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Pharmacophore Definition and Three-Dimensional Quantitative Structure-Activity Relationship Study on Structurally Diverse Prostacyclin Receptor Agonists

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ABSTRACT

Prostacyclin is an endogenous mediator that shows potent platelet inhibitory activity and powerful relaxation of peripheral resistance vessels. Prostacyclin receptor agonists are valuable drugs in the treatment of various vascular diseases spanning primary pulmonary hypertension to Raynaud's syndrome. Although agonists from various structural classes were synthesized, a common pharmacophore was never defined. Therefore, an attempt was made to integrate the different agonists into a single model. A dataset of structurally diverse prostacyclin receptor agonists was tested for its affinity to the human platelet prostacyclin receptor. The dataset included prostanoid and nonprostanoid ligands comprising iloprost, cicaprost, and BMY45778. Extensive conformational analyses were performed for both classes of compounds because of the absence of rigid templates. The search and superimposition procedure yielded a pharmacophore that aligns the essential carboxylate group of the agonists as well as demonstrates that different functional groups in prostanoid and nonprostanoid agonists can be arranged in a uniform conformation. A three-dimensional quantitative structure-activity relationship study was performed using the programs GRID and GOLPE. This analysis yielded a cross-validated correlation coefficient of 0.77. With this model, it is possible to predict the affinity of untested compounds.

Prostacyclin (PGI_2), an endogenous mediator, is synthesized primarily in the vascular endothelium. It plays an important role in the regulation of blood flow; it is a potent vasodilator and inhibits platelet aggregation. Both actions are mediated by a specific G-protein coupled receptor, the prostacyclin receptor (IP receptor). The binding of PGI_2 to this receptor leads to the coupling of G_s protein to adenylate cyclase and subsequent elevation of intracellular cAMP levels.

PGI_2 is a clinically useful agent for the precise control of platelet function. Its use is impaired by its instability: the enol ether linkage of PGI_2 is spontaneously hydrolyzed with a half-life of 3 min (Stehle, 1982; Armstrong, 1996), limiting therapeutic application to parenteral administration. Much effort was therefore directed toward developing metabolically stable and orally available IP-receptor agonists. These can be divided into two groups: the so-called prostanoid agonists preserve the characteristic structural features found in PGI_2, namely the carboxylate group and hydroxyl functions at C-9 and C-15 (see Fig. 1), whereas the nonprostanoid agonists do not show any structural similarities with PGI_2 except for the essential carboxylate group. As can be seen in Fig. 2, the skeletons of the nonprostanoid IP receptor agonists differ considerably. In some compounds, putative hydrogen bond acceptors such as heterocycles (i.e., oxazole) or oxime groups serve instead of the hydroxyl groups.

Several groups have previously attempted to define a pharmacophore for prostacyclin receptor agonists (Tsai et al., 1991; Meanwell et al., 1993a,b), and structure-activity relationships have been described in great detail for both types of compounds (Skuballa and Vorbruggen, 1983; Nickolson et al., 1985; Armstrong et al., 1986; Tsai and Wu, 1989; Meanwell et al., 1992a,b,c, 1993a,b, 1994a,b; Jones et al., 1993; Muir et al., 1993), but no pharmacophore that integrates both groups of compounds has previously appeared.

Correlation of the previous attempts in conjunction with incorporation of new compounds of different structural classes were united into a common agonistic pharmacophore. Extensive conformational searching was required in the absence of a rigid lead compound. The superimposition of one prostanoid [(*S*)-iloprost] and one nonprostanoid agonist (BMY45778) formed the basis of the pharmacophore, and all other compounds were modeled on these two agonists. Hu-

ABBREVIATIONS: PGI_2, prostacyclin; IP, receptor prostacyclin receptor; 3D-QSAR, three-dimensional quantitative structure-activity relationships; MEP, molecular electrostatic potential; q^2, cross-validated correlation coefficient; r^2, correlation coefficient.

man platelet affinity data served as input for a 3D QSAR study to quantify the structure affinity relationships. The resulting 3D-QSAR model showed a cross-validated correlation coefficient of 0.77. The model predicts the binding affinity for untested compounds and serves as a tool for the development of new high affinity ligands.

Materials and Methods

Materials. Cicaprost, iloprost, and nileprost were provided by Dr. F. M. McDonald (Schering, Berlin, Germany). Prostaglandin E_1 was from Dr. P. Ney (Schwarz Pharma, Monheim, Germany). All BMY compounds were from Dr. N. A. Meanwell (Bristol Myers Squibb, Wallingford, CT). ONO-1301 was obtained from Dr. K. Kondo (ONO Pharmaceuticals, Osaka, Japan). Dr. R. A. Armstrong (University of Edinburgh, Edinburgh, UK) provided EP 157.

Binding Assay. Human platelet membranes were prepared as described previously (Kaczmarek et al., 1993) and suspended in 100 mM NaCl, 20 mM Tris-HCl, 5 mM $CaCl_2$, and 10 mM glucose, with pH adjusted to 7.4. For ligand binding analysis, 200-μl aliquots of platelet membrane suspension were incubated with 10 nM [^{3}H]iloprost (Amersham Biosciences, Braunschweig, Germany) and 1 nM to 1 μM concentrations of the respective compounds. Nonspecific binding was measured in the presence of 10 μM iloprost. Equilibration was allowed for 90 min at 15°C. Thereafter, bound radioactivity was separated from free by rapid filtration (GF/C filters; Whatman, Maidstone, UK) and 3 washes with 4 ml of suspension buffer (4°C). Radioactivity was determined with standard liquid scintillation techniques.

Platelet PGI_2 receptor affinity (K_I) for iloprost was determined by nonlinear fitting analysis of the data obtained from the displacement of [^{3}H]iloprost by iloprost. The K_I values of all other compounds were calculated by the formula: $K_I = EC_{50}/[1 + (\text{concentration radioligand}/K_I \text{ radioligand})]$, where EC_{50} is the concentration of the ligand under investigation required to displace 50% of radioligand ([^{3}H]iloprost) from specific binding. The K_I values obtained for the individual compounds (Figs. 1 and 2) are means from at least three independent measurements.

