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IDENTIFICATION OF A NEW CLASS OF ET_A SELECTIVE ENDOTHELIN ANTAGONISTS BY PHARMACOPHORE DIRECTED SCREENING

Ming Fai Chan[a]*, Ilya Okun[b], Fiona Lytton Stavros[b], Emily Hwang[b], Manfred E. Wolff[d] and V.N. Balaji[c]*

Departments of [a]Medicinal Chemistry, [b]Biochemical Pharmacology and [c]Computer Assisted Drug Design
[d]ImmunoPharmaceutics, Inc.,
11011 Via Frontera, San Diego, CA 92127

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SUMMARY: Novel endothelin antagonists were identified through a "pharmacophore directed screening" strategy. The sulfanilamide antibacterial agent sulfisoxazole was found to be a good endothelin receptor antagonist (IC_{50}'s of 0.60 μM and 22 μM for the ET_A and ET_B receptors, respectively). The structurally similar sulfamethoxazole was found to be a weaker antagonist (IC_{50} for ET_A 16 μM and for ET_B 230 μM). These compounds represent a new class of low molecular weight and ET_A-selective non-peptide endothelin antagonists.

The endothelins (ET) are a family of potent vasoactive peptides first isolated from the supernatant of cultured porcine aorta cells (1). Currently, three members of the endothelin family, namely ET-1, 2 and 3, are known. Elevated levels of ET-1 have been found in patients with myocardial infarction, vasospastic angina, cardiogenic and septic shock, sub-arachnoid hemorrhage and renal failure (2). These findings suggest that endothelins are associated with the pathophysiology of certain cardiovascular and renal diseases. The development of potent, subtype selective and orally active endothelin antagonists would be of great interest for several major clinical indications including hypertension, renal failure, asthma and circulatory conditions.

Endothelins are formed from the corresponding big endothelins through the action of endothelin converting enzymes (3). They exert their actions via specific membrane receptors. Two endothelin receptor subtypes have been isolated and cloned. The ET_A receptor has high affinity for ET-1 and is found predominantly in atrial and cardiovascular tissue (4). The ET_B receptor which binds endothelins-1, 2 and 3 as well as sarafotoxin 6c

* Authors to whom correspondence should be addressed. Fax: (619) 679-0105.

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equally, is found in brain, lung, kidney and adrenal tissue (5). Evidence for a third receptor subtype which is selective for ET-3 has recently been presented (6).

A number of peptide endothelin antagonists have been described. BQ-123 (7a), a cyclic pentapeptide and FR 139317 (7b), a tripeptide derivative are potent ET_A-selective antagonists while the hexapeptide PD 142839 (7c) is non-selective. Non-peptide endothelin antagonists have also been reported. These include the potent and ET_A-selective antagonist myriceron caffeoyl ester (50-235) (7d), the anthraquinone WS009 (7e) and the diphenyl ether TAN-1415 (7f). More recently, the non-selective sulfonamide antagonist Ro 46-2005 has been shown to be orally active in animal models (8). Several indane derivatives were also reported as endothelin antagonists although their activity and selectivity are not known (9). The only known ET_B-selective antagonist is IRL 1038 which is a truncated ET (10).

We have been interested in the *de novo* design of subtype selective non-peptide ET antagonists. Using pharmacophores generated from the X-ray crystallographic structure of ET-1 and computer assisted rational drug design techniques, we have discovered several selective ET_A antagonists. The results are reported below.

MATERIALS AND METHODS

Materials: Sulfamethoxazole and sulfisoxazole were purchased from Aldrich Chemical Co. (Milwaukee, WI). Sulfadiazine, sulfadimethoxine, sulfamethoxypyridazine and sulfamonomethoxine were acquired from Sigma Chemical Co. (St. Louis, MO). ET-1 was obtained from Clinalfa Co. (Laufelfingen, Switzerland) and ET-3 from American Peptide Co. (Sunnyvale, CA). [^{125}I]ET-1 was obtained from Amersham (Arlington Heights, IL). All other chemicals and biochemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

