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Nitrophenyl Derivatives as Aldose Reductase Inhibitors

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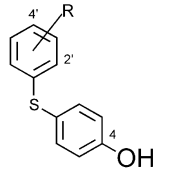
Abstract—Nitrophenyl derivatives were recently discovered as a new class of ALR2 inhibitors by means of docking and database screening of the National Cancer Institute database of organic molecules. The nitro group was predicted to bind to the Tyr48 and His110 active site residues of the enzyme, the site where acidic ALR2 inhibitors such as carboxylic acids bind in their anionic form. Given the novelty of these compounds, we decided to expand their structure–activity relationships by synthesizing and testing a series of derivatives and the corresponding compounds having a carboxylic group instead of the nitro moiety; the results obtained were rationalized by means of docking and molecular dynamics simulations. On the whole there is an agreement between inhibitory data and the results of molecular modeling experiments, supporting the hypothesized binding mode of these compounds.

Introduction

Aldose reductase (ALR2) is an enzyme that catalyzes the reduction of a variety of carbonyl compounds to the corresponding alcohols. It is the first enzyme of the so called 'polyol pathway' and it is able to reduce glucose to sorbitol under hyperglycemic conditions. The activity of this metabolic pathway is believed to be linked to long-term diabetic complications such as cataract, retinopathy, nephropathy and neuropathy. Thus, aldose reductase inhibitors (ARIs) represent a potential therapeutic strategy for preventing the onset or the progression of these complications.[1]

Several studies showed that the inhibitor binding site of ALR2 possesses two subsites, able to accommodate the two main pharmacophoric elements of ARIs. The first, mainly hydrophilic, accommodates acidic functionalities and is constituted by Tyr48, His110 and the oxidized nicotinamide ring of the cofactor, $NADP^+$; the second binds the hydrophobic fragments of ARIs and is mainly constituted by Trp111, Leu300 and Phe122.[2–6]

Recently, simple aromatic nitrocompounds of general formula (**A**) were discovered as a new class of ARIs as a result of a molecular docking and database screening study of the National Cancer Institute database of organic molecules into the crystal structure of ALR2.[7]



R = 2'-NO_2, 4'-NO_2

(A)

Among the compounds with the NO_2 moiety at 2′ or 4′ position, only the NO_2 moiety at 2′-position was able to hydrogen bond efficiently with the Tyr48, His110 and Trp 111 residues of the active site. This conclusion was drawn from the structure of ALR2 complexed with 4-(2′,4′-dinitro-anilino)phenol.[7] In addition, the phenolic ring interacts with the hydrophobic pocket of the enzyme, and the hydroxyl hydrogen bonds to Thr113, contributing favorably to inhibitory activity. The most active compound thus discovered was 4-(2′-nitrophenylmercapto)phenol (R = 2′-NO_2).[7]

In this work we extend the SAR in this class of compounds by firstly synthesizing the 3′-nitro derivative. Then, the nitro group at position 2′, 3′ and 4′ was substituted with carboxylic acid moieties (Table 1). These substitutions were made in order to make a comparison between the inhibitory activities of our new compounds with the activities of a well-known class of ARIs, that is, that of the carboxylic acid-containing ARIs, in which the carboxylate hydrogen bonds to the Tyr48 and His110 residues of the enzyme. The carboxylic acid

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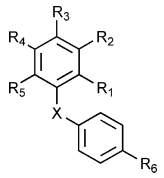
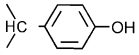
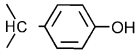
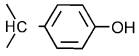
Table 1. Enzyme inhibition data



Compound	R_1	R_2	R_3	R_4	R_5	R_6	X	IC_{50}[a]
1	H	NO_2	H	H	H	OH	S	4.9 (3.9–6.2)
2	H	COOH	H	H	H	OH	S	21 (18–25)
3	H	CH_2COOH	H	H	H	OH	S	14 (11–18)
4	COOH	H	H	H	H	OH	S	78 (63–96)
5	CH_2COOH	H	H	H	H	OH	S	35 (29–43)
6	H	H	COOH	H	H	OH	S	18 (15–21)
7	H	H	CH_2COOH	H	H	OH	S	22 (18–27)
8	NO_2	H	H	H	H	OH		69 (59–81)
9	COOH	H	H	H	H	OH		17% at 64 μM
10	CH_2COOH	H	H	H	H	OH		16 (14–18)
11	NO_2	H	OH	H	H	OH	S	3.5 (2.9–4.2)
12	NO_2	H	$COOC_2H_5$	H	H	OH	S	2.30 (1.95–2.71)
13	NO_2	H	$CONH_2$	H	H	OH	S	2.35 (2.02–2.73)
14	NO_2	H	NH_2	H	H	OH	S	3.48 (2.91–4.16)
15	NO_2	H	COOH	H	H	OH	S	0.99 (0.81–1.21)
16	NO_2	CH_3	H	H	H	OH	S	14 (11–17)
17	NO_2	H	CH_3	H	H	OH	S	2.96 (2.52–3.48)
18	NO_2	H	H	CH_3	H	OH	S	1.57 (1.31–1.88)
19	NO_2	H	H	H	CH_3	OH	S	17% at 80 μM
20	NO_2	H	H	R_4,R_5: 		H	S	0% at 15 μM
21	H	H	NO_2	R_4,R_5: 		H	S	0% at 15 μM
22	NO_2	H	NO_2	R_4,R_5: 		OH	S	0% at 15 μM
23	NO_2	H	R_3,R_4: 		H	OH	S	2.40 (1.86–3.09)
24	NO_2	H	H		H	H	OCH_3	20 (18–23)
Sorbinil								1.68 (1.44–1.96)
Tolrestat								0.057 (0.046–0.070)

[a]IC_{50} values (μM) (95% confidence limits) or % inhibition at a given concentration. The reported concentrations are the highest possible before precipitation of the compounds in the assay solution.

