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Interaction between platelet receptor and iloprost isomers

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Iloprost, a stable analog of prostacyclin, has been used for studying the interaction between prostacyclin and its effector cells such as platelets and vascular cells. The compound is usually prepared as a mixture of 16(*S*) and 16(*R*) stereoisomers. In this work, we compared the biological activity and platelet receptor binding characteristics between the two isomers. The 16(*S*) isomer was 20-times more potent than the 16(*R*) in inhibiting collagen-induced platelet aggregation. Equilibrium binding of iloprost isomers to platelet membrane receptors measured by rapid filtration method revealed that the specific binding data of 16(*S*) isomer was fit for a single binding species with K_d of 13.4 nM and B_{max} 665 fmol / mg protein. By contrast, the K_d and B_{max} of 16(*R*) isomer were 288 nM and 425 fmol / mg, respectively. To further assess different binding behavior of these two isomers, association rate was measured. The observed association rate of the *S* isomer was 0.036 s^{-1} and 0.001 s^{-1} for the *R* isomer at 15 nM iloprost and 2 mg / ml platelet membrane proteins. We postulate that the striking difference in the association rate with resultant difference in binding affinity and biologic activity between the two isomers was due to fitting of the molecule to the receptor channel. The 16(*S*) form has a more favorable orientation for fitting into the receptor. We conclude that the two iloprost isomers must be considered as two entirely different compounds when iloprost is used as the ligand for quantifying prostacyclin receptor binding.

Introduction

Prostacyclin (PGI_2) is a potent inhibitor of platelet aggregation [1] and a strong vasodilator [2]. Investigation of its biological activity and clinical efficacy is difficult due to its chemical instability [3]. The half-life of prostacyclin is only a few minutes in an aqueous environment and a neutral pH [3]. Because of its potential value in therapy, tremendous efforts have been directed towards the synthesis of stable and biologically active analogues [4,5]. Among the stable analogues, iloprost has been extensively investigated. It is a carbacyclin derivative of prostacyclin (Fig. 1). It differs from prostacyclin in three structural features: (1) the ether oxygen between C-6 and C-9 is replaced by a carbon which contributes to high chemical stability, (2) an additional methyl group attached to C-16 and (3) the single bond between C-18 and C-19 is replaced by a triple

Abbreviations: Buffer A, 20% acid-citrate-dextrose buffer; ACD-Tyrode's buffer (Buffer B); Ca^{2+}-free Tyrode's buffer (137 mM NaCl, 2.68 mM KCl, 0.42 mM NaH_2PO_4, 1.7 mM $MgCl_2$, 11.9 mM $NaHCO_3$, 1 g/l glucose), pH was adjusted by Buffer A to 6.8.

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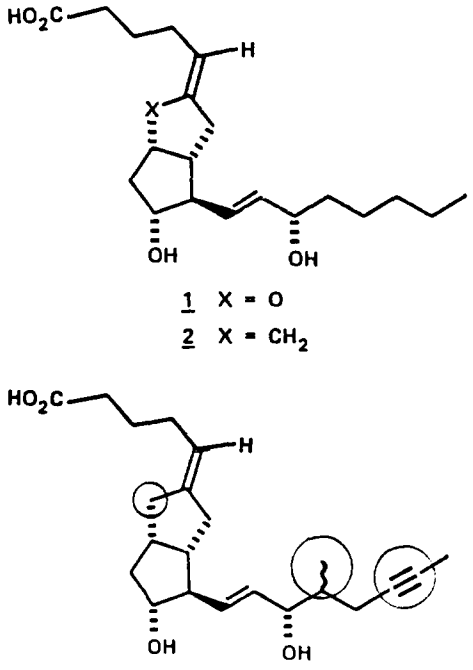

Fig. 1. Structures of prostacyclin (1), carbacyclin (2) (top) and iloprost (bottom). The 16-methyl group indicated by the middle circled area is present as *R* or *S* forms in the synthesized compound. Differences are highlighted by circles.

bond. The last two modifications yield a significant increase in the biological potency. For inhibition of platelet aggregation, iloprost is as potent as prostacyclin and is 10-times more active than carbacyclin. Iloprost has become a very useful tool for quantitation of prostacyclin interaction with platelet receptors, blood vessel walls and serum binding protein(s) [6–8]. Preparations used in most studies contain 16(*S*) and 16(*R*) stereoisomers. As these two isomers may exhibit distinct biological activity and binding characteristics, the purpose of this paper is to characterize the quantitative difference between these two isomers in inhibiting platelet aggregation and binding to platelet receptors. Our data indicate that the 16(*S*) isomer is much more potent than the 16(*R*) isomer by at least an order of magnitude.

