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Synthesis and biological activities of aldose reductase inhibitors bearing acyl benzenesulfonamides as carboxylic acid surrogates

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Abstract – We have synthesized alrestatin derivatives **1–11** possessing acyl benzenesulfonamide groups as surrogates for the carboxylic acid moiety of alrestatin. Most of the compounds were inactive as aldose reductase inhibitors compared to alrestatin, however, some of them demonstrated selectivity towards inhibition of rat kidney aldehyde reductase compared to rat lens aldose reductase suggesting that structural differences may exist between the carboxylic acid binding domains of these closely related enzymes. The chemoreactive derivatives **9** and **10** suggested the presence of a nucleophile(s) at the carboxylic acid binding site on aldose reductase. © Elsevier, Paris

aldose reductase inhibitor / diabetic complication / carboxylic acid surrogate / acyl benzenesulfonamide / alrestatin

1. Introduction

Aldose reductase is an NADPH-linked oxidoreductase that catalyzes the reduction of aldehydic substrates. It catalyzes the conversion of glucose to sorbitol which in turn is converted to fructose by sorbitol dehydrogenase. These two enzymes constitute what has been termed the 'polyol pathway'. Because the K_m of aldose reductase for glucose is relatively high [1, 2], it has been hypothesized that significant flux of glucose through the polyol pathway occurs only at abnormally high glucose levels such as occur in diabetes mellitus [3]. Since sorbitol is a polar compound that does not readily diffuse out of cells, it has been hypothesized that accumulation of sorbitol in tissues such as lens, nerve, or retina contributes to the development of chronic complications of diabetes mellitus, such as cataracts, neuropathy, or retinopathy [1, 2]. Recent results from the Diabetes Control and Complications Trials [4] which concluded that very tight control of hyperglycemia is beneficial in delaying the onset and progression of diabetic complications support this hypothesis. Inhibitors of aldose reductase are therefore of value in preventing or treating diabetic complications. Epalrestat (*figure 1*) is currently marketed in Japan for the treatment of diabetic neuropathy [5], and zopolrestat is currently undergoing clinical trials [6].

Various compounds have been synthesized over the past thirty years and studied as aldose reductase inhibitors. In 1965 several simple fatty acids and their derivatives were found to inhibit calf lens aldose reductase [7]. Since then a plethora of compounds of varying structures have been reported to inhibit the enzyme. Structure–activity relationship studies have revealed that all aldose reductase inhibitors to date possess an anionic functionality [8]. The presence of an anionic binding site on the aldose reductase enzyme has been revealed by X-ray crystallographic studies involving the ternary complex between the enzyme, NADPH and zopolrestat [9]. In this complex, the carboxylic acid group of zopolrestat was found to bind at an anionic binding site on the enzyme. Our interest in studying the molecular determinants for binding to the aldose reductase inhibitor site led us to synthesize and study the aldose reductase inhibitory activity of compounds **1–11** (*figure 2*) possessing acyl benzenesulfonamides as carboxylic acid surrogates. Sarges and coworkers [10] used the hydantoin moiety as a carboxylic acid bioisostere to design alrestatin analogue **12** (*figure 1*). This compound and others were found to have aldose reductase inhibitory activity that roughly paralleled that of their parent compounds.

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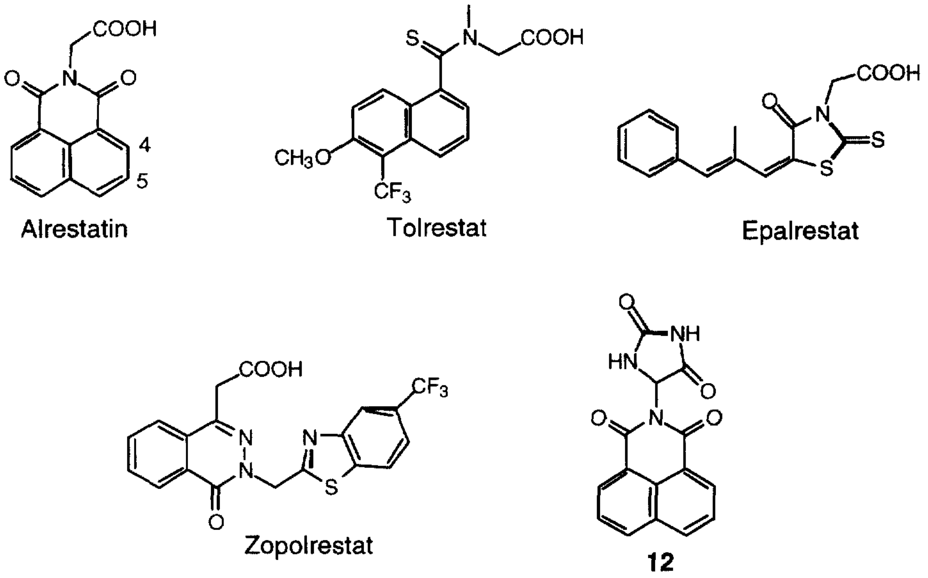


Alrestatin

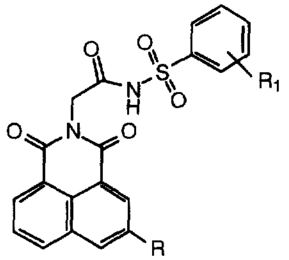
Tolrestat

Epalrestat

Zopolrestat

12

Figure 1.