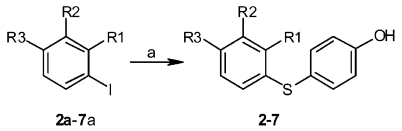
derivatives, being completely ionized at physiological pH, confer to ARIs an in vivo activity which is generally lower than that of less ionized compounds such as spirohydantoins (Sorbinil). While this effect is probably due to an impaired penetration of physiological membranes of ionized compounds,[8] the nitro derivatives here developed are neutral at physiological pH.[7] The same pattern of substitutions, namely, the bioisosteric substitution between the nitro group and the carboxylic moiety, was applied to triphenylmethane derivatives (Table 1). These last compounds were selected because benzylic and diphenilmethane moieties of previously studied compounds were shown to possess similar ALR2 inhibitory activity.[9]

Finally, we decided to introduce substituents of various nature at position 4′ of 4-(2′-nitrophenylmercapto)phenol in order to investigate their effects in a portion of the ligand which was predicted to be mainly exposed to solvent in the ALR2-inhibitor complex.[7] We also introduced a methyl group at positions 3′, 4′, 5′ and 6′ in order to investigate the steric effects of a methyl probe with the active site residues, as well as a benzene ring condensed at positions 5′ and 6′ (Table 1).

Results and Discussion

Chemistry

The (4-hydroxyphenylthio)-benzoic and -phenylacetic acids (compounds **2**–**7**) were prepared by the reaction between the corresponding aryl iodide and 4-hydroxythiophenolate in presence of a catalytic amount of Cu, (Scheme 1), following the general procedure reported.[10]

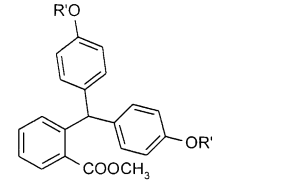


2a-7a **2-7**

a: 4-hydroxythiophenol, KOH, Cu°, Δ

Comp.	R_1	R_2	R_3	Comp.
2a	H	COOH	H	**2**
3a	H	CH_2COOH	H	**3**
4a	COOH	H	H	**4**
5a	CH_2COOH	H	H	**5**
6a	H	H	COOH	**6**
7a	H	H	CH_2COOH	**7**

Scheme 1.



a: **10a** R = $COOCH_3$; R' = H
b: **10b** R = $COOCH_3$; R' = $CH_2C_6H_5$
c: **10c** R = CH_2OH; R' = $CH_2C_6H_5$
d: **10d** R = CH_2Br; R' = $CH_2C_6H_5$
e: **10e** R = CH_2CN; R' = $CH_2C_6H_5$
f: **10f** R = CH_2CONH_2 ; R' = $CH_2C_6H_5$
g: **10g** R = CH_2CONH_2; R' = H
10 R = CH_2COOH; R' = H

a: $C_6H_5CH_2Br$, K_2CO_3; b: $LiAlH_4$; c: PBr_3; d: NaCN; e: NaOH; f: H_2/Pd-C; g: NaOH.

Scheme 2. (a) $C_6H_5CH_2Br$, K_2CO_3; (b) $LiAlH_4$; (c) PBr_3; (d) NaCN; (e) NaCN; (e) NaOH; (f) H_2/Pd-C; (g) NaOH.

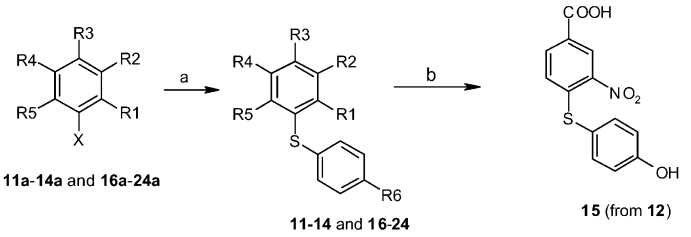
The 2-(4,4′-dihydroxybenzidryl)phenylacetic acid **10** was prepared starting from the methyl ester of phenolphtalin **10a** (Scheme 2). After the protection of the two phenolic groups as benzyl derivative (**10b**), the ester group was reduced with $LiAlH_4$ (**10c**), the alcohol was converted into the bromoderivative by treatment with PBr_3 in CH_2Cl_2 (**10d**), then reaction with NaCN in DMSO at 70 °C afforded the corresponding nitrile **10e**. The subsequent hydrolisis with refluxing NaOH 50% afforded only the corresponding amide **10f**. After deprotection of the two phenolic hydroxy groups by means of H_2/Pd-C, alkaline hydrolysis of the amide afforded **10**.

The 2′-nitro-4′-methoxydiphenylsulphide (**24**), the 4-(nitrosubstituted arylmercapto)-phenols and -naphtalenes (compounds **12**–**14**, **16**–**23**) were obtained by the reaction of the appropriate nitroaryl halide with the corresponding thiophenol in alkaline medium (Scheme 3); likewise, compound **11** was obtained starting from 4-chloronitrophenol **11a** protected as acetylderivative (Scheme 3). Compound **15** was obtained by means of alkaline hydrolysis of compound **12**.

Aldose reductase inhibition

Starting from the lead compound 4-(2′-nitrophenylmercapto)phenol (general formula A, R = 2′-NO_2) with an IC_{50} of 4.8 μM,[7] several new derivatives were synthesized and tested (Table 1). We previously reported that when the nitro substituent is placed at position 4′ instead of 2′, the compound is about six times less active (IC_{50}: 30 μM).[7] This finding was predicted by the remarkably stronger ability of the nitro group at position 2′ to hydrogen bond to Tyr48, His110 and Trp111 residues of the active site.[7] In order to test the effect of a nitro moiety at position 3′, compound **1** was synthesized and found to have an inhibitory activity which is very close to 4-(2′-nitrophenylmercapto)phenol (IC_{50}: 4.9 μM, Table 1). Docking into ALR2 and molecular dynamics refinement of the ALR2-inhibitor complex of 4-(2′-nitrophenylmercapto)phenol and **1** have been performed, and the resulting structures have been compared, in order to explain the activity of **1**. Figure 1 reports the structures of the ALR2 complexes with the lead inhibitor 4-(2′-nitrophenylmercapto)phenol (Fig. 1a) and with compound **1** (Fig. 1b). 4-(2′-nitrophenylmercapto)phenol binds ALR2 by adopting an orientation which is almost identical to that previously described for its congener 4-(2′,4′-dinitroanilino)phenol.[7] The 2′-nitro group hydrogen bonds to Tyr48, His110 and Trp111, and the phenolic ring is inserted into the additional hydrophobic pocket of ALR2 making Van der Waals contacts with Trp111, Leu 300, Phe122 and Cys303. Moreover, the hydroxyl substituent hydrogen bonds to Thr113. The orientation of the 3′-nitro derivative **1** in the active site is such that compound **1** is still able to hydrogen bond to Tyr48, His110, Trp111 and Thr113 (Fig. 1b). The main difference between compound **1** and 4-(2′-nitrophenylmercapto)phenol is in the positioning of the two sulphur atoms with respect to the active site residues, difference which does not prevent the binding of the phenol ring into the hydrophobic pocket. Therefore, structures nicely explain why the 2′- and 3′-nitro derivatives are both apt to bind ALR2, and in a similar way.

