QSAR and the Rational Design of Long-Acting Dual D_2-Receptor/β_2-Adrenoceptor Agonists

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This paper describes the development of a QSAR model for the rational control of functional duration of topical long-acting dual D_2-receptor/β_2-adrenoceptor agonists for the treatment of chronic obstructive pulmonary disease. A QSAR model highlighted the importance of lipophilicity and ionization in controlling β_2 duration. It was found that design rules $\log D_{7.4} > 2$, secondary amine $pK_a > 8.0$, yielded ultra-long duration compounds. This model was used successfully to guide the design of long- and ultra-long-acting compounds. The QSAR model is discussed in terms of the exosite model, and the plasmalemma diffusion microkinetic hypothesis, for the control of β_2 duration. Data presented strongly suggests that β_2 duration is primarily controlled by the membrane affinity of these compounds.

Introduction

Chronic obstructive pulmonary disease (COPD) and asthma are diseases that are characterized by a marked increase in the hyperreactivity of the airways.[1] In the disease state, this hyperreactivity is an inappropriate response to a variety of stimuli, that leads to distressing exacerbations of the symptoms of COPD and asthma (bronchoconstriction, dyspnoea, cough, and mucus production).[2] While topically administered β_2-adrenoceptor agonists are the most commonly prescribed antibronchoconstrictor agents, and topically applied steroids reduce airway inflammation, there has been relatively little attention paid to the investigation of mechanisms that could specifically reduce airways hyperreactivity.

We have recently described an approach to control the underlying hyperreactivity of the lung based on modulation of sensory nerve traffic controlled through dopamine receptor activation.[3] Our working hypothesis has been that the stimulation of D_2 receptors on afferent nerves should lead to the inhibition of afferent nerve activity in the lung. Given the known emetic properties of D_2-receptor agonists, we proposed to administer our compounds topically to the lung and thus minimize systemic exposure. From our understanding of receptor pharmacology alone it seemed unlikely that D_2-receptor agonist activity would also afford useful antibronchoconstrictor activity. Consequently we set out to discover compounds that were dual D_2-receptor and β_2-adrenoceptor agonists with duration of action suitable for twice daily administration. While other papers have described our attempts to optimize D_2/β_2 and minimize unwanted α_1-adrenoceptor activity, this paper describes our attempts to control duration of action by D_2/β_2 agonists by rational drug design.

The control of functional duration of action remains one of the most difficult aspects of drug design. Functional duration is controlled by many factors including dose, potency, clearance, distribution, and sometimes the off-rate from the receptor itself. At the time of the onset of the project, salmeterol was already being viewed as the archetypal long-acting β_2 agonist. Its very long duration of action was attributed to binding to an exosite on the β_2-adrenoceptor, near to the agonist binding site.[4−6] Apart from defining a pharmacophore for long duration of action based on SAR, in vitro studies showed that while the agonist activity could be displaced by the β-adrenoceptor antagonist sotalol, agonism returned on washout of the antagonist from the receptor. This rather unusual persistence of agonism led to the development of the exosite concept. Molecular modeling studies and recent mutagenesis experiments have apparently identified the putative location of the duration-controlling exosite on the β_2-adrenoceptor.[7,8]

A second long duration β_2 agonist, formoterol, appeared to violate the exosite duration hypothesis.[9] Formoterol lacked the long lipophilic side chain of salmeterol, yet still showed similar persistence of agonism on washout to that of salmeterol and can show long functional duration in vivo. On the basis of studies with formoterol, it has been proposed that bulk lipophilicity and dose are the features that convey long duration.[9,10]

With what were apparently contradictory data published in the literature, we embarked on our own optimization exercise in attempt to control D_2 and β_2 functional duration and onset time based on a QSAR approach to compound design.

Results and Discussion

Our aim was to design compounds that would possess structural features that would allow us to control duration at both D_2- and β_2-adrenoceptors. While D_2- and β_2-adrenoceptors are distantly structurally related, it would be very likely that exosite control of duration would follow different structure−activity relationships at each receptor. Salmeterol has already been suggested to show selectivity of functional duration in vitro of β_2- (long) over β_1-adrenoceptors.[11,12] Our lead compound, **2**, Table 1, appeared not to fit the pharmacophore defined by salmeterol for accessing the exosite, but still showed salmeterol-like duration of action in the dura-

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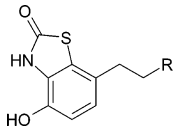
Table 1. Physicochemical Properties and β_2 Potency and in Vitro Duration Data for Compounds Studied