Molecular Modeling. All structures were generated using the SYBYL software package (SYBYL 6.5; Tripos Inc., St. Louis, MO). The carboxylate group was always deprotonated to imitate physiological conditions. Partial atomic charges were calculated with the Gasteiger-Hückel method (Gasteiger and Marsili, 1980). Energy minimizations and conformational analyses also employed the SYBYL software. Energy minimizations were performed using first the steepest descent method (500 steps) and then refined using conjugate gradient to a gradient of 0.05 kcal/mol Å.

For the conformational analysis no rigid template was available; therefore, 16*S*-iloprost and BMY45778 were chosen because of their structural diversity. A comparison of the conformational possibilities of the compounds was used to outline the conformational space both can occupy. By this procedure, a template for the fitting of the other agonists was created. Extensive conformational analyses were performed to determine the putative binding conformations of the two ligands.

The conformational space of BMY45778 was studied using systematic conformational analysis. Because of the huge number of possible conformations, not all rotatable bonds were treated at once. Initially only the bonds between phenyl and oxazole rings were rotated using a 10° increment to assure high accuracy; the side chain remained in an extended conformation. More than 300,000 conformations were obtained in this way. They could be classified into 45 families. The lowest energy conformer was chosen as representative of each family and minimized.

Approximately half of the family representatives could be eliminated because their carboxylate group was near one of the phenyl rings. Because this carboxylate group is essential for high affinity (Tsai and Wu, 1989) it has to be accessible to the interacting amino acid (Arg279). The lowest energy conformer of each of the eight remaining collections was selected for further consideration.

In preparation for systematic analysis of the side chain, a preliminary fit with the molecular features of 16*S*-iloprost (i.e., the bicyclooctane ring and the omega chain) was carried out (for nomenclature, see Fig. 3). Because a systematic conformational search is not an effective method to explore cyclic systems, Random Search, a Monte Carlo method, was chosen (SYBYL 6.5). The Random Search was performed with a 16*S*-iloprost fragment (Fig. 4) and was repeated seven times with different starting conformations to insure that all low-energy conformations were found.

Random Search was also used for analysis of cicaprost and isotetralynaprost (the side chains were truncated to speed up the calculation). Seven runs with different starting structures were done for each compound, each run consisting of 1000 cycles. In a cycle, the bonds defined as rotatable were set to random torsion angles and the compounds minimized subsequently. The resulting conformers were compared with those already found. Side chains were then reintroduced to the conformers chosen for superimposition with BMY45778.

A systematic search was performed with the omega chains of both iloprost conformations that were chosen. Using an increment of 30°, it was possible to cover the entire conformational space with reasonable accuracy. For both 16*S*-iloprost conformers more than 10,000 side chain conformations were classified into 89 (85) families using IXGROS (Sippl, 1997). Again, all family representatives were minimized to a gradient of 0.05 kcal/mol Å using the Conjugate Gradient method.

Further reduction could not be achieved without taking into account the conformational space of BMY45778. In this study, family representatives of both compounds were superimposed with each other using the fit points 1 to 5 depicted in Fig. 3; the root-mean-square deviation was restricted to ≤1.5. This preliminary superimposition reduced the number of conformers to be further considered to 4 for BMY45778 and 24 (23) for 16*S*-iloprost. Each of these conformers was then subjected to an incremental (30°) systematic search of the (α) side chain. The conformers obtained were again

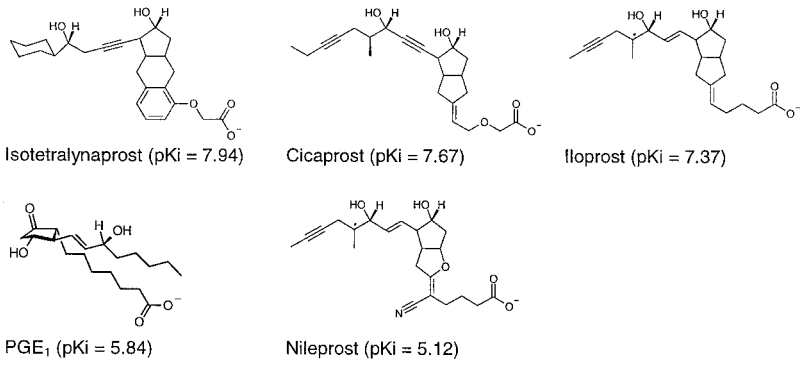


Fig. 1. Prostanoid prostacyclin receptor agonists (including affinity data).