Membrane Preparation: A membrane preparation containing human ET_A receptor was prepared from TE 671 (ATCC # HTB 139). Cells, grown to confluence, were harvested using a rubber policeman and centrifuged at 190 X g for 10 min at 4° C. The pellet was resuspended in 5 mM HEPES (pH 7.4) containing 5 mM EDTA and 100 KIU aprotinin and homogenized using a Tenbroeck homogenizer. The suspension was centrifuged at 57,800 X g for 15 min at 4° C , the pellet resuspended in 5 ml of 5 mM HEPES buffer, pH 7.4, containing 10 mM $MnCl_2$ to which 5 ml of a 0.001% deoxyribonuclease Type 1 was added. The suspension was mixed, incubated at 37° C for 30 min and then centrifuged at 57,800 X g for 15 min at 4° C. The pellet was then washed twice with 5 mM HEPES buffer containing 5 mM EDTA before finally being resuspended in 30 mM HEPES buffer, pH 7.4, containing aprotinin (100 KIU/ml) to give a final protein concentration of 2 mg/ml. Aliquots of membrane were stored at -70° C until use. Protein determinations were done using the Pierce BCA assay with BSA as a standard.

A membrane preparation containing human ET_B receptors was prepared as described above from COS 7 cells which were transfected with DNA encoding the human ET_B receptor (11).

Ligand Binding Studies: Binding studies were performed using a 30 mM HEPES buffer, pH 7.4, containing 150 mM NaCl, 5 mM $MgCl_2$, and 0.05% bacitracin. Test compounds were dissolved in DMSO and diluted with the assay buffer to give a final concentration of 0.25% DMSO. Displacement experiments were performed in triplicate in a final volume of 200 µl containing 4 pM [^{125}I]ET-1 (1.6 nCi). Non specific binding was determined in

the presence of 100 nM ET-1. Samples were incubated for 16 - 18 hr at 4° C. 1 ml of ice cold PBS was then added and the assay centrifuged at 2,000 X g for 25 min at 4° C. The supernatant was decanted and the membrane bound radioactivity counted on a Genesys gamma counter.

Phosphoinositide Hydrolysis in Cells: TE 671 or transfected COS 7 cells described above were grown to confluence in 6 well plates. Sixteen hours prior to use, the media in each well was replaced with 2 ml inositol-free RPMI-164 (IF-RPMI) media containing 10% inositol-free FCS and 2 μCi 1-[^{3}H]myo-inositol, and incubated at 37° C in the presence of 6% CO_2. The media was aspirated and the cells washed twice with PBS. Cells were preincubated for 10 min in 1 ml Lithium buffer (15 mM HEPES, pH 7.4, 145 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 1.0 mM NaH_2PO_4, 11.2 mM Glucose, 20 mM LiCl) with or without test compound prior to the addition of 100 μl of ET-1 at different concentrations. Cells were then incubated for a further 45 min. The buffer was discarded and the accumulated inositol phosphates extracted with ice cold methanol and measured according to the method of Berridge (12). The total cell protein in each well was measured using the Pierce BCA assay after solubilizing the cells in 0.1 M NaOH.

RESULTS

Receptor Binding

The inhibitory effect of the sulfanilamides on the binding of [^{125}I]ET to the ET_A and ET_B receptors is shown in Table 1. Of the known sulfanilamides tested, sulfisoxazole is most active with IC_{50}'s of 0.60 μM and 22 μM for the ET_A and ET_B receptors, respectively. The Hill coefficient for both receptor subtypes did not differ significantly from unity. The corresponding values for sulfamethoxazole are 15 μM and 230 μM. The other compounds have measurable IC_{50}'s for the ET_A receptor only and these values range from 22 to 100 μM. The binding inhibition curves of sulfisoxazole and sulfamethoxazole are shown in Fig. 1 and Fig. 2.

Phosphoinositide Hydrolysis

ET-1 causes a dose dependent accumulation of phosphoinositide metabolites in TE 671 cells (Fig. 3) and in the transfected COS 7 cells in the presence of lithium (11). Addition of sulfisoxazole resulted in a parallel shift to the right of the ET-1 dose response curve in the TE 671 cells indicating that sulfisoxazole acts as a functional antagonist of ET-1 in this system. The K_D value for sulfisoxazole calculated from the inhibition of ET-1 stimulated phosphoinositide hydrolysis was 1.2 μM. A similar inhibition was observed in the transfected COS 7 cells but this required a tenfold increase in the dose of sulfisoxazole.