Materials and Methods

Materials. Human serum albumin, crystalline, was purchased from Sigma. Free acid iloprost was a generous gift of Dr. E. Schillinger, Schering Laboratories, F.R.G. The trometamol salt of iloprost and [11-^{3}H]iloprost (14.8 Ci/mmol) were purchased from Amersham, Chicago, IL. Collagen (1 mg/ml in isotonic glucose) was the product of Hormon-Chemie Co. (Munich, F.R.G.).

Platelet preparations. Platelet-rich plasma was prepared from venous blood collected in 3.8% sodium citrate and centrifuged at $200 \times g$ for 10 min at 23° C. Platelet concentration was adjusted to $3 \cdot 10^8$/ml with autologous platelet-poor plasma. To prepare washed platelets, blood was collected in 20% acid-citrate-dextrose buffer (Buffer A). Platelet-rich plasma obtained by centrifugation was washed with ACD-Tyrode's buffer (Buffer B) (pH 6.8), and spun down against a 5% albumin cushion ($1000 \times g$, 15 min, 23° C). The washing process was repeated two times in Buffer B using Ficoll-Hypaque as cushion in the centrifugation step. Washed platelets were counted and adjusted to $3 \cdot 10^8$/ml with Buffer B. Crude platelet membrane fractions were prepared from washed platelets according to Tsai and Lefkowitz [9]. The final membrane pellet was resuspended to a protein concentration around 2 mg/ml as measured by Bradford [10] using bovine serum albumin as standard.

Inhibition of collagen-induced platelet aggregation by iloprost was performed by turbidometric method using a Chronolog aggregometer [11].

Separation of the stereoisomers of iloprost. [^{3}H]Iloprost or a mixture of iloprost and [^{3}H]iloprost (1000 : 1 molar ratio) was separated into the *R* and *S* isomers by a reverse phase HPLC C18 column (Radial-Pak Cartridge contained in a *Z* module radial compression separation system, Waters, MA).

The composition of the mobile phase is given in Table I. These two isomers were eluted at a water (containing 0.1% acetic acid): acetonitrile ratio of 60 : 40. The two eluted radioactive peaks were carefully separated and reinjected into the HPLC to check for any possible cross contamination of the isomers (Fig. 2). Each isomer fraction was pooled and dried under vacuum and redissolved in 50 mM phosphate (pH 7.4). The concentration of each isomer was determined by its radioactivity.

Binding of iloprost to platelet receptors. Binding experiments were performed by a slightly modified method of the fast filtration method de-

scribed by Siegl [12]. For binding equilibrium measurements, 0.2 ml of platelet membrane suspensions prepared as described above were incubated with serial concentrations of [^{3}H]iloprost for 20 min at 23°C. 4 ml of ice-cold incubation buffer (40 mM Tris, 10 mM $MgCl_2$, and 5 mM EDTA (pH 7.4)) was added to the reaction mixture which was quickly loaded onto a Millipore AP15 glass filter (25 mm diameter) in a Millipore 5025 Sampling Manifold (Millipore Corporation, Bedford, MA). The mixture was rapidly filtered under vacuum, and the filters were then rinsed with 5 ml ice-cold incubation buffer by five consecutive 1 ml washes. After washing, the filters were dried with a 250 W infrared heat lamp and placed in plastic scintillation vials with 7 ml Liquescent (National Diagnostics, Manville, NJ). Radioactivity was determined by a Beckman LS6800 scintillation counter with a counting efficiency of 63%. Nonspecific binding was obtained by performing parallel binding experiments in the presence of 10 μM nonlabelled iloprost. Specific binding was quantitated by subtracting the nonspecific binding from the total binding. The data were analyzed by a nonlinear regression analysis using a model of one class of receptor as well as by Scatchard method (STATGRAPHICS, STSC Inc., Rockville, MD). The time course for the association kinetics was obtained by quantitation of the bound radioligand at different time points, from immediately after the addition of 15 nM [^{3}H]iloprost to 10 min after the addition of radioligand. Nonspecific association of iloprost was obtained as described above using membrane preparations preincubated with 10 μM nonlabeled iloprost. The nonspecific association of iloprost was subtracted from the total binding for each time point to yield the time course of the specific binding. Because the quantity of ligand is always in excess to the number of receptors, the association process therefore follows pseudo first-order kinetics. In most cases, the observed rate of association was determined by fitting the data to a one-exponential model ($cpm_t = cpm_e\ [1 - \exp(-k \cdot t)]$ where cpm_t is the radioactivity at time t and cpm_e is the radioactivity after the system is equilibrated. k is the association rate constant) by a Maquardt nonlinear regression method. Data obtained using iloprost mixture was fitted to two-exponential model due to its biphasic behavior.