The study of compounds **2**–**10** (Table 1) served to investigate the differences in inhibitory activity between nitrophenyl derivatives and the corresponding carboxylic acids (the acetic acid moiety was selected because the most active ARIs possess this group).[11] In the class of diarylsulphides, the nitro derivatives 4-(2′-nitrophenylmercapto)phenol (IC_{50} 4.8 μM[7]) and 4-(3′-nitrophenylmercapto)phenol **1** proved to be more active than the corresponding benzoic and phenylacetic acid derivatives **2**–**5** (Table 1) while for position 4′, the carboxylic acids **6,7** are as active as the nitrophenyl derivative (4-(4′-nitrophenylmercapto)phenol, IC_{50}: 30 μM).[7] Within the triphenylmethanes **8**–**10**, the nitro compound **8** is still more active than the benzoic derivative **9**, but it is four times less active than the acetic acid **10**. Thus the activity exerted by the nitro group, in comparison to the carboxylic acid derivatives, depends



11a-14a and **16a-24a**

11-14 and **16-24**

15 (from **12**)

a: 4-methoxythiophenol (for **24**), thiophenol (for **20,21**) and 4-hydroxythiophenol for **11-14,16-19**, **22**, **23**, KOH (for **20**, **21**, **24**), K_2CO_3 (for **11-14,16-19,22,23**), Δ; b: NaOH

Comp.	X	R_1	R_2	R_3	R_4	R_5	R_6	Comp.
11a	Cl	NO_2	H	OH	H	H	OH	**11**
12a	Cl	NO_2	H	$COOC_2H_5$	H	H	OH	**12**
13a	Cl	NO_2	H	$CONH_2$	H	H	OH	**13**
14a	Cl	NO_2	H	NH_2	H	H	OH	**14**
16a	Cl	NO_2	CH_3	H	H	H	OH	**16**
17a	Br	NO_2	H	CH_3	H	H	OH	**17**
18a	Cl	NO_2	H	H	CH_3	H	OH	**18**
19a	Br	NO_2	H	H	H	CH_3	OH	**19**
20a	Cl	NO_2	H	H	R_4,R_5: 		H	**20**
21a	Cl	H	H	NO_2	R_4,R_5: 		H	**21**
22a	Cl	NO_2	H	NO_2	R_4,R_5: 		OH	**22**
23a	Br	NO_2	H	R_3,R_4: 		H	OH	**23**
24a	Br	NO_2	H	H	H	H	OCH_3	**24**

Scheme 3.

on the nature of the substrate (diarylsulphide or triphenylmethane) and on the position in which this moiety is inserted.

In order to test the hypothesis derived from modeling experiments that position 4′ of diarylsulphides is directed toward the opening of the active site of the enzyme, compounds **11**–**15** were synthesized. As expected, the activity of these compounds is rather close to the activity of 4-(2′-nitrophenylmercapto)phenol (Table 1). Moreover, we introduced a methyl probe at positions 3′, 4′ 5′ and 6′ (compounds **16**–**19**). While **17** and **18** turned out to be active as 4-(2′-nitrophenylmercapto)phenol, compound **16** is 3-times less active and **19** is almost inactive (Table 1). Remarkably, the trend of inhibitory activity is in full agreement with the mode of binding predicted for this class of compounds. The molecular surface of the ALR2–4-(2′-nitrophenylmercapto)phenol active site drawn in Figure 1c unequivocally shows that while positions 3′ and 6′ are sterically hindered and cannot accept substituents, positions 4′ and 5′ are directed toward the opening of the active site, and can therefore be substituted without loss of activity. Based on structures, a methyl at position 3′ would be in steric conflict with the Tyr48 and Val47 side chains, while a methyl at position 6′ would be in steric conflict with the Leu300 and Trp219 side chains (Fig. 1a and c). As a further evidence that position 6′ is in steric conflict with the enzyme, compounds **20**–**22**, which have a bulkier benzene ring condensed at positions 6′ and 5′, are completely inactive (Table 1). On the contrary, compound **23**, having a bulkier pyrazine ring which is condensed at positions 4′ and 5′, is as active as 4-(2′-nitrophenylmercapto)phenol, again in agreement with the proposed binding mode.

Finally, the substitution of the hydroxy group in 4-(2′-nitrophenylmercapto)phenol with a methoxy group (**24**) lead to a less active compound (IC_{50}: 20 μM, Table 1). The structure reported in Figure 1a shows, in fact, that this hydroxyl hydrogen bonds to Thr113, contributing favorably to inhibitory activity.[7]

In conclusion, we found a qualitative agreement between inhibitory data and molecular modeling experiments; the results here obtained will be useful for the synthesis of new ARIs neutral at physiological pH.