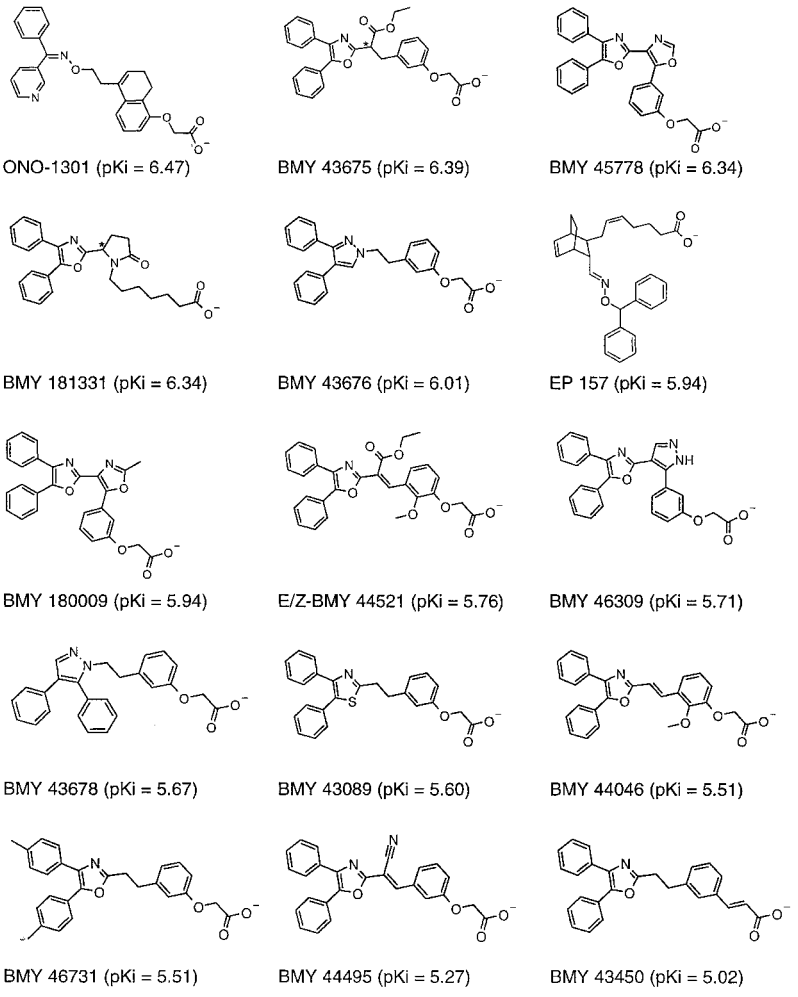


Fig. 2. Nonprostanoid prostacyclin receptor agonists (including affinity data).

divided into families and each of the 14 family representatives of BMY45778 was superimposed with each of the 21 + 20 representatives of 16*S*-iloprost. For this final superimposition, all six fit points were considered and a tighter root-mean-square deviation of ≤1.1 was applied.

The chosen fit points considered both steric correspondences as well as the existence of a similar hydrogen bond pattern. For the fit points 1 and 2, the ends of the corresponding lone pairs were used to insure that both functional groups could interact with the same amino acid in the receptor.

For the other ligands, a shorter procedure was used, only cicaprost was treated as extensively as 16*S*-iloprost to test the proceeding. The diphenyloxazole derivatives and PGE_1 were integrated into the pharmacophore using the Multifit option of SYBYL.

Multifit is a flexible fitting method in which pairs of atoms are forced onto each other by a force constant during a minimization procedure. Because the pairing atoms have to be chosen explicitly, this method is applicable only for very similar compounds. The reference ligand was always treated as a rigid entity; the force constants used were high (50 kcal/mol $Å^2$) for the very important fit points chosen in analogy to Fig. 3 and more relaxed for all others (20 kcal/mol $Å^2$).

For isotetralynaprost (Jakubowski et al., 1994), Random Search was applied to find favorable ring conformations. The conformation that is similar to the one used for 16*S*-iloprost was chosen and side chains were adjusted using Multifit. To integrate 16*R*-iloprost into the pharmacophore, the conformation of 16*S*-iloprost was adopted, position 16 was inverted, and the epimer was minimized subsequently.

For EP 157 and ONO-1301, a Multifit was not possible because their structural homology with other ligands is insufficient. Inclusion of these latter two structures was accomplished using the pharmacophoric superimposition program FLEXS (Lemmen et al., 1998a). The validity of this approach was verified by fitting (*S*)- and (*R*)-nileprost on the reference ligand 16*S*-iloprost. FLEXS (Lemmen and Lengauer, 1997; Lemmen, 1998a,b) was designed to flexibly superimpose pairs of molecules. One of the molecules, the reference ligand, is kept rigid. The other one is split into several fragments that are fitted incrementally on the reference ligand, starting with the base fragment. Many different solutions are obtained and are ranked by a scoring function. All compounds had Gasteiger-Hückel charges and the carboxylate group was chosen as the base fragment. The best 20 solutions were inspected visually, and the selected solution was energy minimized.

To ensure that the conformation obtained by Multifit or FLEXS was not dramatically changed, parts of the ligand were fixed in the beginning of the minimization and only relaxed step-by-step using Steepest Descent and Conjugate Gradient.

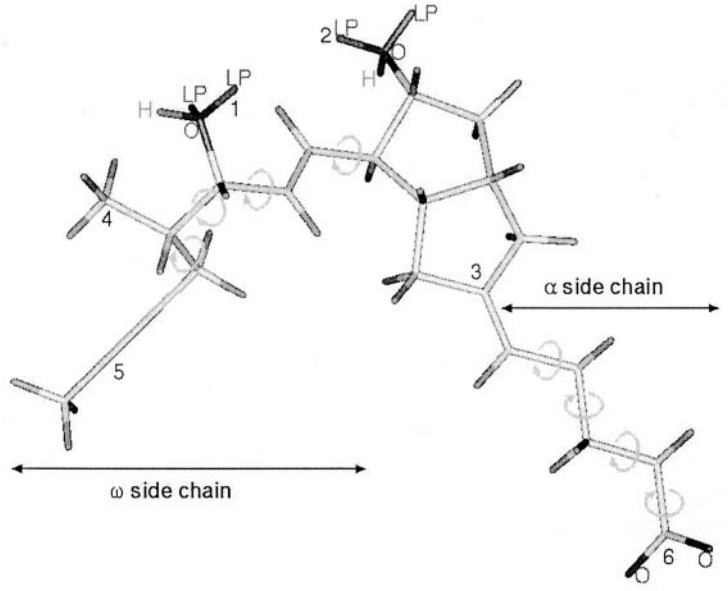
MEPs. Molecular electrostatic potentials (MEPs) were computed with the HF3–21G* basis set using SPARTAN 5.1.1 (Wavefunction Inc., Irvine, CA).