Compd.	R	R_1
1	H	H
2	NO_2	H
3	H	$4\text{-}NO_2$
4	H	$3\text{-}NO_2$
5	H	$2\text{-}NO_2$
6	H	$3\text{-}NH_2$
7	H	$4\text{-}NH_2$
8	NO_2	$4\text{-}NH_2$
9	H	4-NHCOCH_2Cl
10	H	4-NHCOCH_2I
11	H	$4\text{-NHCOCH}_2\overset{+}{N}(CH_3)_3Cl$

Figure 2.

2. Chemistry

Attempts to synthesize lead compound **1** by reacting the potassium salt of 1,8-napthalimide with chloroacetamidobenzenesulfonamide **15** failed (*figure 3*). The failure of this reaction could be due to the acidity of the sulfonamide which exceeds that of the naphthalimide and results in precipitation. With the exception of compound **6** an alternative route employing a facile procedure reported by Matassa and coworkers [11] for the conversion of carboxylic acids to *N*-sulfonamides using water-soluble carbodiimide in methylene chloride was adopted to synthesize target compounds **1–8**. Slight modifications were made to the Matassa procedure, i.e. the water soluble 1-[3 (dimethylamino)-propyl]-3-ethylcarbodiimide hydrochloride was added to an ice-cooled suspension of the acid [alrestatin, or 5-nitroalrestatin **18**], the appropriately substituted benzenesulfonamide, and *N*,*N*-dimethylamino pyridine in methylene chloride under argon (*figure 4*). When the reactants were mixed at room temperature, or in a different order, the reaction did not proceed as cleanly, and tarry products were obtained. With this procedure the only work-up required was vigorous shaking with 1 N HCl. The products either fell out of solution or remained in the organic layer, while the unreacted materials were removed in the aqueous layer. The synthesis of compounds **7** and **8** involved protection of 4-aminobenzenesulfonamide (sulfanilamide) as the triflate before applying Matassa's procedure. The protecting group was conveniently cleaved

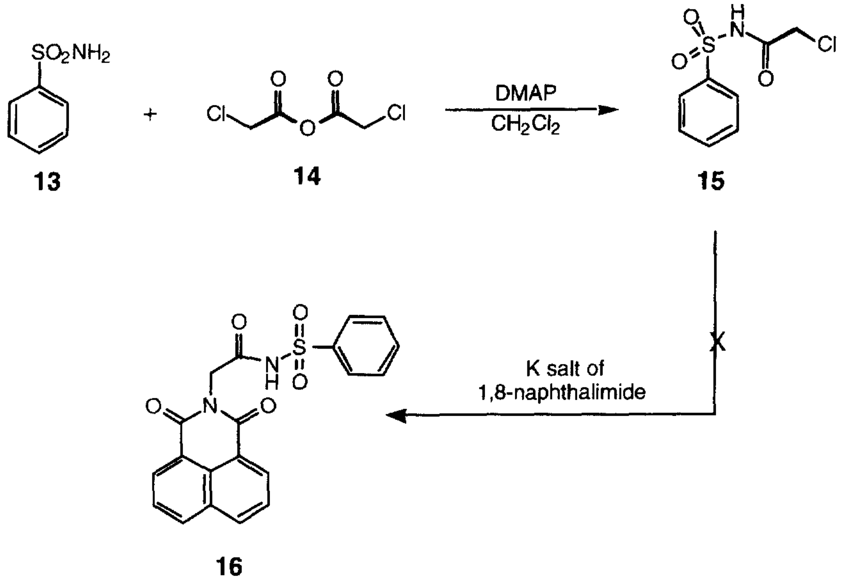


Figure 3.