Experimental

Chemistry

Melting points were determined on a Buchi 510 capillary melting point apparatus and are uncorrected. Elemental analyses for the tested compounds were within +/−0.4% of the theoretical values. ^{1}H NMR spectra (200 MHz) were recorded on a Bruker AC200 spectrometer; chemical shifts are reported as as δ (ppm) relative to TMS.; DMSO-d_6 was used as solvent unless otherwise noted. TLC on silica gel plates was used to check product purity. Silica gel 60 (Merck, 70–230 mesh) was used for

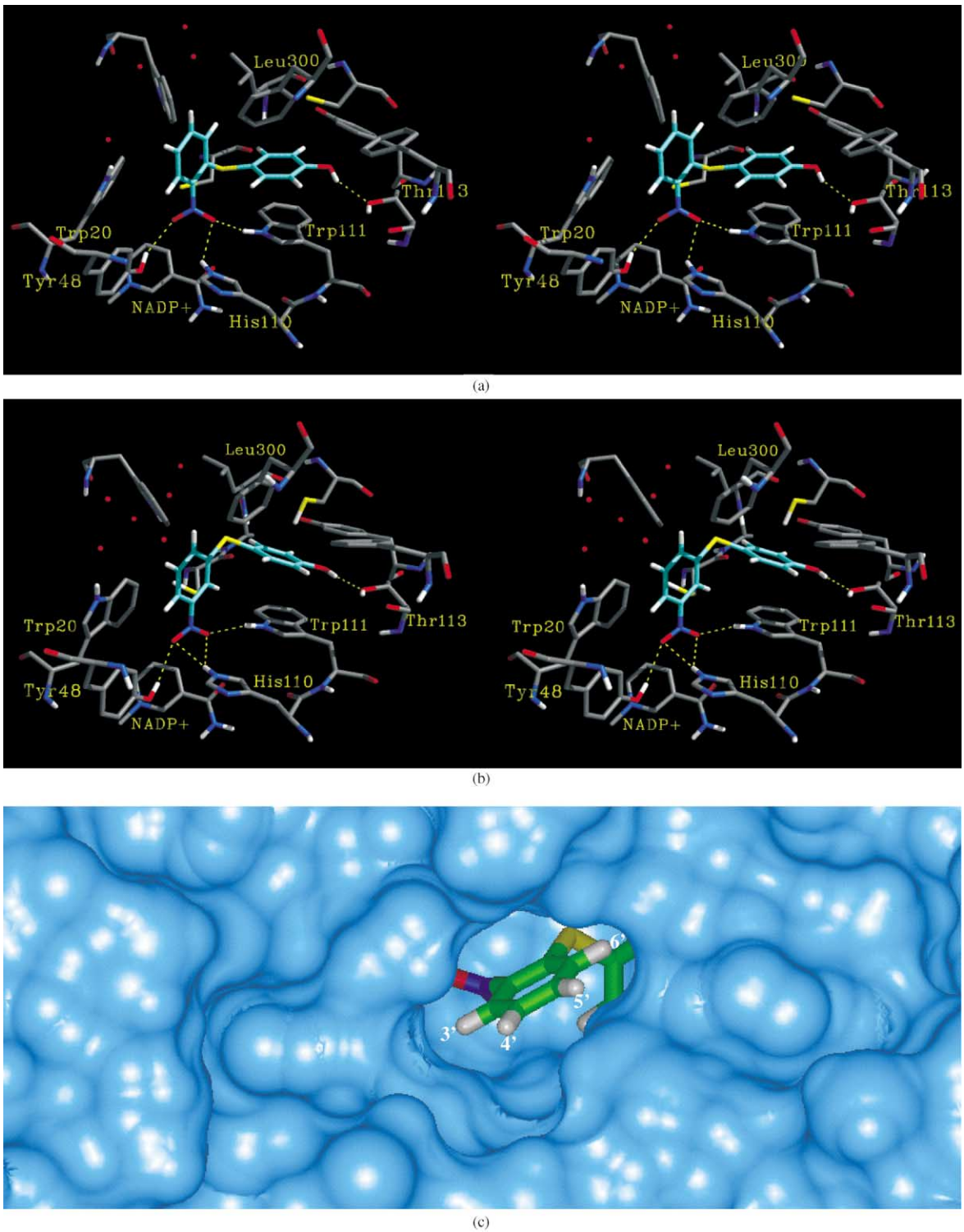


Figure 1. (a) Stereoview of active site residues of ALR2 interacting with the lead inhibitor 4-(2′-nitrophenylmercapto)phenol after refinement with MD. (b) Stereoview of active site residues of ALR2 interacting with the inhibitor 4-(3′-nitrophenylmercapto)phenol **1** after refinement with MD. For clarity, only the polar hydrogens are shown, and hydrogen bonds are shown as dotted lines. Water molecules are shown as dots. (c) Molecular surface of ALR2 with the inhibitor 4-(2′-nitrophenylmercapto)phenol bound at the active site. Positions substituted in the present work are numbered from 3′ to 6′.

column chromatography. Microanalyses were carried out in the Microanalysis Laboratory of the Dipartimento di Scienze Farmaceutiche, Modena University. 4-(3′-nitrophenyl)mercaptophenol **1** was synthesized according to ref 12, 4,4′-(nitrobenzyliden)diphenol (**8**) and 2-(4,4′-dihydroxybenzidryl)benzoic acid **9** according to ref 13 1-Chloro-2-nitronaphtalene **20a** and 1-chloro-4-nitronaphtalene **21a** were synthesized according to ref 14; 2,4-dinitro-1-chloronaphtalene **22a** was synthesized accordingto ref 15; 3-chloro-2-nitrotoluene **16a** and 3-chloro-4-nitrotoluene **18a** were synthesized according to refs 16,17.

(4-Hydroxyphenylthio)-benzoic and-acetic acids (2–7). To a stirred mixture of the appropriate iodobenzoic or iodophenylacetic acid (4.00 mmol) and 4-hydroxythiophenol (4.00 mmol) in water (30 mL), KOH was added (8.00 mmol), and Cu° (0.31 mmol). The reaction mixture was then refluxed for 12 h, cooled, filtered and the filtrate acidified. The precipitate thus formed was collected, washed with water and purified as described.