Using the program GRID 16 (Molecular Discovery Ltd., London, Great Britain; Goodford, 1985), interaction energies between a compound and a probe can be calculated. A wide variety of probes with very different chemical features are available. They are designed to cover the different qualities of atoms in a protein-binding site so that the surroundings can be mapped in an indirect way. The probe is placed on each point of a grid that is created around the ligand and the interaction energy is then calculated. The types of noncovalent

interaction accounted for in the GRID program are steric, electrostatic, and hydrogen-bonding energies. The results can be visualized by contouring isoenergy levels.

For each compound, a grid with a spacing of 1 Å was generated and GRID fields were calculated using an amidic NH probe (N1, simulating a hydrogen bond donor), the carbonyl probe (a hydrogen bond acceptor), and the amidine probe. With the latter, interactions between the essential carboxylate group of the ligands and an arginine of the binding pocket (Arg279) were simulated. Best results for hydrophobic interactions were received with the dry probe (a "dry" water molecule).

The Program GOLPE 4.0 (Multivariate Infometric Analysis, Perugia, Italy) is used to statistically analyze three-dimensional molecular fields and to correlate the important data points with biological data. For this 3D-QSAR analysis, interaction fields calculated with GRID were used as input. Several probes were tested in order



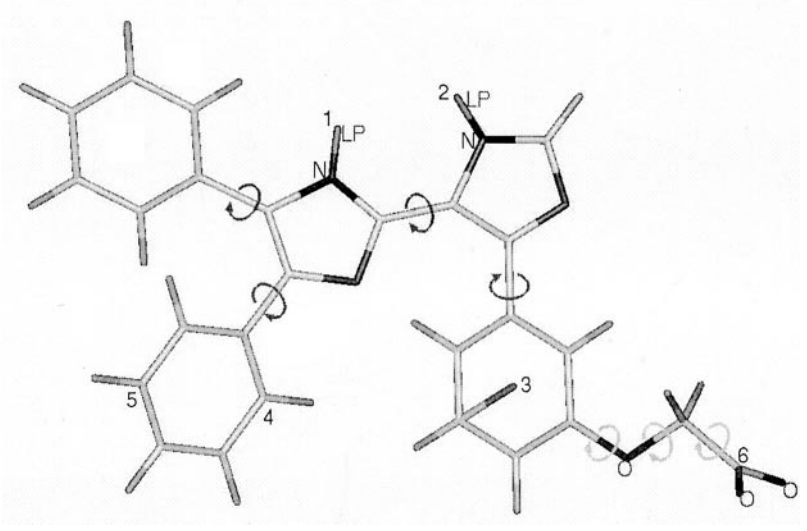


Fig. 3. Conformational analysis of 16*S*-iloprost and BMY45778. Numbers, superimposed atoms; dark arrows, increment 10°; lighter arrows, increment 30°; LP, lone pair.

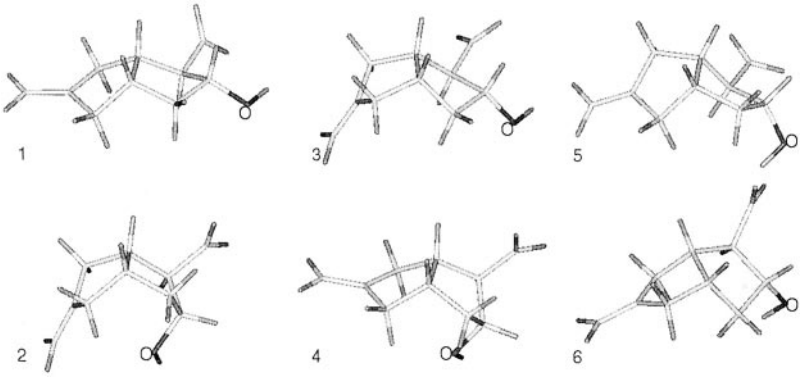


Fig. 4. The six conformations for the bicyclooctane ring system of iloprost received with Random Search.

to determine which was best suited for the description of the differences between the compounds. Principal component analysis showed that of all probes used the NH = probe (sp^2 hybridized NH with a lone pair) could distinguish best between the compounds.

The interaction field between the NH = probe and the ligands was calculated as described before: the pure enantiomers were placed into a standardized grid (grid spacing 1 Å) and interactions were computed with different GRID probes. The interaction energies obtained between each compound and the probe as well as the affinity to the prostacyclin receptor served as input for GOLPE. The preliminary model calculated with this probe contained 10,098 x variables for each compound (interaction values, x variables; affinity, y variable). Most of these variables are not meaningful for the explanation of the differences in affinity and introduced noise into the statistical PLS analysis. This noise was eliminated during the data pretreatment procedure for variable selection.

Data Pretreatment. GRID points with interaction energies near to zero (≤0.03) as well as those with a very low standard deviation of ≤0.02 were eliminated. Grid points where all but one compound have the same interaction value (2-level variables) as well as 3- and 4-level variables were discarded. By this procedure the number of x variables was reduced from 10098 per ligand to 4473.

The approach using D-optimal preselection of variables searches for the most informative variables since most grid points do not contain information with relevance for biological data. Applying this method enabled a further reduction of x-variables to 2236. The "smart region" definition (Pastor et al., 1997) was subsequently employed to reduce the number of groups from 1009 to 740. In the last step, a fractional factorial design procedure was employed to optimize the predictability of the model. The final model contained 959 x variables, with three principal components being used.

Cross validation of the model was done using the leave-one-out method and the leave-20%-out method (five random groups). For the first method, one compound is not used for the generation of a new model and its affinity is predicted using that new model. The model building and prediction cycle is repeated until each compound was left out once. A correlation coefficient q^2 is calculated from the correlation between experimental and predicted pK_I values. The second method works the same way but the compounds are distributed randomly into five groups and each group is left out once.

The remaining compounds were used as an external data set. The isomers/enantiomers were treated separately and the average of the predictions for both isomers was calculated and compared with the binding data.

Results

Binding Assay. The experiments showed clearly that all compounds displaced [^{3}H]iloprost in a competitive way from the binding site. This is exemplified in Fig. 5 for isotetralynaprost, iloprost, BMY45778, and BMY43450.