TABLE I

COMPOSITION OF THE MOBILE PHASE

Elution time (min)	Acidified H_2O (%)	Acetonitrile (%)
0	74	26
3	65	35
25	65	35
30	50	50
45	0	100

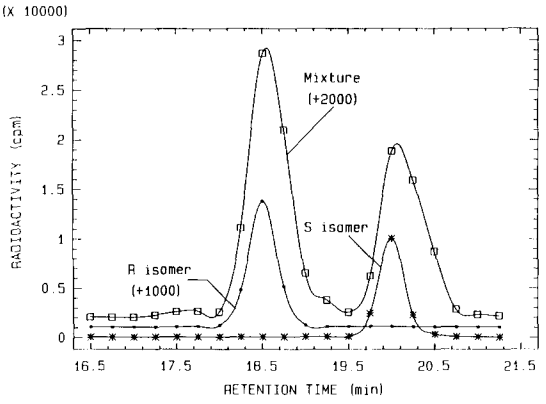

Fig. 2. Separation of the R and S isomers of iloprost by HPLC. 45 μl of [^{3}H]iloprost (6.8 μM) was injected in the C-18 column with a flow rate of 2 ml/min (0.5 ml per fraction). The fractions corresponding to a retention time of 18 to 19 min and of 19.7 to 20.7 min were pooled and concentrated. One third of each concentrated pooled fraction was reinjected to the same HPLC column. □———□, [^{3}H]iloprost racemic mixture. ■———■, reinjection of the R isomer after being purified from the first HPLC. *———*, reinjection of the S isomer after being isolated from the first HPLC run. The numbers in the parenthesis indicate baseline adjustments to show clearly the individual chromatogram.

Results

Our HPLC analysis of the iloprost preparation revealed two peaks with a molar ratio of 58 : 42 (Fig. 2). Rechromatography of each peak yielded a single peak indicative of no cross contamination. Our results matched those of Skuballa and Vorbruggen who identified the 16(R) and 16(S) isomers by using synthesized individual stereo-

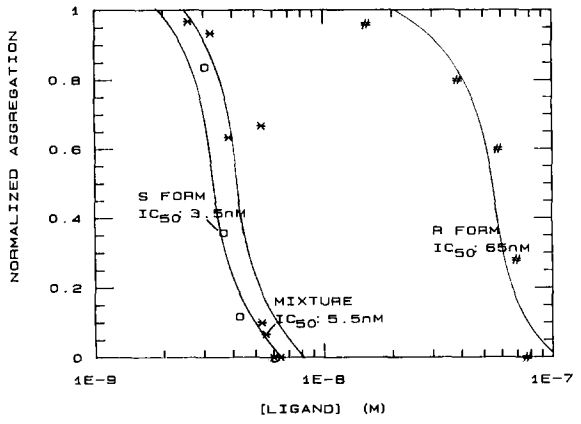

Fig. 3. Biological activity of iloprost (individual isomer and original mixture) as determined by inhibition of platelet aggregation. Collagen (10 μg/ml) was added to 0.5 ml of platelet-rich plasma ($3 \cdot 10^8$ cells/ml). The potency of each chemical is expressed as IC_{50} as shown in the figure. Two complete sets of data were obtained using different blood donors. Only one set of data is shown. Both sets of data are very similar.