3-(4′-Hydroxyphenylmercapto)benzoic acid (2). Yield 86%; mp 174–175 °C (EtOAc/petroleum ether); ^{1}H NMR: δ 12.95 (1H, s), 9.90 (1H, s), 7.70 (1H, m), 7.59 (1H, m), 7.38 (4H, m), 6.88 (2H, m). Anal. calcd for $C_{13}H_{10}O_3S$: C 63.40; H, 4.09; found: C, 63.01, H, 4.15.

3-(4′-Hydroxyphenylmercapto)phenylacetic acid (3). Yield 58%; mp 150–152 °C (EtOAc/petroleum ether); ^{1}H NMR: δ 11.00 (2H, broad s), 7.35 (2H, m), 7.20 (1H, m), 7.06 (2H, m), 6.96 (1H, m), 6.84 (2H, m), 3.50 (2H, s). Anal. calcd for $C_{14}H_{12}O_3S$: C 64.60; H, 4.65; found: C, 64.32, H, 4.78.

2-(4′-Hydroxyphenylmercapto)benzoic acid (4). Yield 80%; mp 238–240 °C (acetone/petroleum ether) (193 °C ref. [18]); IR (nujol) (cm^{-1}): 3622, 3356, 2649, 2360, 1673; ^{1}H NMR: δ 13.12 (1H, s), 9.99 (1H, s), 7.96 (1H, d, *J* 7.56), 7.36 (3H, m), 7.13 (1H, t), 6.92 (2H, m), 6.64 (1H, d, *J* 8.00). Anal. calcd for $C_{13}H_{10}O_3S$: C 63.40; H, 4.09; found: C, 63.06, H, 4.15.

2-(4′-Hydroxyphenylmercapto)phenylacetic acid (5). Yield 67%; mp 172–173 °C (acetone/petroleum ether); ^{1}H NMR: δ 12.00 (1H, s), 9.50 (1H, s), 7.23 (5H, m), 7.00 (1H, m), 6.81 (2H, m), 3.78 (2H, s). Anal. calcd for $C_{14}H_{12}O_3S$: C 64.60; H, 4.65; found: C, 64.51, H, 4.68.

4-(4′-Hydroxyphenylmercapto)benzoic acid (6). Yield 75%; mp 192–194 °C (diethyl ether/petroleum ether); IR (cm^{-1}) (Nujol): 3328, 2677, 2565, 1684; ^{1}H NMR: δ 7.05 (2H, m), 6.60 (2H, m), 6.30 (2H, m), 6.12 (2H, m), 4.06 (2H, s). Anal. calcd for $C_{13}H_{10}O_3S$: C 63.40; H, 4.09; found: C, 63.36, H, 4.19.

4-(4′-Hydroxyphenylmercapto)phenylacetic acid (7). Yield 81%; mp 185–187 °C (EtOAc/petroleum ether); ^{1}H NMR: δ 12.25 (1H, s), 9.80 (1H, s), 7.33 (2H, m), 7.18 (2H, m), 7.06 (2H, m), 6.84 (2H, m), 3.60 (2H, m). Anal. calcd for $C_{14}H_{12}O_3S$: C 64.60; H, 4.65; found: C, 64.33, H, 4.78.

4-(2′-Nitrosubstitutedphenylmercapto)phenols (11–14, 15–19 and 22, 23). To a stirred solution of the appropriate aryl halide **11a–14a, 15a–19a** and **22a, 23a** (chloride or bromide) (2.36 mmol) in EtOH (10 mL), a solution of 4-hydroxythiophenol (7.93 mmol) in EtOH (2 mL) was added, followed by K_2CO_3 (7.93 mmol). The reaction mixture was then refluxed for 6 h, (in the case of 2,4-dinitro-1-(4′-hydroxyphenylthio)naphthalene **22** the reaction was performed at 0 °C for 30 min, in the case of 7-nitro-6-(4′-hydroxyphenylmercapto)quinoxaline **23** at 40 °C for 1 h), then cooled. Water was added; the pH was brought to 6.00 with HCl 1 N. Then the reaction mixture was extracted with EtOAc (3 × 25 mL), the organic layer was dried (Na_2SO_4) and the solvent removed under reduced pressure. The residue thus obtained was purified as described.

4-(2′-Nitro-4′-hydroxyphenylmercapto)phenol (11). This compound was prepared as described above, starting from 4-(2′-nitro-4′-acetoxyphenylmercapto)phenol, which in turn was prepared from 4-chloro-3-nitrophenol **14a**, following standard procedures. Purification by means of column chromatography (CH_2Cl_2/CH_3OH 97.5/2.5, then cyclohexane/EtOAc 60/40). Yield 6%; mp 178–181 °C (diethyl ether/petroleum ether); ^{1}H NMR: δ 10.35 (1H, s), 10.00 (1H, s), 7.55 (1H, d, *J* 2.00), 7.38 (2H, m), 7.06 (1H, dd, *J* 2.00, *J* 8.28), 6.90 (2H, m), 6.75 (1H, d, *J* 2.00). Anal. calcd for $C_{12}H_9NO_4S$: C 54.75; H, 3.45; N, 5.32; found: C, 54.96, H, 3.36; N, 5.20.

Ethyl 3-nitro-4-(4′-hydroxyphenylmercapto)benzoate (12). Purification by means of column chromatography (CH_2Cl_2/CH_3OH 95/5). Yield 60%; mp 148–150 °C (diethyl ether/petroleum ether); ^{1}H NMR ($CDCl_3$): δ 8.86 (1H, d, *J* 1.83), 7.95 (1H, dd, *J* 8.60, *J* 1.83), 7.45 (2H, m), 7.05 (2H, m), 6.95 (1H, d, *J* 8.78), 6.11 (1H, broad s), 4.45 (2H, q), 1.43 (3H, t). Anal. calcd for $C_{15}H_{13}NO_5S$: C 56.42; H, 4.10; N, 4.39; found: C, 56.20, H, 4.18; N, 4.48.