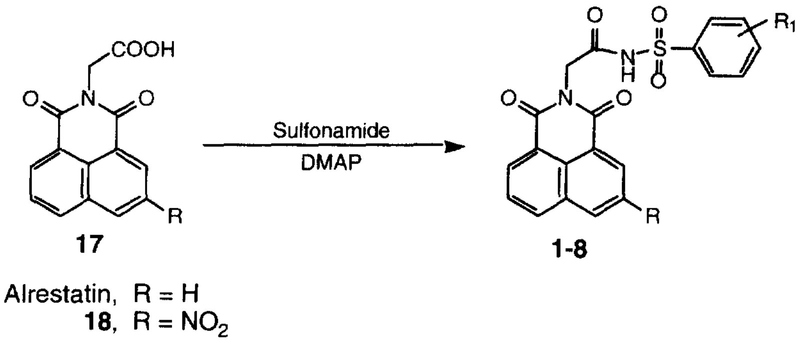


Figure 4.

during the acidic work-up. Synthesis of compound **6** was accomplished via catalytic hydrogenation of compound **4** over 10% palladium on carbon (*figure 5*). The synthesis of affinity labels **9–11** derived from the benzenesulfonamide carboxylic acid surrogates is outlined in *figure 6*. Sulfanilamide **19** was chloroacetylated by treatment with chloroacetic anhydride (**14**) in methylene chloride with pyridine as base to give *N*-4-chloroacetamido benzenesulfonamide **20**. Reaction of **20** with alrestatin in Matassa's conversion procedure [11] afforded compound **9** in good yield. The 4-iodoacetamido benzenesulfonamide analogue **10** was prepared by treatment of **9** with excess potassium iodide in acetone under Finkelstein halide exchange reaction conditions [12]. Trimethylamine displacement of the chloride atom in **9** afforded the quaternary ammonium analogue **11**.

3. Results and discussion

A number of structurally diverse compounds possessing acidic functionalities have been reported to inhibit aldose reductase [8]. Many of these are not totally specific for aldose reductase [13–15]. Kinetic

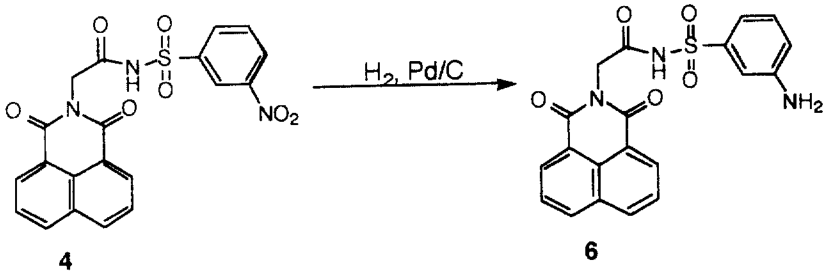


Figure 5.