isomers of iloprost [13]. Based on careful comparison of the data, we were confident that the two peaks were the 16(R) and 16(S) isomers, respectively. Further physicochemical characterization was deemed unnecessary. The 16(R) and 16(S) fractions were collected and their biological properties were evaluated. Their potency in inhibiting collagen-induced platelet aggregation was strikingly different (Fig. 3). The 16(S) isomer was much more potent than the 16(R) isomer. The IC_{50} of the 16(S) and 16(R) isomers differed by at least one order of magnitude. The difference in their biological activity appeared to be more significant than previously reported [13]. In fact, the potency of the original iloprost mixture was very close to that of the 16(S) isomer (Fig. 3), suggesting that the difference in the biological activity of the isomers might be related to different affinities of these two isomers for the platelet receptor. To confirm this, we performed equilibrium binding measurements using the individual isomers and the mixture as the ligand. We first established our binding equilibrium method using the racemic iloprost. A typical binding curve between the racemic iloprost and the platelet membrane preparation is shown in Fig. 4A. The data of specific binding were nicely fit for a single-binding species with K_d of 14 nM and a capacity of 984 fmol/mg membrane. Data analysis by a Scatchard plot yielded similar values of binding parameters (Fig. 4B). Statistical evaluation of five measurements performed on three different membrane preparations yields a K_d of 16.5 ± 6.1 nM ($\pm$S.D.) and a capacity of 743 ± 200 fmol/mg membrane protein.

The specific binding data of the 16(S) isomer (Fig. 5) were also fit for a single-binding species with a K_d and B_{max} similar to those of the racemic iloprost, i.e. 13.4 nM and 665 fmol/mg, respectively. By contrast, binding of the 16(R) isomer to the platelet membrane prostacyclin receptors was

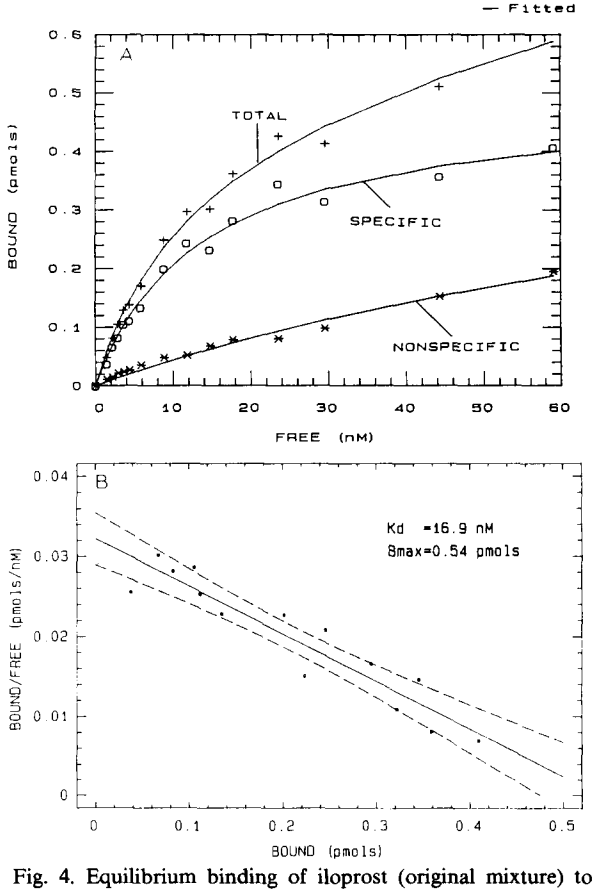

Fig. 4. Equilibrium binding of iloprost (original mixture) to platelet receptors. Each point represents an average of two measurements. Temperature, 23 ± 1° C. Nonspecific binding of iloprost with the filter membrane was tested to be less than 0.4% after five wash cycles. (A) Nonlinear regression analysis using single binding species for the data of 'nonspecific' and 'specific' binding and two species model for the data of 'Total' binding. (B) A transformation of the data of 'specific' binding by Scatchard. K_d and B_{max} were given in the figure.

much less avid with a K_d (288 nM) of at least one order of magnitude higher than the 16(S) isomer. The B_{max} (425 fmol/mg) was, on the other hand, comparable to the B_{max} of the 16(S) isomer (Fig. 5). The IC_{50} for the platelet antiaggregation activity, using either pure isomer or the isomer mixture, was smaller than the corresponding K_d values for receptor binding, suggesting a significant number of spare receptors.

To further assess the different binding behavior of these two stereoisomers, we carried out kinetic binding measurements. The 16(R) and 16(S) isomers exhibited surprisingly different association rates (Fig. 6). Association rates were measured at a membrane protein concentration of 2 mg/ml and an iloprost concentration of 15 nM. The observed association rate of the S isomer and the original mixture was very close (0.036 vs. 0.024 s^{-1} at 23° C). The association rate of the R isomer at the same membrane protein and ligand concentrations is as low as 0.001 s^{-1}. The noise associated with the kinetic binding data of the 16(R) isomer are probably contributed by the slow nonspecific binding processes to the membrane surface. Although the predominant association rate of the iloprost mixture was close to that of the (S) isomer, we did observe a second slower phase of association after the first fast phase was almost completed (data not shown). When the data were fitted by a two-exponential model, the rate obtained for the second slow phase was very similar to that obtained using R isomer alone. This second phase of association was therefore attributed to the binding of the 16(R) isomer.