4-(4′-Carboxamide-2′-nitrophenylmercapto)phenol (13). Yield 10%; mp 250–252 °C; ^{1}H NMR: δ32;10.16 (1H, s), 8.72 (1H, d, *J* 2.03), 8.15 (1H, s), 7.98 (1H, dd, *J* 2.00, *J* 8.78), 7.55 (1H, s), 7.45 (2H, m), 6.95 (2H, m), 6.85 (1H, d, *J* 8.60). Anal. calcd for $C_{13}H_{10}N_2O_4S$: C 53.79; H, 3.47; N, 9.65; found: C, 53.70, H, 3.55; N, 9.60.

4-(4′-Amino-2′-nitrophenylmercapto)phenol (14). Yield 5%; mp 172–175 °C; ^{1}H NMR: δ 9.88 (1H, broad s), 7.30 (3H, m), 6.85 (2H, m), 6.78 (1H, d, *J* 2.56), 6.68 (1H, d, *J* 8.60), 5.73 (2H, s). Anal. calcd for $C_{12}H_{10}N_2O_3S$: C 54.95; H, 3.84; N, 10.68; found: C, 54.59, H, 3.90; N, 10.88.

3-Nitro-4-(4′-hydroxyphenylmercapto)benzoic acid (15). The ethyl 3-nitro-4-(4′-hydroxyphenyl)benzoate **15** (0.10 g, 0.31 mmol) was treated with NaOH 2 N (3 mL) and EtOH (3 mL) at rt with stirring. After 15 min the solution thus obtained was acidified and the solid thus formed was collected and washed with water. Yield 0.040 g (44%); mp 238–240 °C (acetone/petroleum ether); ^{1}H NMR: δ 13.40 (1H, s), 10.05 (1H, s), 8.66 (1H, d, *J* 1.80), 8.04 (1H, dd, *J* 1.80, *J* 8.49), 7.45 (2H, m), 6.95 (3H, m). Anal. calcd for $C_{13}H_9NO_5S$: C 53.61; H, 3.11; N, 4.81; found: C, 53.36, H, 3.20; N, 4.99.

4-(3′-Methyl-2′-nitrophenylmercapto)phenol (16). Purification by means of column chromatography (CH_2Cl_2/CH_3OH 97.5/2.5 then cyclohexane/EtOAc 75/25). Yield 5%; ^{1}H NMR ($CDCl_3$): δ7.44 (2H, m), 7.20 (1H, t), 7.10 (1H, d), 6.94 (1H, d), 6.88 (2H, m), 5.33 (1H, s), 2.40 (3H, s). Anal. calcd for $C_{13}H_{11}NO_3S$: C 59.76; H, 4.24; N, 5.36; found: C, 59.98, H, 4.21; N, 5.30.

4-(4′-Methyl-2′-nitrophenylmercapto)phenol (17). Purification by means of column chromatography

(CH_2Cl_2:CH_3OH 95/5). Yield 81%; mp 124–1236 °C (128 °C ref [19]); ^1H NMR ($CDCl_3$): δ 8.06 (1H, d, *J* 0.70), 7.45 (2H, m), 7.18 (1H, dd, *J* 0.70, *J* 8.74), 7.00 (2H, m), 6.77 (1H, d, *J* 8.74), 4.95 (1H, broad s), 2.38 (3H, s). Anal. calcd for $C_{13}H_{11}NO_3S$: C 59.76; H, 4.24; N, 5.36; found: C, 59.58, H, 4.36; N, 5.45.

4-(5′-Methyl-2′-nitrophenylmercapto)phenol (18). Purification by means of column chromatography (cyclohexane/EtOAc 80/20). Yield 24%; mp 142–145 °C; ^1H NMR: δ 7.25 (1H, d, *J* 8.20), 6.58 (2H, m), 6.20 (1H, dd, *J* 2.00, *J* 8.20), 6.13 (2H, m), 5.80 (1H, d, *J* 2.00), 4.05 (1H, s), 1.38 (3H, s). Anal. calcd for $C_{13}H_{11}NO_3S$: C 59.76; H, 4.24; N, 5.36; found: C, 59.70, H, 4.26; N, 5.55.

4-(6′-Methyl-2′-nitrophenylmercapto)phenol (19). Purification by means of column chromatography (cyclohexane/EtOAc 80/20). Yield 50%; mp 88–91 °C; ^1H NMR: δ 9.66 (1H, broad s), 7.70 (1H, m), 7.55 (2H, m), 7.02 (2H, m), 6.70 (2H, m), 2.32 (3H, s). Anal. calcd for $C_{13}H_{11}NO_3S$: C 59.76; H, 4.24; N, 5.36; found: C, 59.91, H, 4.20; N, 5.28.

2,4-Dinitro-1-(4′-hydroxyphenylthio)naphthalene (22). Purification by means of column chromatography (cyclohexane/EtOAc 75/25). Yield 78%; mp 148–150 °C (EtOAC/cyclohexane); ^1H NMR: δ 9.78 (1H, s), 8.84 (1H, s), 8.74 (1H, m), 8.39 (1H, m), 7.94 (2H, m), 7.20 (2H, m), 6.71 (2H, m). Anal. calcd for $C_{16}H_{10}N_2O_5S$: C 56.14; H, 2.94; N, 8.18; found: C, 56.10, H, 2.98; N, 8.11.

7-Nitro-6-(4′-hydroxyphenylmercapto)quinoxaline (23). Purification by means of column chromatography (CH_2Cl_2/CH_3OH 95/5). Yield 15%; mp 260–262 °C; ^1H NMR: δ 10.20 (1H, s), 8.95 (3H, m), 7.51 (2H, m), 7.28 (1H, s), 7.02 (2H, m). Anal. calcd for $C_{14}H_9N_3O_3S$: C 56.18; H, 3.03; N, 14.04; found: C, 55.96, H, 3.15; N, 14.20.