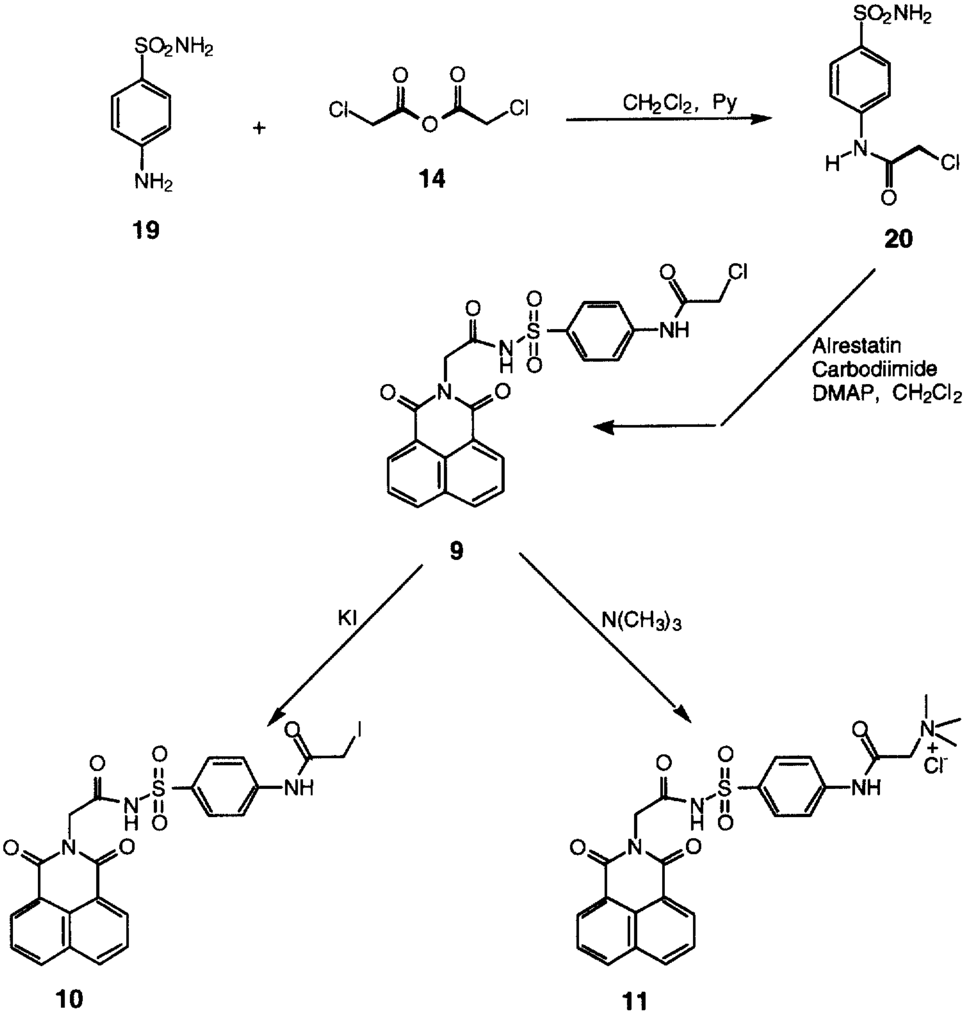


Figure 6.

studies [16] suggest that these inhibitors bind to an inhibitor binding site on the enzyme that is distinct from the substrate and cofactor binding sites. Using a radiolabeled analogue of the irreversible inhibitor, iodoacetamidoalrestatin, Kador and others [17] located an inhibitor binding site on aldose reductase distinct from the substrate binding site. This finding is consistent with kinetic data which indicates that aldose reductase inhibitors demonstrate either noncompetitive or uncompetitive inhibition kinetics [16]. X-ray crystallographic studies however revealed only two binding sites on the enzyme, a substrate site and a cofactor site [9]. Near the substrate site an anionic binding domain that binds citrate, cacodylate, glucose-6-phosphate, and the carboxylate group of zopolrestat has been located [9, 18]. Compounds **1–11** were synthesized as alrestatin analogues possessing acyl benzenesulfonamide groups as carboxylic acid bioisosteres to further explore the inhibitor site of aldose reductase. The acyl benzenesulfonamide moiety is acidic in nature and as such can potentially bind to the anionic site on the enzyme. Using NAP-5 desalting columns, compounds **9** and **10** which possess chemoreactive functionalities were found to only marginally inhibit aldose reductase irreversibly (*table I*). These columns rapidly separate enzymes from reversibly bound or unreacted inhibitors. The compounds were evaluated [19] against cloned rat lens aldose reductase and rat kidney aldehyde reductase. The aldose reductase inhibitory activities of the compounds are summarized in *table I*. Clearly, the acyl bezenesulfonamide moiety is a poor bioisosteric replacement for the carboxylic acid moiety of alrestatin (IC_{50} of alrestatin is 1.6 mM compared to an IC_{50} of 37 mM for **1**). However, substitution of a nitro moiety at the 5-position of **1** gave compound **2** with comparable aldose reductase inhibitory activity as alrestatin. Affinity labels derived from the acyl benzenesulfonamide carboxylic acid surrogates of alrestatin (compounds **9** and **10**) irreversibly inhibited aldose reductase suggesting the presence of a nucleophilic residue(s) at or near the carboxylic acid binding domain of alrestatin. The less reactive chloroacetamide analog **9** was a better irreversible inhibitor than the more reactive iodoacetamide derivative **10**. This suggests that the alkylation process was not a function of the reactivity of the electrophilic group present in the molecule. Alternatively, steric factors could play a role such that **10** is precluded from proper alignment for optimum interaction with the nucleophile on the enzyme. Compound **11**, a permanently charged analog of alrestatin, showed no enhanced inhibitory activity compared to alrestatin. This suggests that the nucleophile(s) at the inhibitor binding site is probably not an anionic amino acid residue such as glutamate or aspartate.

Table I. IC_{50} values and percent irreversible inhibitory activities of acyl benzenesulfonamide carboxylic acid surrogates (**1–11**).

Compound	IC_{50} (mM)	% Irreversible inhibition at 0.1 mM
Alrestatin	1.5 ± 0.79	0.0
1	37 ± 13	3.3
2	2.4 ± 2.1	0.0
3	>100	2.3
4	11 ± 8	0.0
5	>100	0.0
6	3.0 ± 8.4	0.0
7	101 ± 16	0.0
8	5.5 ± 8	7.7
9	51 ± 64	22.3
10	22 ± 47	13.5
11	49 ± 9	0.0