Pharmacophore Development. To define a common pharmacophore for prostanoid and nonprostanoid agonists, a set of structurally diverse agonists was chosen and their affinity to the prostacyclin receptor was measured using human platelet membranes. The 21 compounds used (five of which are mixtures of isomers) are listed in Figs. 1 and 2 together with the corresponding affinity data.

The conformational analysis of the BMY45778 (leaving out the side chain) resulted in 45 family representatives. A superimposition of all 45 conformations is shown in Fig. 6. The phenyl rings of the biphenyl oxazole moiety show two distinct arrangements, both leading to maximum planarity.

For the two remaining torsion angles (one between the oxazole rings and one between the second oxazole ring and the side chain phenyl ring), only a small number of low energy conformations was observed (Fig. 6). The two oxazole rings always lie in the same plane but the nitrogen atoms point in the same or in opposite directions. The side-chain phenyl ring also arranges itself in a pseudoplanar fashion but real planarity is not possible because of steric repulsion. Therefore, the side chain is found pointing toward the biphenyl oxazole ("closed conformation") or outward ("elongated conformation").

Initially, the conformational analysis of 16*S*-iloprost was restricted to the central bicyclooctane ring. Six conformations were detected (Fig. 4). To reduce this number further, a comparison with crystal structures for similar fragments was done. Of 16 hits found in the Cambridge Structural Database (Allen and Kennard, 1993; Bruno et al., 1997), 10 could be assigned to the conformations **3** and **4,** whereas two hits were found for conformations **1**, **2**, and **6**. There were no hits for conformation **5**; therefore, this conformation was eliminated. As mentioned above, all conformers of BMY45778 showed an arrangement that was as planar as possible. 16*S*-iloprost has to fit into the same binding pocket as BMY45778 and should therefore adopt a conformation that presents the same overall shape. Because of these restrictions, side chains were added only to the more planar bicyclooctane conformations **1** and **3**. After the conformational search of the side chains, all conformers of BMY45778 and 16S-iloprost were superimposed with each other. The superimposition with the best match is shown in Fig. 7.

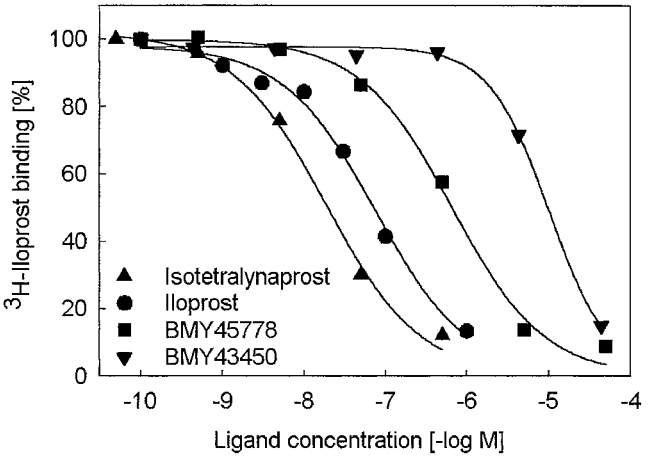


Fig. 5. Displacement of [^{3}H]iloprost by isotetralynaprost, iloprost, BMY45778, and BMY43450.

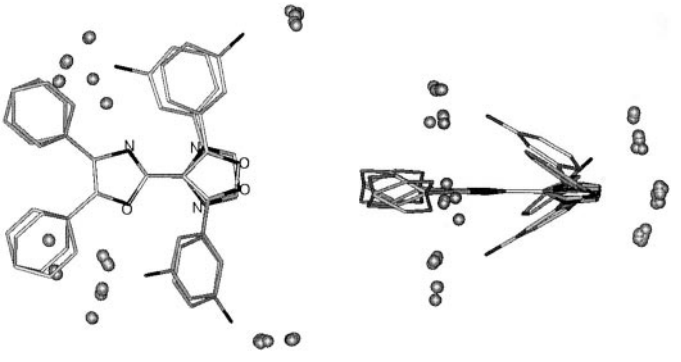


Fig. 6. Superimposition of 45 family representatives of BMY45778. To ensure clearness only a sphere indicates the position of the carboxylate group for most structures. Left, top view; right, side view.

The conformation of all other compounds was compared to either BMY45778 or to 16S-iloprost and a superimposition procedure was performed. The superimposition of all compounds is shown in Fig. 8.

To complement these efforts, the MEPs of all compounds were computed (data not shown). The electronic properties of BMY45778 and 16*S*-iloprost are quite similar: Both MEPs are dominated by the strong negative potential of the carboxylate group, with the areas near the oxazole nitrogens and around the hydroxyl groups also presenting negative character.

To verify the superimposition of the ligands, GRID calculations were performed. The results obtained with the amidine probe confirmed good superimposition of the carboxylate groups; very similar interaction fields were found for all ligands. More diverging results were obtained with the N1 Probe (amidic nitrogen, hydrogen bond donor). Figure 9 shows that here, again, the interaction fields around the carboxylate groups are very similar but differences can be observed in other areas. The high affinity agonist Cicaprost possesses two distinctive fields around its hydroxylate groups; in contrast to BMY45778, which shows only one field located in the prolongation of the nitrogen lone pairs. With the other hydrophilic probes, similar results were obtained, whereas the fields produced by the hydrophobic probe did not present any interpretable variations (data not shown).

To quantify the results, a 3D-QSAR study was carried out using GOLPE. Because some of the compounds listed in Figs. 1 and 2 are mixtures of isomers, only the 15 pure enantiomers could be included in the data set. For details regarding the experimental data, see *Materials and Methods*. Examples of four displacement experiments are shown in Fig. 5. The model calculated uses three principal components and achieves a correlation coefficient r^2 of 0.96. The correlation after cross-validation with the leave-one-out method is shown in Fig. 10; the cross-validated correlation coefficient q^2 obtained with this method was 0.77 (SD of error prediction = 0.37). The cross-validation with the leave-20%-out method yielded a correlation coefficient q^2 of 0.68.