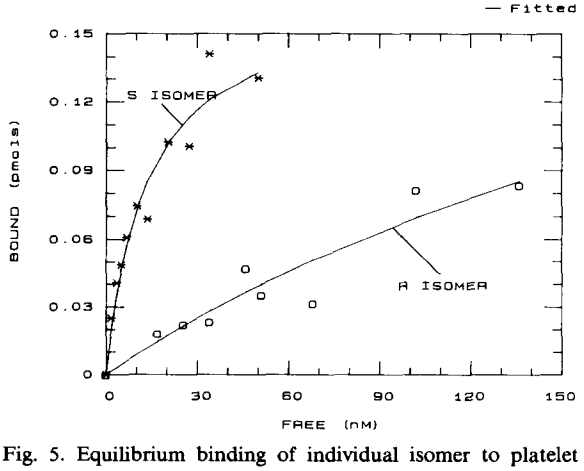

Fig. 5. Equilibrium binding of individual isomer to platelet receptors. Only specific binding is shown. Experimental conditions were the same as in Fig. 4.

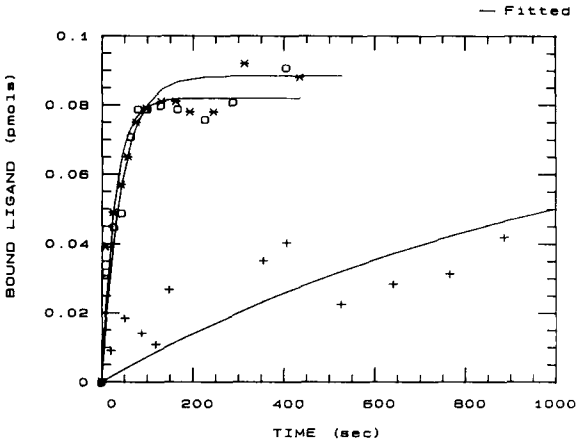

Fig. 6. Association of iloprost mixture (○——○), 16(S) isomer (*——*), and 16(R) isomer (+——+) with the platelet receptors. Nonspecific binding for each set of data was also obtained in the presence of 10 μM iloprost and was subtracted from the total binding to yield the specific association. Exponential fitting for the data using iloprost mixture was only performed for the first fast phase.

Discussion

We demonstrated a significant difference in the biological activity and binding equilibrium measurements between the 16(S) and 16(R) isomers of iloprost. The 16(S) isomer exhibits a significantly higher affinity for platelet membrane prostacyclin receptor and a more potent anti-aggregatory activity. The binding affinity of the 16(S) isomer differs from that of the 16(R) isomer by a factor of about 20. This large difference in the binding affinity between the R and S isomers indicates that the biologically efficient species in the iloprost mixture is the 16(S) isomer. The 16(R) isomer which constitutes about 60% of the entire iloprost molarity behaves as a 'spectator' due to its weak affinity. Thus, the potency of iloprost is attributable to the 16(S) isomer which constitutes only 40% of the molarity. To date all of the reported binding affinity measurements have been performed with the mixture of iloprost isomers, therefore the affinity data have generally been overestimated by a factor of 2. Hence, these

two isomers must be considered as two entirely different compounds when iloprost is used as the ligand for quantifying prostacyclin receptor binding. This is particularly important when the binding kinetics are determined. Otherwise, the data become difficult to interpret.

When the apparent free ligand concentration is adjusted for the actual concentration of the 16(*S*) isomer (which makes up 40% of the total) then the binding equilibrium measurements of the 16(*S*) iloprost match those employing the original iloprost mixture. Moreover, we have observed a single class of binding by either the 16(*S*) or the mixture. These results indicate that the 16(*R*) isomer contributes little to the binding nor does it interfere with the binding activity of the 16(*S*) isomer. Our binding equilibrium data are similar to those reported for platelets [6] and NCB-20 cells [6,14] but differ from those for bovine coronary arteries [15]. The latter tissue exhibits a heterogeneity of iloprost receptors. Although the reason for the discrepancy is not entirely clear, it may be speculated that these two stereoisomers may exhibit binding affinities that are not as different in the bovine coronary artery receptors as for platelet receptors. Consequently the use of a mixture of the two stereoisomers as the ligand may lead to the observed heterogeneity of iloprost receptors.