It has been shown that aldose reductase inhibitors are nonspecific and also that they inhibit the closely related enzyme aldehyde reductase (EC 1.1.1.20) [13–15]. Due to the close homology between the primary sequence of the two enzymes, it has been suggested that the inhibitor binding site of the enzymes are structurally similar [20, 21]. Compounds **3–7** were therefore assayed for their ability to inhibit aldehyde reductase to determine structural similarities/differences between the inhibitor binding sites of the two enzymes. We have previously demonstrated that structural differences may exist at the inhibitor binding sites of these enzymes using an iodoacetamide analogue of alrestatin [22]. Results summarized in *table II* indicate that compounds **3**, **5** and **7** are more selective towards inhibition of rat kidney aldehyde reductase than rat lens aldose reductase. This suggests that the carboxylic acid binding domain on rat kidney aldehyde reductase can tolerate bulky substituents better than the corresponding domain on rat lens aldose reductase. Comparison of the inhibitory activities of compounds **3** and **7** suggest that an electron-donating substituent at the *para* position of the aromatic ring of the sulfonamide moiety enhances binding to rat kidney aldehyde reductase and also increases selectivity for this enzyme. The position of the electron-donating substituent appears to play a critical role in determining selectivity of inhibition. Thus, *meta*-amino substitution (as in compound **6**) increases inhibition of both enzymes resulting in decreased selectivity. However, *para*-amino substitution (as in compound **7**) enhances inhibition as well as selectivity versus rat kidney aldehyde reductase. These differences could be exploited in the development of selective inhibitors of aldose reductase.

Table II. Inhibitory activities of selected alrestatin analogs against RLAR[a] and RKALR[b].

Compound	RLAR IC_{50} (mM)	RKALR IC_{50} (mM)	RLAR/RKALR[c]
3	>100	37 ± 9	>2.7
4	11 ± 8	26 ± 17	0.42
5	>100	14 ± 29	>7.1
6	3.0 ± 8.4	5.9 ± 4.7	0.51
7	101 ± 16	8.8	11.5

[a]Rat lens aldose reductase; [b]rat kidney aldehyde reductase; [c]selectivity ratios.

4. Experimental protocols

4.1. Chemistry

Melting points were determined on a Thomas–Hoover capillary melting point apparatus and are uncorrected. Infrared (IR) spectra were recorded on a Perkin Elmer System 2000 FT-IR spectrophotometer. Proton nuclear magnetic resonance (^{1}H-NMR) spectra were recorded on a Bruker AX 300 spectrometer. Chemical shift values are reported in parts per million (δ) relative to tetramethylsilane (TMS) as an internal standard. Spectral data are consistent with assigned structures. Elemental analyses were performed by Atlantic Microlab Inc., Norcross, GA, and experimentally determined values are within ± 0.4% of the theoretical values (*table III*). Routine thin-layer chromatography (TLC) was performed on silica gel GHIF plates (Analtech Inc., Newark, DE). Flash chromatography was performed on silica gel (Merck, grade 60, 230–400 mesh, 60 Å). Acetonitrile (MeCN) was dried by distillation, dimethylformamide (DMF) was dried by distillation from P_2O_5. All solvents (except anhydrous MeCN and DMF) were stored over 3 Å or 4 Å molecular sieves. Analyses indicated by the symbols of the elements were within ± 0.4% of theoretical values.

4.1.1. 1,3-Dioxo-1H-benz[de]isoquinoline-2(3H)-acetic acid (Alrestatin)

A suspension of 1,8-naphthalic anhydride (5.0 g, 25 mmol) and glycine (1.89 g, 25 mmol) in DMF (50 mL) was heated under reflux for 2 h. The reaction mixture was treated with activated charcoal, filtered and poured over crushed ice while hot. The separated heavy off-white product (6.1 g, 95%) was collected by filtration and dried. Purity of the product was sufficient for subsequent reactions; m.p. 268 °C; ^{1}H-NMR (DMSO-d_6: 8.48–8.55 (m, 4H, ArH), 7.86–7.95 (dd, 2H, ArH), 4.73 (s, 2H, NCH_2CO).

Table III. Elemental analyses of compounds **1**–**11**.

Compound	Calculated			Found		
	C	H	N	C	H	N
1	60.90	3.57	7.10	61.19	3.72	7.24
2	54.67	2.98	9.56	54.70	3.21	9.70
3	54.66	2.98	9.57	54.39	3.20	9.60
4	54.66	2.98	9.57	54.87	3.18	9.64
5	53.04	2.89	9.27	53.02	3.06	9.38
6	58.67	3.69	10.26	58.69	3.57	10.27
7	58.67	3.69	10.26	58.68	3.59	10.24
8	52.34	3.18	12.21	52.39	3.16	11.92
9	53.88	3.39	8.57	53.93	3.51	8.50
10	45.77	2.79	7.28	46.17	2.90	7.14
11	55.10	4.62	10.28	55.08	4.88	10.38

*4.1.2. Synthesis of acyl benzenesulfonamide carboxylic acid surrogates **1–5***

Compounds **1–5** were synthesized by coupling an appropriately 5-substituted alrestatin derivative with an appropriately substituted benzenesulfonamide. The synthesis of **1** is described below as an example of the general procedure used for the synthesis of these compounds.