Because the data set was rather small, it could not be divided into a training set and a test set. To prove the predictive power of the model, the binding affinities of the four racemic compounds and the E/Z-mixture not included in the QSAR analysis were also calculated. The results obtained for the isomers are shown in Table 1. For iloprost, BMY44521,

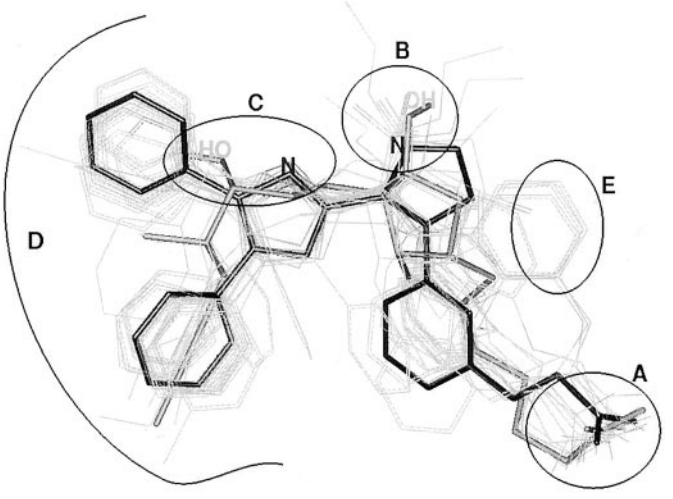


Fig. 8. Superimposition of all 25 prostacyclin receptor agonists (15 compounds and 5 isomeric mixtures). Dark gray, BMY 45778; light gray, 16*S*-iloprost. A, essential carboxylate groups; B and C, hydrogen bonding groups; D and E, lipophilic areas.

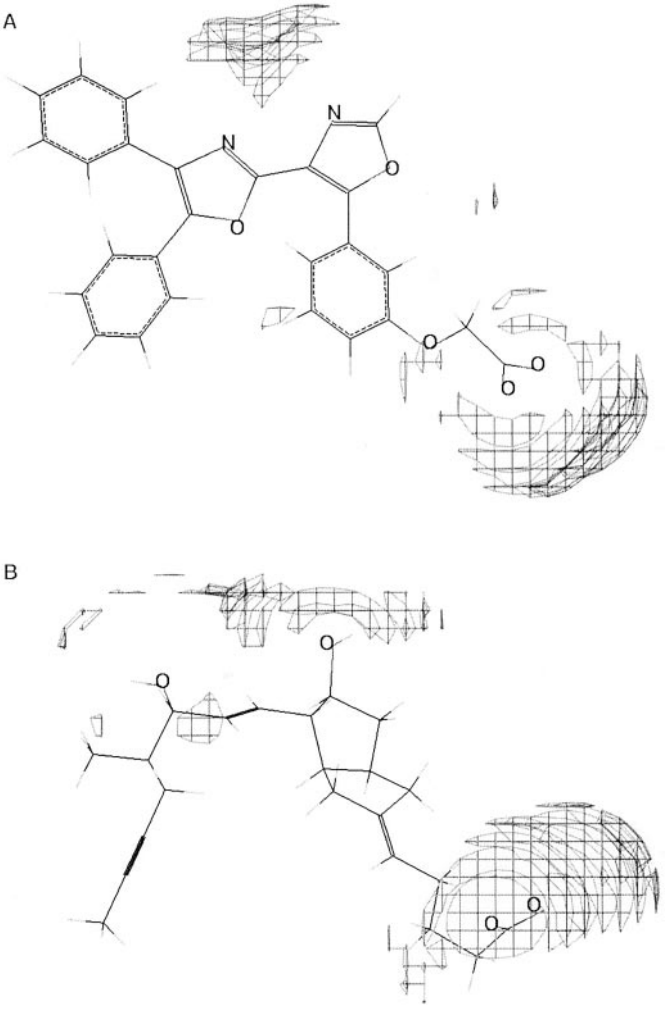


Fig. 9. GRID fields obtained with a N1 probe (contoured at −5 kcal/mol). A, BMY45778; B, cicaprost.

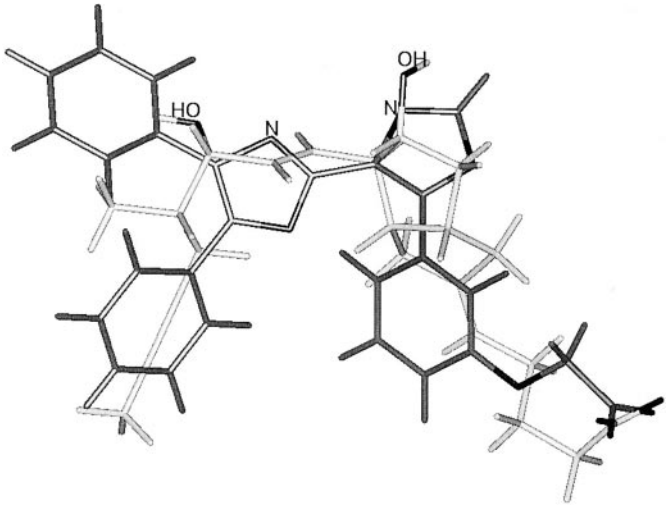


Fig. 7. Final superimposition of BMY45778 and 16*S*-iloprost.

BMY43675, and BMY181331, deviations between the average of the predicted pK_I values and the experimental value of 0.12 to 0.86 were found. Only nileprost shows a greater deviation of 1.89.