Methyl 2-(4,4′-dibenzyloxybenzidril)benzoate (10b). To a solution of methyl 2-(4,4′-dihydroxybenzidril)benzoate (**10a**) (2.83 g, 8.47 mmol) in anhyd acetone (50 mL) benzyl bromide was added (1.38 mL, 16.95 mmol) and K_2CO_3 (2.34 g, 16.93 mmol) at rt and under stirring; the mixture was refluxed for 18 h, then cooled and filtered; the solvent was removed under reduced pressure and the residue was purified by column chromatography (cyclohexane/EtOAc 87.5/12.5). Yield 3.69 g (85%); ^1H NMR ($CDCl_3$): δ 7.82 (1H, m), 7.40 (12H, m), 7.00 (9H, m), 6.55 (1H, s), 5.02 (4H, s), 3.78 (3H, s).

2-(4,4′-Dibenzyloxyibenzidril)benzylalcohol (10c). A solution of methyl 2-(4,4′-dibenzyloxybenzidril)benzoate **10b** (3.69 g,7.18 mmol) in anhyd diethyl ether (30 mL) was added to a suspension of $LiAlH_4$ (0.20 g, 5.38 mmol) in anhyd diethyl ether (70 mL) under stirring. The mixture was stirred at 30 °C for 90′, then ethyl acetate was added, followed by water (30 mL) and H_2SO_4 (10%, 50 mL). The mixture was then extracted with diethyl ether, the organic layer was dried (Na_2SO_4) and the solvent removed under reduced pressure. Yield 3.00 g (86%), oil; ^1H NMR: δ 7.40 (12H, m), 6.92 (10H, s), 5.72 (1H, s), 5.22 (1H, t), 5.06 (4H, s), 4.45 (2H, d).

2-(4,4′-Dibenzyloxybenzidril)benzyl bromide (10d). To a solution of 2-(4,4′-dibenzyloxybenzidril)benzylalcohol **10c** (3.0 g, 6.17 mmol) in anhyd CH_2Cl_2 (20 mL) at 0 °C and under stirring, a solution of PBr_3 in CH_2Cl_2 (1 M, 2.5 mL, 2.5 mmol) was added, then the solution was maintained at rt for 2 h. After, the solution was cooled at 0 °C, ice was added and extracted with CH_2Cl_2 (3×40 mL). The organic layer was dried (Na_2SO_4), the solvent removed under reduced pressure and the residue purified by column chromatography (ciclohexane/EtOAc 90/10). Yield 2.90 g (86%), oil; ^1H NMR: δ 7.80 (12H, m), 7.00 (10H, s), 5.90 (1H, s), 5.05 (4H, s), 4.61 (2H, s).

2-(4,4′-Dibenzyloxybenzidril)benzyl cyanide (10e). To a solution of NaCN (0.39 g, 8.00 mmol) in DMSO (2 mL) at 70 °C a solution of 2-(4,4′-dibenzyloxybenzidril)benzyl bromide **10d** (2.90 g, 5.28 mmol) in DMSO was added and the mixture was stirred for 90 min. Then water was added, the mixture was extracted with diethyl ether (3×50 mL), the organic layer was dried (Na_2SO_4) and the solvent removed under reduced pressure. The residue thus obtained was purified by means of column chromatography. (cyclohexane/EtOAc 90/10). Yield 2.2 g (84%), oil; IR (cm^{-1}): 2248; ^1H NMR: δ 7.40 (12H, m), 7.00 (10H, s), 5.72 (1H, s), 5.05 (4H, s), 3.86 (2H, s).

2-(4,4′-Dibenzyloxybenzidril)phenylacetamide (10f). A suspension of 2-(4,4′-dibenzyloxybenzidril)benzyl cyanide **10e** (1.0 g, 1.78 mmol) in NaOH 50% (5.0 mL, 37.50 mmol) was refluxed for 12 h, then cooled. The aqueous layer was discarted, and the organic layer was extracted with CH_2Cl_2 (3×50 mL), the organic layer was dried (Na_2SO_4) and the solvent removed under reduced pressure. The residue thus obtained was purified by column chromatography (CH_2Cl_2/CH_3OH 87.5/12.5). Yield 0.60g (58%); IR (Nujol) (cm^{-1}): 1665.

2-(4,4′-Dihydroxybenzidril)phenylacetamide (10g). A solution of 2-(4,4′-dibenzyloxybenzidryl)phenylacetamide **10f** (0.60 g, 1.17 mmol) in methanol (30 mL) was hydrogenated at 3 atm for 3 h in presence of Pd/C 10% (50 mg). At the end of the reaction, the mixture was filtered and the solvent was removed under reduced pressure. Yield 0.30 g (77%); ^1H NMR: δ 9.30 (2H, s), 7.40 (1H, s), 7.10 (3H, m), 6.90 (1H, s), 6.82 (5H, m), 6.65 (4H, m), 5.72 (1H, s), 3.22 (2H, s).

2-(4,4′-Dihydroxybenzidryl)phenylacetic acid (10). A suspension of 2-(4,4′-dihydroxybenzidryl)phenylacetamide **10g** (0.30 g, 0.90 mmol) in NaOH 30% was refluxed for 4 h; afer cooling, the solution was acidified (HCl 5 N) and the precipitate thus formed was collected and washed with water. Yield 0.20 g (67%); mp 180–181 °C; IR (Nujol) (cm^{-1}): 1689; ^1H NMR: δ 12.15 (1H, s), 9.05 (2H, s), 7.15 (3H, m), 6.78 (9H, m), 5.56 (1H, s), 3.48 (2H, s). Anal. calcd for $C_{21}H_{18}O_4$: C, 75.43; H, 5.43; found: C, 75.63, H, 5.40.

2-(20) and 4-Nitro-1-phenylthionaphtalene (21). 2-nitro-4′-methoxydiphenylsulphide (24). A solution of thiophenol (for **20** and **21**) or 4-methoxythiophenol (for **24**) (5.63

mmol) in EtOH (2 mL) was added to a solution of the appropriate aryl halide (5.00 mmol) in EtOH (10 mL), then pulverized KOH (5.63 mmol) was added. The solution was warmed at 70 °C for 4 h, cooled and filtered. Then water was added to the filtrate and the mixture was extracted with CH_2Cl_2 (2 × 20 mL); the organic layer was washed with KOH 2 N, dried (Na_2SO_4) and the solvent removed under reduced pressure. The residue was then purified by means of column chromatography (cyclohexane/EtoAc 87.5/12.5).