*4.1.3. 1,3-Dioxo-1H-benz[de]isoquinoline-2(3H)-acetyl acyl benzenesulfonamide **1***

1-[3-(Dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (0.4 g, 2 mmol) was added to a stirred ice-cooled suspension of alrestatin (0.5 g, 1.95 mmol), benzenesulfonamide (0.33 g, 2 mmol) and DMAP (0.26 g, 2 mmol) in dry CH_2Cl_2 (200 mL) under argon. The reaction mixture was allowed to warm to room temperature and stirring was continued under argon for 18 h. The mixture was then poured into 1 N HCl (100 mL), the separated aqueous layer was extracted with CH_2Cl_2 (2 x 50 mL), and the combined extracts were washed with water, dried and evaporated. The residue was recrystallized from DMF/H_2O to give 0.65 g (84%) of **1**; m.p. 284–285 °C. ^{1}H-NMR (DMSO-d_6): 8.51–8.42 (m, 4H, ArH), 7.97–7.89 (m, 4H, ArH), 7.75–7.60 (m, 3H, ArH), 4.72 (s, 2H, NCH_2CO). Anal. $C_{20}H_{14}N_2SO_5$ (C, H, N).

*4.1.4. 5-Nitro-1,3-dioxo-1H-benz[de]isoquinoline-2(3H)-acetyl-benzene-sulfonamide **2***

Pure crystals of **2** were obtained following recrystallization from DMF/H_2O mixture in 84% yield; m.p. 296–298 °C. ^{1}H-NMR (DMSO-d_6): 9.51 (d, 1H, ArH), 8.93 (d, 1H, ArH), 8.80 (d, 1H, ArH), 8.60 (d, 1H, ArH), 8.06 (t, 1H, ArH), 7.90 (d, 2H, ArH), 7.72 (m, 1H, ArH), 7.62 (t, 2H, ArH), 4.75 (s, 2H, NCH_2CO). Anal. $C_{20}H_{13}N_3SO_7$ (C, H, N).

*4.1.5. 1,3-Dioxo-1H-benz[de]isoquinoline-2(3H)-acetyl-(4-nitrobenzene)-sulfonamide **3***

MeOH was used to recrystallize **3** in quantitative yield; m.p. 275 °C. ^{1}H-NMR (DMSO-d_6: 8.50–8.41 (m, 6H, ArH), 8.17 (d, 2H, ArH), 7.86 (t, 2H, ArH), 4.76 (s, 2H, NCH_2CO). Anal. $C_{20}H_{13}N_3SO_7$ (C, H, N).

*4.1.6. 1,3-Dioxo-1H-benz[de]isoquinoline-2(3H)-acetyl-(3-nitrobenzene)-sulfonamide **4***

Compound **4** was recrystallized from acetone in quantitative yield; m.p. 275–277 °C. ^{1}H-NMR (DMSO-d_6): 8.72 (d, 1H, ArH), 8.59–8.53 (m, 2H, ArH), 8.49–8.42 (m, 4H, ArH), 8.32 (d, 1H, ArH), 7.94 (t, 1H, ArH), 7.86 (t, 1H, ArH), 4.75 (s, 2H, NCH_2CO). Anal. $C_{20}H_{13}N_3SO_7$ (C, H, N).

*4.1.7. 1,3-Dioxo-1H-benz[de]isoquinoline-2(3H)-acetyl-(2-nitrobenzene)-sulfonamide **5***

Crude **5** was recrystallized from acetone to afford a quantitative yield of pure **5**; m.p. 285 °C. ^{1}H-NMR (DMSO-d_6): 8.48–8.44 (m, 4H, ArH), 8.14 (d, 1H, ArH), 8.06 (d, 1H, ArH), 7.97–7.82 (m, 4H, ArH), 4.80 (s, 2H, NCH_2CO). Anal. $C_{20}H_{13}N_3SO_7 \cdot 0.75H_2O$ (C, H, N).

*4.1.8. 1,3-Dioxo-1H-benz[de]isoquinoline-2(3H)-acetyl-(3-aminobenzene)-sulfonamide **6***

Compound **4** (0.5 g, 1.13 mmol) in acetone was hydrogenated over 10% Pd/C at 50 psi for 4 h. The mixture was filtered, acetone was removed under vacuum, and the yellow residue was recrystallized from DMF/H_2O mixture to give **6** in 64% yield; m.p. 264–265 °C. ^{1}H-NMR (DMSO-d_6): 12.18 (s, 1H, SO_2NHCO), 8.72 (d, 1H, ArH), 8.51–8.45 (m, 4H, ArH), 7.92–7.85 (dd, 2H, ArH), 7.34–7.42 (m, 1H, ArH), 7.19–7.28 (m, 1H, ArH), 7.08–7.12 (m, 1H, ArH), 4.75 (s, 1H, NCH_2CO). Anal. $C_{20}H_{15}N_3SO_5$ (C, H, N).