Discussion

A pharmacophore for prostacyclin receptor agonists was defined on the basis of a data set of 15 structurally diverse compounds. The superimposition was based on structural analyses of two agonists: the prostanoid compound 16*S*-iloprost was chosen because of its high affinity and the nonprostanoid BMY45778 because of its noticeable structural dissimilarity and its relative rigidity. The conformations found for BMY45778 (excluding the side chain) showed a relatively planar arrangement, which is also found in the crystal structure of BMY45778 (Meanwell et al., 1993a). Using these results, the conformational space of 16*S*-iloprost could be restricted so that only very few ring conformations for the central bicyclooctane ring were possible. After adding the side-chains to both compounds and exploration of their conformational space, it became clear that the elongated conformations were preferred. The conformations of BMY45778 and 16*S*-iloprost that agreed best were used as the basis for the fitting of all other compounds. The carboxylate groups of both ligands could be overlaid very well. The C-11 hydroxyl group of 16*S*-iloprost is in a similar position as one of the oxazole nitrogens of BMY45778. For this nitrogen, an important function as hydrogen-bond acceptor was described in the structure-activity relationships published earlier (Meanwell et al., 1993a).

The other compounds were superimposed with either BMY45778 or 16*S*-iloprost and good results were also obtained for them. Figure 8 shows the pharmacophore deduced from the superimposition: the carboxylate group (region A) is an essential feature of all agonists. In a distance of 8 to 11 Å, a hydrogen bond accepting and/or donating group is important (region B). This group is a hydroxyl group in the prostanoid agonists and can be a heterocyclic nitrogen, an oxime nitrogen, or an ester in the nonprostanoid compounds.

For the second hydroxyl group (at C-15) of the prostanoid agonists, no clear superimposition with other hydrogen bond accepting (or donating) groups could be found because the distance between both hydroxyl groups is larger than that between the heterocyclic nitrogens present in some of the nonprostanoid compounds (region C). D indicates an extended lipophilic area that is formed by aromatic or aliphatic side chains. Region E is occupied only by side chain phenyl rings of those nonprostanoid ligands that contain an E-configurated double bond (BMY44046, BMY44495, E-BMY44521; see Fig. 2).

To complement this information, the environment of the agonists was scanned. The results obtained with GRID can be used to deduct some information about the binding pocket in the IP receptor. It is well known that in many prostaglandin receptors, an arginine of the seventh transmembrane helix (position 7.40; for nomenclature, see Ballesteros and Weinstein, 1995) is important for the binding of the ligands (Negishi et al., 1995; Huang and Tai, 1995; Audoly and Breyer, 1997; Chang et al., 1997; Kedzie et al., 1998). Because all prostaglandin receptor ligands possess a carboxylate group, it can be assumed that a charged hydrogen bond between this carboxylate group and the arginine is formed. Because no experimental results regarding the IP receptor were available, the amidine GRID probe was used to mimic this interaction. Not surprisingly, the fields that were found for the different ligands were very similar. This supports the assumption that Arg279 (7.40) of the IP receptor is the binding partner of the carboxylate group.

The results obtained with the hydrogen bond donating group are very similar in the area around the carboxylate group, but important differences can be found in the interaction fields induced by the central parts of the compounds. The high-affinity agonists produce two distinctive interaction fields around their hydroxyl groups (which are rotated by GRID). These two fields allow the conclusion that two amino acids in the binding pocket interact with these compounds and lead to their good binding properties. The compounds with medium affinity (i.e., BMY45778) are able to form only one interaction by accepting a hydrogen bond. Some low-affinity agonists do not show any interaction fields in this area because no suitable functional groups are found. Based on the GRID results, a hypothesis can thus be established: the higher affinity that is found for most prostanoid compounds can be explained by the interaction with two additional binding partners in the receptor. In the nonprostanoid compounds, only one or even none additional interaction field can be found, and their affinity is much lower than that of most prostanoid agonists. The low affinity measured for nileprost does not fit into this scheme, but because the cyano group protrudes from the common pharmacophoric volume, it can be assumed that this compound cannot be accommodated easily by the ligand-binding pocket.

Because there are no mutagenesis studies published for the IP receptor itself, it is not easy to phrase a hypothesis regarding the interacting amino acids in the binding pocket. Kedzie et al. (1998) stated that iloprost did not activate the EP2 receptor wild-type, whereas activity was measured for the L304Y mutation. This leads to the conclusion that the corresponding Tyr281 in the prostacyclin receptor could be important for agonist binding and/or the activation of the receptor.

In all prostaglandin receptors, a conserved Ser/Thr can be found in the second extracellular loop. For the EP2 and EP4 receptors, it has been shown that the mutation of this amino acid to Ala decreased the ligand binding affinity considerably, whereas a mutation from Thr to Ser had no effect (Stillman et al., 1998). In the IP receptor, a Ser (Ser168) can be found at the corresponding position.

Both amino acids are plausible binding partners for the prostanoid and nonprostanoid compounds (hydroxyl groups, heterocycles, oxime functions; see Fig. 8, region B and C). The GRID probes used to calculate the fields shown in Fig. 9 correspond well with these experimental results. A more detailed analysis of ligand receptor interactions will only be possible after the construction of a prostacyclin receptor model, but it has to be mentioned that even then, far-reaching conclusions about the stimulus transfer will not be conceivable.

It is achievable, however, to quantify the structure-activity relationships and predict the affinity of untested compounds using QSAR methods. The basis of the 3D-QSAR approach used here (program GOLPE) is a statistical analysis of GRID fields. It could be shown in many cases that areas found to be important for the explanation of affinity differences can be superimposed with amino acids of the binding site when the