This study provides a model for understanding the mechanism governing the potency of synthetic stable prostacyclin analogues. Our kinetic data show that the interaction between the stereoisomers and the platelet receptors is dependent on the rate of association. This is unconventional because the decreased binding affinity of 16(*R*) isomer results from hindered accessibility of the ligand toward its receptors rather than a fast dissociation of the ligand from its receptors. Previous studies indicate that the molecular moiety beyond C-15 of prostacyclin is not important for the overall receptor interaction [4,5]. On the other hand, addition to the carbacyclin molecule of a methyl group at the C-16 position and introduction of a triple bond between C-18 and C-19 is reported to enhance the biological potency over the parent molecule [5,13]. Our findings indicate that molecular modifications beyond C-15 have an important impact on the biological activity as well as receptor binding. To illustrate this, we have prepared the spatial models for the *R* and *S* isomers by first acquiring the spatial coordinates for each atom through a molecular mechanics calculation program PCMODEL (Serena Soft-

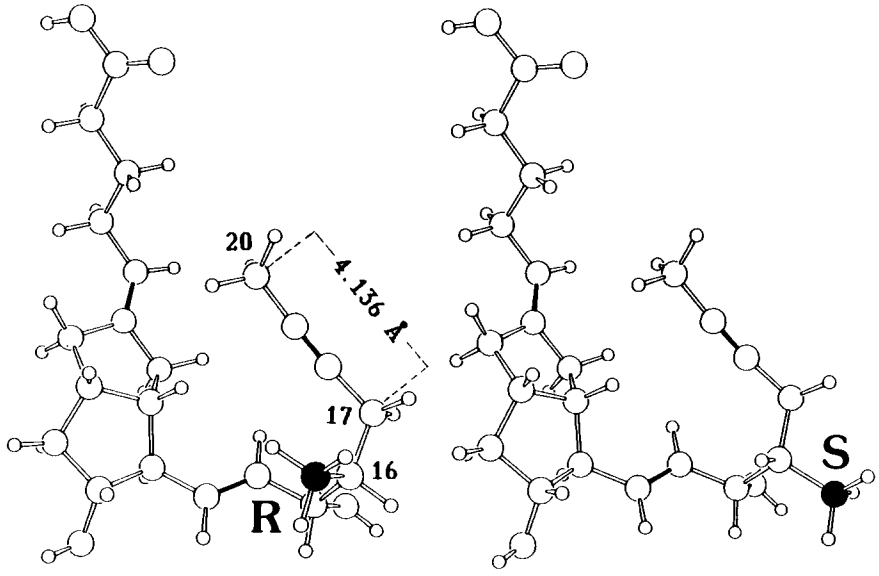

Fig. 7. Spatial model of the 16(*R*) and 16(*S*) stereoisomers of iloprost. The 16-methyl carbon atom is highlighted in both isomers. Multiple bonds are also highlighted. The distance between C-17 and C-20 was calculated based on the coordinates generated by PCMODEL.

ware, Bloomington, IN) and then drawing the spatial model using a transformed coordinate and an ORTEP graphic software. The spatial models suggest that the potency of the stereoisomers may depend on the orientation of the C-16 methyl group and its relationship to the triple bond at C-18 position (Fig. 7). Introduction of this triple bond results in a stiff linear segment about 4.5 Å extended from C-17 to C-20 tetrahedral protons. The presence of the methyl group at C-16 and the hydroxyl group at C-15 seems to limit the flexibility of the methyl end of iloprost (C-17–C-20). Since the 16(*S*) isomer exhibits much higher biological activity and binding affinity for prostacyclin receptors than the parent carbacyclin [16,17] and the 16(*R*) isomer, we postulate that the 16(*S*) stereoisomer possesses a more favorable configuration at the $-CH_3$ tail region than carbacyclin or the 16(*R*) form. We would suggest that the limited flexibility resulting from the (*S*)-methyl group can facilitate the binding of the entire iloprost molecule and permits the close interaction between the active functional groups of iloprost and the binding domain of the receptor. The 16(*R*)-methyl group, on the other hand, hinders the fitting of the iloprost molecule due to an abnormal orientation of the isomer in the receptor. Consequently, the association rate is reduced resulting in a low binding affinity for the receptor. The geometry of the C-17–C-20 tail of prostacyclin and its stable analogs is hence an important determinant for their interaction with their receptors on platelet membranes.

Acknowledgements

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