*4.1.9. 1,3-Dioxo-1H-benz[de]isoquinoline-2(3H)-acetyl-(4-aminobenzene)-sulfonamide **7***

A mixture of sulfanilamide (0.85 g, 4.9 mmol), tritylchloride (1.5 g, 5.4 mmol) and triethylamine (1.0 g, 9.8 mmol) in dry CH_2Cl_2 (20 mL) was stirred overnight at room temperature. The solvent was evaporated under vacuum and the residue was washed with 2% acetic acid and H_2O followed by drying to give the N_4-protected sulfanilamide. The N_4-protected sulfanilamide (1.78 g, 4.3 mmol) was mixed with alrestatin (1.0 g, 3.9 mmol) and DMAP (0.52 g, 4.3 mmol) in dry CH_2Cl_2 (200 mL) and cooled in an ice-bath under argon. 1-[3-(Dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (0.82 g, 4.3 mmol) was added and the mixture was stirred overnight under argon at room temperature. The resulting solution was shaken with 1 N HCl (100 mL) and the separated aqueous layer was extracted with CH_2Cl_2 (2 x 100 mL). The combined organic phases were washed with water and evaporated under vacuum. The resulting residue was washed with MeOH and recrystallized from DMF/H_2O to give **7** (1.3 g, 81%); m.p. 268–270 °C. ^{1}H-NMR (DMSO-d_6): 12.17 (s, 1H, SO_2NHCO), 8.51–8.46 (m, 4H, ArH), 7.88 (t, 2H, ArH), 7.51 (d, 2H, ArH), 6.58 (d, 2H, ArH), 6.18 (s, 2H, NH_2), 4.67 (s, 2H, NCH_2CO). Anal $C_{20}H_{15}N_3SO_5$ (C, H, N).

*4.1.10. 5-Nitro-1,3-dioxo-1H-benz[de]isoquinoline-2(3H)-acetyl-(4-aminobenzene)-sulfonamide **8***

Compound **8** was synthesized as described for **7** using 5-nitro alrestatin **18** in place of alrestatin. The product was washed with Et_2O and MeOH followed by recrystallization from DMF/H_2O to give **8** in 30% yield; m.p. 255–256 °C. ^{1}H-NMR (DMSO-d_6): 12.19 (s, 1H, SO_2NHCO), 9.51 (d, 1H, ArH), 8.94 (d, 1H, ArH), 8.81 (d, 1H, ArH), 8.67 (d, 1H, ArH), 8.06 (t, 1H, ArH), 7.51 (d, 2H, ArH), 6.58 (d, 2H, ArH), 6.18 (s, 2H, NH_2), 4.70 (s, 2H, NCH_2CO). Anal. $C_{20}H_{14}N_4SO_7 \cdot 0.25H_2O$ (C, H, N).

*4.1.11. N4-(Chloroacetyl)sulfanilamide **20***

A solution of chloroacetic anhydride (5.6 g, 32.7 mmol) in dry CH_2Cl_2 (10 mL) was added dropwise to a suspension of sulfanilamide (5.0 g, 29 mmol) and pyridine (5.0 g, 63 mmol) in dry CH_2Cl_2 (100 mL). The mixture was heated under reflux for 4 h. The product that separated out was recovered by filtration, washed with 1 N HCl and recrystallized from MeOH to give 6.7 g (quantitative yield) of **20** as colorless crystals; m.p. 218–220 °C ([23, 24]: m.p. 217 °C).

*4.1.12. 1,3-Dioxo-1H-benz[de]isoquinoline-2(3H)-acetyl-(4-chloroacetamido)-benzenesulfonamide **9***

Compound **9** was synthesized as described for **1** using **20** in place of benzenesulfonamide. Recrystallization from MeOH gave 0.76 g (80%) of **9**; m.p. 276–278 °C. ^{1}H-NMR (DMSO-d_6): 12.59 (broad s, 1H, $CONHSO_2$), 10.75 (s, 1H, NHCO), 8.52–8.42 (m, 4H, ArH), 7.91–7.75 (m, 6H, ArH), 4.71 (s, 2H, NCH_2CO), 4.30 (s, 2H, $ClCH_2CO$). Anal. $C_{22}H_{16}N_3SO_6Cl \cdot 0.25H_2O$ (C, H, N).