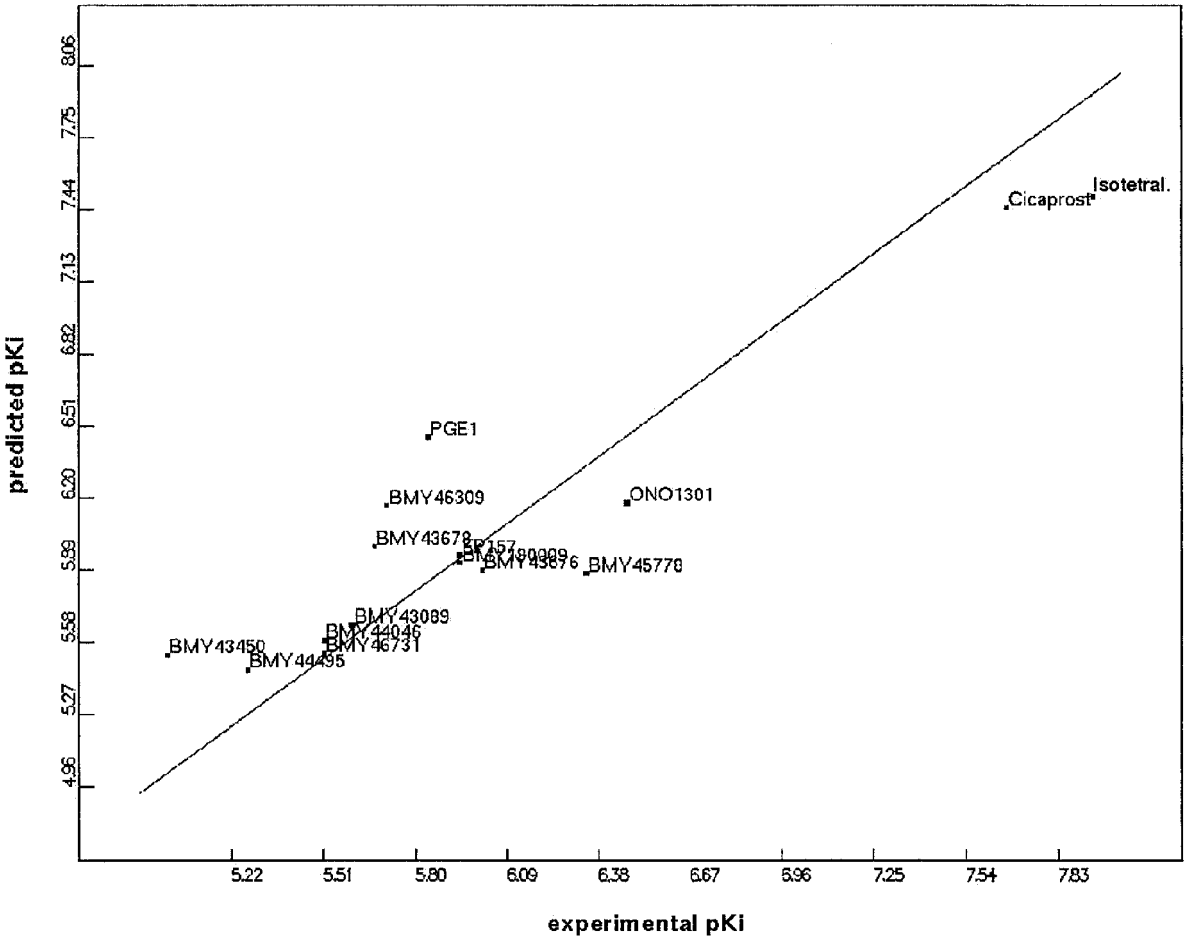


Fig. 10. Correlation of 15 prostacyclin receptor agonists (q^2 = 0.77, validation method: leave-one-out).

TABLE 1
Experimental and predicted binding affinities for external test set (isomeric mixtures)

Compound	pK_i		Average of Predicted pK_i (B)	Difference (|A–B|)
	Experimental (Mixtures, A)	Predicted (Isomers)		
BMY181331	6.34	R: 6.05 S: 5.87	5.96	0.38
BMY43675	6.39	R: 5.43 S: 5.62	5.53	0.86
BMY44521	5.76	Z: 5.72 E: 5.55	5.64	0.12
Iloprost	7.37	R: 7.17 S: 7.33	7.25	0.12
Nileprost	5.12	R: 6.90 S: 7.12	7.01	1.89

structure of the protein is known (for example, see Sippl and Höltje, 2000).

The results of the 3D-QSAR study can be regarded as very satisfactory for a data set that is as heterogeneous as the one used here. A good correlation of r^2 = 0.96 could be obtained and the cross-validated correlation coefficient (q^2) of 0.77 (leave-one-out) documents the predictive power and significance of the model. A more-demanding cross-validation using the five random groups method still gave very satisfactory results with a q^2 of 0.68. It has to be admitted that enlargement of the training set would improve the reliability of the model. Especially interesting would be the incorporation of compounds that cover the gap in affinities between the two existing clusters. Unfortunately, such compounds were not available to us.

Unfortunately, the data set was too small to extract an independent test set. Because of this, predictions were made for racemates and isomeric mixtures. The average of these predictions was compared with the binding data. This was possible because the difference in affinities between the two predicted isomers amounts in all cases only to about 0.2 pK_I units. The results obtained still have to be treated with some caution although they give an indication of the predictability of the model.

Very good predictions could be obtained for most compounds. The deviations of iloprost, BMY181331, BMY43675,

and BMY44521 can be considered well within experimental tolerance. Nileprost, however, does not fit into the 3D-QSAR model. Because the most remarkable structural variation compared with iloprost or cicaprost is the introduction of the cyano moiety, it can be assumed that this group causes steric repulsion in the binding pocket of the protein, which may lead to the poor affinity measured for nileprost.

It was possible for the first time to define a common pharmacophore for prostanoid and nonprostanoid agonists of the prostacyclin receptor. This pharmacophore was supplemented by molecular electrostatic potentials showing that the compounds studied possess similar steric and electronic properties. It can be deduced from these findings that both classes of agonists show a similar binding mode. This result was complemented by the computation of GRID fields with a number of probes that scanned the different properties of the compounds. A possible explanation for the higher affinity of most prostanoid agonists probably results from their ability to form an additional hydrogen bond to the receptor. A 3D-QSAR study quantified the results. The final model had a q^2 of 0.77, which is considered a significant correlation for the structurally heterogeneous data set investigated in this study.

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