARI lidorestat, derivative of indol-1-yl acetic acid. Stobadine (1a) as an efficient ROS scavenger was extensively studied in multiple models of diabetic complication with the aim to attenuate the oxidative component of glucose toxicity. Lidorestat belongs to a broad class of acidic aldose reductase inhibitors with characteristic carboxymethyl pharmacophore.

At the very beginning of the drug design, we employed the hexahydropyridoindole moiety of stobadine (1a) as a starting scaffold, which was sequentially endowed with the carboxymethyl pharmacophore in three different synthetically accessible positions (2, 5 and 8). SAR study of the novel derivatives in relation to the carboxymethyl pharmacophore regiosomerization and core scaffold modification resulted in developing of several promising series of aldose reductase inhibitors/antioxidants as summarized in Figure 17.
Starting from the efficient hexahydropyridoindole antioxidant stobadine (1a), two series of hexahydro- and tetrahydropyridoindoles carboxymethylated in position 8 were synthesized and characterized as AR inhibitors with negligible (e.g., compound 1b) or mild (e.g., compounds 2b,c) efficacy and selectivity yet with significant antioxidant (AO) effect as an additional biological activity. The hexahydropyridoindole scaffold was excluded from further drug design since the marked antioxidant activity of the hexahydropyridoindoles was combined with only minor AR inhibition (e.g., compound 1b), which was explained by space distortion of the hexahydropyridoindole structure not fitting properly the AR binding pocket. Tetrahydropyridoindole congeners with a planar tricyclic moiety appeared more promising route in designing efficient ARIs. Notably, elimination of the basicity center at N2 position, which prevented zwitterions formation, significantly improved AR inhibition as shown in the case of compound 2e.

Shifting the carboxymethyl pharmacophore from position 8 to position 2, yielded derivatives with markedly decreased inhibition efficacy, as exemplified by compound 2g, therefore this route of drug designing was not followed further.

Structure optimization of the tetrahydropyridoindole scaffold by transferring the carboxymethyl pharmacophore from position 8 to position 5, yielded derivatives with markedly enhanced inhibition efficacy and selectivity, yet with abolished AO activity (e.g., compounds 3a,d,f). In this series, the AR inhibition efficacy markedly increased with decreasing basicity of N2 nitrogen as documented by compound 3f.

Thioxotriazine structural alternatives yielded highly efficient AR inhibitors (e.g., compound 5a) with high selectivity and reasonable AO activity. In further structure optimization efforts, isosteric replacement of sulfur in compound 5a with oxygen provided compound 7a with increased AR inhibition efficacy and markedly improved selectivity, yet with diminished AO activity.

Interestingly, the structural features of the most efficient ARIs resulting from the above optimization procedure, namely compounds 2c, 3f, 5a, and 7a (IC50(ALR2) = 18,000; 13; 97; 42 nM, respectively) still match the strict criteria of the “rule of three”, which points to their excellent “lead-likeness” with prospects of further structure optimizations. Based on the experimental data available, summarized in Table 15, the lead-likeness score is favoring compounds 5a (cemtirestat, CMTI) and 7a (OTI) as promising scaffolds for further structure optimization.
Table 15. Lead likeness of compounds 2c, 3f, 5a and 7a (IC50(ALR2): 18,000; 13; 97 and 42 nM, respectively).

| Property/Compound | 2c | 3f | 5a (CMTI) | 7a (OTI) |
|-------------------|----|----|-----------|----------|
| Rule of 3         |    |    |           |          |
| AR inhibition in vitro |    |    |           |          |
| Inhibition of sorbitol accumulation in the eye lens ex vivo |    |    |           |          |
| Inhibition of sorbitol accumulation in the sciatic nerve in vivo |    |    |           | n.d.     |
| DPPH scavenging   |    |    |           |          |
| Inhibition of AAPH-induced peroxidation of DOPC liposomes |    |    |           |          |

- excellent; - medium; - poor; n.d.–not determined.

Four major routes of possible structural modifications of the leads with the aim to increase inhibition activity, to improve selectivity, bioavailability, and ADME properties, have been envisaged:

Replacement of the carboxymethyl pharmacophore by bioisosteric group of lower acidity, with the aim to improve bioavailability while binding affinity is preserved.

Introduction of additional substituents with a variable H-bonding ability and lipophilicity into N2 position. Additional interactions with the unoccupied interactive pocket of ALR2 are expected to increase the selectivity as suggested by Hlavac et al. [80].

Aromatic substitutions by OH and/or OCH3 groups in variable positions relative to the indole nitrogen, with the aim to obtain bifunctional derivatives combining AR inhibition with antioxidant activity.

Syntheses of both symmetrical and asymmetrical disulfides of variable lipophilicity in the array of 5-carboxymethyl thioxotriazinoidoles. As a result, the disulfides may serve as prodrugs of efficient ARIs with an opportunity of targeted delivery into e.g., cancer cells endowed with high GSH. Therapeutic potential of the disulfide prodrugs in relation to several types of chronic inflammation-related cancer is expected (P5).

Among the novel derivatives, compound 5a was patented (P3). At the present time, it is in the stage of complex preclinical evaluation under the name of centristat [76,78,87–89,94–100]. Apparently, there are several advantages of centristat over the clinically used epalrestat, namely lower molecular weight, better water solubility, higher AR inhibition activity recorded both at the level of isolated enzyme (Tables 2 and 4) and at the organ level of isolated rat eye lenses (Table 13) and additional AO action (Tables 10 and 11, Figures 15 and 16). Recently, antioxidant activity of centristat was corroborated by a study of Valachova et al. [96] reporting ability of the drug to protect hyaluronan against oxidatively induced degradation. In addition, protective effects of centristat in neuron-like PC12 cells and BV2 rodent microglial cells exposed to toxic models of oxidative stress were recently documented [97,98]. Studies in vivo in rat models of diabetes revealed ability of centristat (5a) to attenuate symptoms of peripheral neuropathy with high significance [89,95,100]. Moreover, our very recent study [89] proved that the direct ROS scavenging activity of centristat (5a) is complemented by its ability to restore thiol-disulfide homeostasis by releasing free GSH from the pool of endogenously bound disulfides. In addition, ceasing aldose reductase activity by centristat spares NADPH, which is needed for recycling of GSH by glutathione reductase. Considering the above-mentioned complementary activities (Figure 18), centristat (5a) represents a practical example of a
therapeutic strategy against chronic complications in diabetes based on multiple pharmacological activities. Yet, in-depth studies of the indicated molecular mechanisms and their mutual interactions are still needed.

Figure 18. Multiple pharmacological activities of cemtirestat (5a). Reprinted with permission from [89]. Copyright (2020) Elsevier.

5. Patents

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