*4.1.13. 1,3-Dioxo-1H-benz[de]isoquinoline-2(3H)-acetyl-(4-iodoacetamido)-benzenesulfonamide **10***

A suspension of **9** (0.2 g, 0,4 mmol) and potassium iodide (0.4 g, 2.4 mmol) in acetone (20 mL) was stirred at room temperature for 48 h. The solvent was removed under reduced pressure and the residue was washed with H_2O, dried and recrystallized from DMF/H_2O mixture to give 0.2 g (84%) of **10**, m.p. 258–260 °C. ^{1}H-NMR (DMSO-d_6): 12.59 (broad s, 1H, $CONHSO_2$), 10.75 (s, 1H, NHCO), 8.56–8.45 (m, 4H, ArH), 7.96–7.80 (m, 4H, ArH), 7.77–7.74 (m, 2H, ArH), 4.71 (s, 2H, NCH_2CO), 3.84 (s, 2H, ICH_2CO). Anal. $C_{22}H_{16}N_3SO_6I$ (C, H, N).

4.1.14. 1,3-Dioxo-1H-benz[de]isoquinoline-2(3H)-acetyl-(4-betainylamido)-benzenesulfonamide hydrochloride ***11***

Trimethylamine (0.12 g, 2 mmol) was added to a suspension of **9** (0.24 g, 0.5 mmol) in acetone (20 mL) and the mixture was heated under reflux for 3 h. The separated product was collected by filtration, washed with acetone and dried. The product was dissolved in 1 N NaOH, treated with charcoal, filtrated and reprecipitation with 1 N HCl to give 0.14 g (56%) of **10**; m.p. 278–282 °C. ^{1}H-NMR (DMSO-d_6 + NaOD): 7.88 (d, 1H, ArH), 7.79 (d, 1H, ArH), 7.59–7.46 (m, 4H, ArH), 7.40 (t, 2H, ArH), 7.23 (d, 2H, ArH), 3.88 (s, 4H, NCH_2CON and N^+CH_2CO), 3.17 (s, 9H, $(CH_3)_3N^+$). Anal. $C_{25}H_{25}N_4SO_6Cl$ (C, H, N).

4.2. Biological studies

4.2.1. Enzyme purification

Recombinant rat lens aldose reductase was purified by a series of chromatographic procedures as previously described [19]. Briefly, aldose reductase was released from *E. coli* by sonication and the mixture was centrifuged at 10 000 *g* for 10 min. The supernatant was placed on a Sephadex G-75 column (2.5 x 90 cm) equilibrated with 10 mM imidazole–HCl buffer, pH 7.5 containing 7 mM 2-mercaptoethanol and eluted with the same imidazole buffer. The eluent was collected in 220-drop aliquots (ca. 10 mL) and fractions containing aldose reductase activity were then applied to a Matrex Gel Orange A affinity column (2.5 x 15 cm). The affinity column was washed with the imidazole buffer (ca. 500 mL) and the enzyme was then eluted with the same imidazole buffer containing 0.1 mM NADPH. Fractions eluted with NADPH were chromatofocused on a Mono P (HR 5/20) column developed at a flow rate of 1 mL/min with Polybuffer 74 (diluted 10-fold and containing 7 mM 2-mercaptoethanol). The protein concentration of the eluent was monitored at 280 nm and peaks containing aldose reductase activity were collected and concentrated on Centricon 10 filters.

4.2.2. Enzyme assay

Reductase activity was spectrophotometrically assayed on a Shimadzu UV2100U spectrophotometer by following the decrease in the absorption of NADPH at 340 nm over a period of 4 min with DL-glyceraldehyde as substrate [22]. Each 1.0 mL cuvette contained equal units of enzyme, 0.10 M Na/K phosphate buffer, pH 6.2, 0.3 mM NADPH with/without 10 mM substrate and inhibitor. Appropriate controls were employed to negate potential changes in the absorption of nucleotide and/or protein modification reagents or aldose reductase inhibitors at 340 nm in the absence of substrate.

4.2.3. Affinity binding

Rat lens aldose reductase was passed through NAP-5 desalting columns equilibrated with 0.1 M sodium phosphate buffer, pH 7.6 to remove potentially reactive 2-mercaptoethanol which stabilizes AR [22]. Equal aliquots of enzyme were then combined with either reversible or irreversible inhibitor dissolved in 0.1 M sodium phosphate buffer, pH 7.6 and the reaction was allowed to proceed at room temperature for 15 min. Reversible and unreacted inhibitor was then removed from the protein by gel filtration with a NAP-5 desalting column with 0.1 phosphate buffer, pH 7.0 containing 10 mM 2-mercaptoethanol. Residual aldose reductase activity was spectrophotometrically measured. All experiments were conducted at least in triplicate.

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