Table 1. IC_{50} values for the sulfanilamides on [^{125}I]ET binding to ET_A and ET_B receptors

	Compound	IC_{50} (μM)	
		ET_A	ET_B
1	Sulfisoxazole	0.60	22
2	Sulfamethoxazole	15	230
3	Sulfamethoxypyridazine	22	»100
4	Sulfathiazole	97	»100
5	Sulfadiazine	100	»100

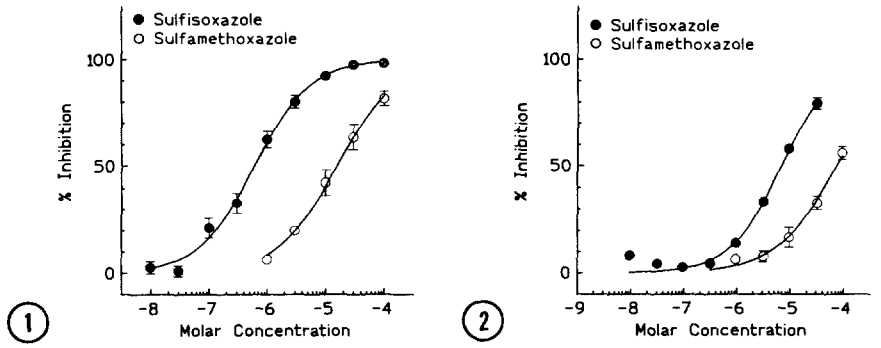


Figure 1. Dose response curve for the inhibition of binding of ^{125}I-ET-1 to the human ET_A receptor. Results are expressed as the mean ± S.E.M. from four experiments.

Figure 2. Dose response curve for the inhibition of binding of ^{125}I-ET-1 to the human ET_B receptor. Results are expressed as the mean ± S.E.M. from four experiments.

DISCUSSION

Rational drug design and random screening of compound libraries are two of the most widely used techniques in the discovery of new drugs. Either technique alone is a powerful means of generating new lead compounds. We have adopted a combination of these two methods in the discovery of the sulfanilamide antagonists. Based on the X-ray crystallographic structure of ET-1 recently determined in our laboratories (13) and the dynamic conformational features of endothelin and its constrained analogs, e.g. BQ-123 (14), a pharmacophore model was developed (15). Potential endothelin antagonist prototypes were identified by examination of this model and 3-D searching. Compounds that satisfy the pharmacophore requirements were acquired from in-house or commercial sources and tested for their ability to inhibit the binding of [^{125}I] ET-1 to the ET_A and ET_B

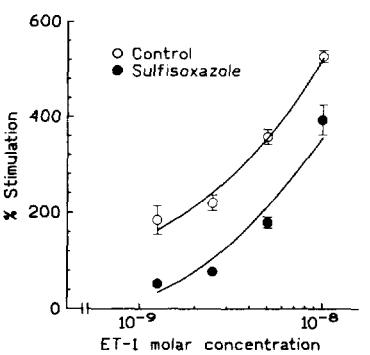
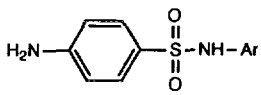
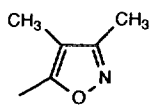
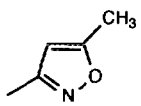
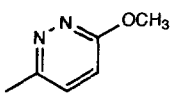
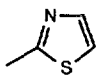
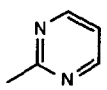
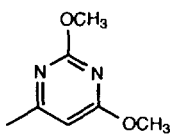
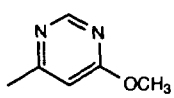


Figure 3. Effect of Sulfisoxazole on ET-1 stimulated phosphoinositide turnover in TE 671 cells.

receptors. Several classes of compounds, including the sulfanilamides, were discovered by this "pharmacophore directed screening" strategy (Scheme 1).

The antibacterial sulfanilamides are structurally related to Ro 46-2005. Although all of these compounds are benzene sulfonamides, the former has a *para*-amino group whereas the latter has a *para-tert*-butyl group. The N-substitution is also very different. Ro 46-2005 has a 4-pyrimidinyl substituent; the more active sulfanilamides, sulfisoxazole and sulfamethoxazole, have 5-isoxazolyl and 3-isoxazolyl groups at the nitrogen, respectively. The presence of a N-4-pyrimidinyl substituent is not essential for activity in the sulfanilamides as demonstrated by the lack of inhibition for sulfadimethoxine **6** and sulfamonomethoxine **7** (results not shown). The activity profiles of these two classes also differ markedly. Ro 46-2005 is a non-selective antagonist (8) but the sulfanilamides are selective ET_A antagonists. These differences imply that the site and/or mode of binding of



Compound Name	Ar
Sulfisoxazole, **1**	
Sulfamethoxazole, **2**	
Sulfamethoxypyridazine, **3**	
Sulfathiazole, **4**	
Sulfadiazine, **5**	
Sulfadimethoxine, **6**	
Sulfamonomethoxine, **7**	

Scheme 1. Chemical structures of the sulfanilamide endothelin antagonists.

the sulfanilamides to the endothelin receptors are different from those of Ro 46-2005. Detailed structure-activity relationship for these sulfanilamides is currently under study in these laboratories.