2-Nitro-1-phenylthionaphtalene (20). Yield 39%; mp 85–88 °C; ^{1}H NMR ($CDCl_3$): δ 8.58 (1H, m), 8.02 (2H, m), 7.66 (3H, m), 7.17 (5H, m). Anal. calcd for $C_{16}H_{11}NO_2S$: C 68.31; H, 3.94; N, 4.98; found: C, 68.00, H, 3.98; N, 5.00.

4-Nitro-1-phenylthionaphtalene (21). Yield 29%; mp 100–103 °C; ^{1}H NMR ($CDCl_3$): δ 8.69 (1H, m), 8.46 (1H, m), 8.09 (1H, d, *J* 8.26), 7.74 (2H, m), 7.53 (5H, m), 7.10 (1H, d, *J* 8.26). Anal. calcd for $C_{16}H_{11}NO_2S$: C 68.31; H, 3.94; N, 4.98; found: C, 68.05, H, 3.81; N, 4.90.

2-Nitro-4′-methoxydiphenylsulphide (24). Yield 78%; mp 95–96 °C; ^{1}H NMR ($CDCl_3$): δ 8.24 (1H, m), 7.54 (2H, m), 7.35 (1H, m), 7.21 (1H, m), 7.04 (2H, m), 6.87 (1H, m), 3.95 (3H, s). Anal. calcd for $C_{13}H_{11}NO_3S$: C 59.76; H, 4.24; N, 5.36; found: C, 59.81, H, 4.22; N, 5.33.

Computational procedure

The aldose reductase structure used for molecular modelling analysis is the one previously obtained after docking and molecular dynamics simulations of a complex between ALR2 and the lead compound 4-(2′,4′-dinitro-anilino)phenol previously described.[7] The structures of the ALR2 complexes with 4-(2′-nitrophenylmercapto)phenol and 4-(3′-nitrophenylmercapto)phenol bound at the active site were firstly energy-minimized with the AMBER 5[20] program using the Cornell[21] et al. force field, and then equilibrated with molecular dynamics. To calculate the partial atomic charges of inhibitors, the geometries of the molecules were completely optimized using the AM1Hamiltonian, and charges were calculated with electrostatic potential fits to a 6-31G* ab-initio wave function using Gaussian98, followed by a standard RESP[22,23] fit. Van der Waals parameters of the inhibitors were assigned to be consistent with the Cornell force field. Parameters for $NADP^+$ were taken from our previous simulations.[7,24,25] Dihedral parameters consistent with the Cornell et al. parameterization were assigned and, in some cases, derived from a conformational analysis performed with AM1. For each molecule, energy minimization was performed to make sure that the optimized conformation was in agreement with the AM1-optimized conformation.

The ALR2-inhibitor complexes were solvated with spherical caps of TIP3P[26] water molecules within 24Å of the inhibitors, resulting in 460 water molecules. Harmonic radial forces (1.5 kcal/mol Å^2) were applied to avoid evaporation. Prior to energy-minimization of the complexes, only the water molecules were energy-minimized and then subjected to 20ps of molecular dynamics at 300 K, in order to let the solvent equilibrate around the enzyme-inhibitor structure. Then, 5000 steps of conjugate gradient minimization were performed, with all protein residues within 10 Å from the inhibitor and all the water molecules allowed to move during minimization. A 10 Å non-bonded cutoff was adopted in the simulations. Molecular dynamics of the ALR2-inhibitor complexes was then performed at 300 K for over 400 ps. The complexes were gradually heated to 300 K during the first 20 ps, in order to avoid abrupt changes of structure. SHAKE[27] was turned on during molecular dynamics, and a time-step of 2.0 fs was used. Coordinates were collected and averaged every 10 ps, using CARNAL. The last 10ps were re-optimized with 5000 steps of conjugate gradient minimization. Structures were visualized with the computer graphics software MidasPlus.[28] Calculations were performed on a IBM-SP3 computer, and graphic display was performed on SGI O2 workstations. Other details can be found in ref 7.

Enzyme section

Sorbinil was a gift from Pfizer; Tolrestat was synthesized according to a published procedure.[29] IC_{50} value for Sorbinil was determined several times ($n = 12$): IC_{50} (+/−S.D.): 1.45 μM (+/−0.33). Calf lenses for the purification of ALR2 were obtained from freshly-slaughtered animals and kept to −20 °C until needed. The capsule was incised and the frozen lenses were suspended in sodium phosphate buffer pH 7.00 containing 5 mM DTT (1 g tissue/3.5 mL) and stirred in an ice-cold bath for 1 h. The suspension was then centrifuged at 22,000 g at 4 °C for 40 min and the supernatant was subjected to ion exchange chromatography (DE52).[9] Enzyme activity was measured by monitoring the change in absorbance at 340 nm which accompanies the oxidation of NADPH catalyzed by ALR2. The assay was performed at 37 °C as previously described,[9] using 4.7 mM D,L-glyceraldehyde as substrate in 0.25 M sodium phosphate buffer pH 6.80, containing 0.38 M ammonium sulphate and 0.11 mM NADPH. The sensitivity of ALR2 inhibition by different ARIs was tested in the above assay conditions by adding a DMSO solution of the inhibitors in the reaction mixture. Mother solutions of inhibitors were prepared in DMSO at such concentration that, after the addition into the assay mixture, DMSO is at constant concentration of 1%. A reference blank containing all the above reagents except the substrate was used to correct for the non-enzymatic oxidation of NADPH. IC_{50} values (the concentration of the inhibitor required to produce 50% inhibition of the enzyme catalyzed reaction) were determined from least squares analyses of the linear portion of the log dose-inhibition curves. Each curve was generated using at least three concentrations of inhibitor causing an inhibition between 20 and 80% with two replicates at each concentration. The 95% confidence limits (95% C.L.) were calculated from *T* values for $n-2$, where n is the total number of determinations.[30]

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