In summary, the sulfanilamides represent a new class of low molecular weight, structurally simple, subtype selective, non-peptide endothelin antagonist. They should prove useful as lead compounds for the development of ET_A-selective endothelin antagonists.

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REFERENCES

1. Yanagisawa, M., Kurihara, H., Kimura, S., Tomobe, Y., Kobayashi, M., Mitsui, Y., Yazaki, Y., Goto, K., and Masaki, T. (1988) Nature, 332, 411-415.
2. Doherty, A.M. (1992) J. Med. Chem. 35, 1493-1508.
3. Opgenorth, T.J., Wu-Wong, J.R. and Shiosaki, K (1992) FASEB J., 6, 2653-2659.
4. Arai, H., Hori, S., Aramori, H., Ohkubo, H. and Nakanishi, S. (1990) Nature, 348, 730-732.
5. Sakuri, T., Yanigasawa, M., Takuwa, Y., Miyazaki, H., Kimura, S., Goto, K. and Masaki, I., (1990) Nature, 348, 732-735.
6. Karne, S., Jayawickreme, C.K., Lerner, M.R. (1993) J. Biol. Chem., 268, 19126-19133.
7. (a) Ihara, M., Noguchi, K., Saeki, T., Fukuroda, T., Tsuchida, S., Kimura, S., Fukami, T., Ishikawa, K., Nishikibe, M. and Yano, M. (1991) Life Sci., 50, 247-255;
   (b) Sogabe, K., Nirei, H., Shoubo, M., Hamada, K., Nomoto, A., Henmi, K., Notsu, Y. and Ono, T. (1992) J. Vasc. Res., 29, 201-2;
   (c) Cody, W.L., Doherty, A.M., He, J.X., DePue, P.M., Rapundalo, S.T., Hingorani, G.A., Major, T.C., Panek, R.L., Haleen, S., LaDouceur, D., Reynolds, E.E., Hill, K.E. and Flynn, M.A. (1992) J. Med. Chem. 35 ,3031-3033;
   (d) Fujimoto M., Mihara, S.-i., Nakajima, S., Ueda, M., Nakamura, M. and Sakurai, K., (1992) FEBS Lett., 305, 41-44;
   (e) Miyata, S., Ohata, N., Murai, H., Musai, Y., Ezaki, M., Takase, S., Nishikawa, M., Kiyoyo, S., Okuhara, M. and Kohsaka, M. (1992) J. Antibiot., 45, 1029-1040;
   (f) Ishimaru, T., Tsuboya, S., Shirafuji, H. and Terashita, Z., Japanese Patent 04134084, 7 May, 1992.
8. Clozel, M., Breu, V., Burri, K., Cassal, J.-M., Fischli, W., Gray, G.A., Hirth, G., Löffler, B-M., Müller, M., Neidhart, W. and Ramuz, H. (1993) Nature, 365, 759-761.
9. Cousins, R.D., Elliott, J.D., Lago, M.A., Leber, J.D. and Peishoff, C.E., World Patent Application WO 93/08799, 13 May, 1993.
10. Urade, Y., Fujitani, Y., Oda, K., Watakabe, T., Umemura, I., Takai, M., Okada, T., Sakaba, I. and Karaki H. (1992) FEBS Lett., 311, 12-16.
11. Stavros, F.D., Hasel, K.W., Okun, I., Baldwin, J. and Freriks, K. (1993) J. Cardiovasc. Pharmacol., 22, Suppl. 8, S34-37.
12. Berridge, J.M., Downes, C.P. and Hanley, M.R. (1982) Biochem. J., 206, 587-595.

13. (a) Waller, D., Cudney, R., Wolff, M., Day, J., Greenwood, A., Larson, S. and McPherson, A. (1992) Acta Cryst., B48, 239-240;
(b) Larson, S., Balaji, V.N., Wolff, M., Waller, D., Cudney, R., Day, J., Greenwood, A. and McPherson, A. (1992) Abstract PB81, American Crystallography Association Annual Meeting, August 9-14, 1992, Pittsburgh, PA.
14. Balaji, V.N., Ramnarayan, K. and Chan, M.F. (1993) Abstract Handbook, Third International Conference on Endothelin, February 15-17, 1993, Houston, TX, p. 13.
15. Unpublished results, details will be published elsewhere.