compd no.	side chain	logD$_{7.4}$	B = pK_a > 8; N = pK_a < 8	β_2 p[A]$_{50}$	int act.[b]	β_2 duration, min
1	$NH(CH_2)_6NH(CH_2)_2$benthiazolone	1[a]	B	7.86	0.40	45
2	$NH(CH_2)_6O(CH_2)_2Ph$	2.73[a]	B	8.23	0.50	>180
3	$NH(CH_2)_5O(CH_2)_2Ph$	2.19	B	7.80	0.32	>180
4	$NHCH(CH_3)(CH_2)_5O(CH_2)_2Ph$	3.03	B	8.08	0.45	>180
5	$NH(CH_2)_5O(CH_2)_3Ph$	2.72	B	7.00	0.30	>180
6	$NH(CH_2)_3SO_2(CH_2)_2NH(CH_2)_2Ph$	0.7	B	7.88	0.37	18
7	$NH(CH_2)_6O(CH_2)_2$(Ph-4-OH)	2.05	B	8.81	0.30	>180
8	$NH(CH_2)_5CONH(CH_2)_2Ph$	1.29[a]	B	7.18	0.35	22
9	$NHC(CH_3)_2(CH_2)_5O(CH_2)_2Ph$	3.43	B	8.42	0.46	>180
10	$NH(CH_2)_6NHCONHPh$	1.26[a]	B	7.01	0.20	60
11	$NH(CH_2)_6SO_2(CH_2)_2Ph$	1.44[a]	B	7.00	0.21	>180
12	$NH(CH_2)_6O(CH_2)_2$-2-pyridyl	1.24	B	8.12	0.45	109
13	$NH(CH_2)_6O(CH_2)_2$-2-thiazole	2.36	B	7.78	0.33	>180
14	$NHCH_2C(CH_3)_2(CH_2)_4O(CH_2)_2$(Ph-4-OH)	2.85	B	9.03	0.44	>180
15	$NH(CH_2)_6O(CH_2)_2$(Ph-4-NH_2)	1.49	B	9.40	0.40	>180
16	$NH(CH_2)_6O(CH_2)_2$(Ph-4-$NHCONH_2$)	1.42	B	9.20	0.49	140
17	$NH(CH_2)_3S(CH_2)_2O(CH_2)_2Ph$	2.31[a]	B	8.02	0.39	>180
18	$NHCH_2C(CH_3)_2(CH_2)_4O(CH_2)_2$(Ph-4-$NO_2$)	3.26	B	6.35	0.23	>180
19	$NH(CH_2)_6NHCH_2CF_2Ph$	1.57[a]	B	6.84	0.17	>180
20	$NHCH_2C(CH_3)_2(CH_2)_4O(CH_2)_2$(Ph-4-$NH_2$)	2.29	B	8.86	0.35	>180
21	$NH(CH_2)_3SO_2(CH_2)_2O(CH_2)_2Ph$	1.37[a]	B	7.93	0.27	140
22	$NH(CH_2)_3O(CH_2)_2O(CH_2)_2Ph$	1.19[a]	B	8.72	0.53	>180
23	$NH(CH_2)_2O(CH_2)_3O(CH_2)_2Ph$	2.06[a]	B	7.16	0.31	>180
24	$NHCH_2C(CH_3)_2(CH_2)_4O(CH_2)_2$(Ph-2-$NH_2$)	2.25	B	7.58	0.32	>180
25	$NHCH_2C(CH_3)_2(CH_2)_4O(CH_2)_2$(Ph-3-$NH_2$)	2.29	B	8.39	0.51	>180
26	$NH(CH_2)_2NHCO(CH_2)_2O(CH_2)_2Ph$	1.34[a]	B	9.27	0.58	57
27	$NH(CH_2)_2SO_2(CH_2)_3O(CH_2)_2Ph$	2.51[a]	N	8.09	0.51	146
28	$NH(CH_2)_2NH(CH_2)_3O(CH_2)_2Ph$	0.88[a]	B	7.01	0.54	32
29	$NH(CH_2)_2CF_2(CH_2)_3O(CH_2)_2Ph$	3.26[a]	B	7.77	0.49	>180
30	$NH(CH_2)_2S(CH_2)_3O(CH_2)_2Ph$	2.86[a]	B	8.36	0.54	>180
31	$NH(CH_2)_2NHSO_2(CH_2)_2O(CH_2)_2Ph$	1.56[a]	B	7.39	0.43	135
32	$NHCH_2C(CH_3)_2CH_2S(CH_2)_2O(CH_2)_2$(Ph-2-$NH_2$)	3.44[a]	B	7.13	0.39	>180
33	$NH(CH_2)_3SO_2(CH_2)_2O(CH_2)_2$(Ph-2-$NH_2$)	1.17[a]	B	7.46	0.26	33
34	$NH(CH_2)_2NHCO(CH_2)_3O(CH_2)_2Ph$	1.55[a]	B	7.56	0.27	71
35	$NH(CH_2)_3SO_2(CH_2)_2O(CH_2)_2$(Ph-2-$NO_2$)	1.37[a]	B	7.45	0.28	50
36	$NH(CH_2)_3SO_2(CH_2)_2NH(CH_2)_2OPh$	0.9[a]	B	6.73	0.45	28
37	$NH(CH_2)_3S(CH_2)_2O(CH_2)$2-pyridyl	0.83[a]	B	7.32	0.40	75
38	$NH(CH_2)_2SO_2(CH_2)_3O(CH_2)_2$-2-thiazolyl	2.35[a]	N	7.72	0.51	45
39	$NH(CH_2)_2SO_2(CH_2)_3O(CH_2)_2$(Ph-4-F)	2.67[a]	N	7.92	0.49	67
40	$NH(CH_2)_2NHCO(CH_2)_2O(CH_2)_3Ph$	1.91[a]	B	8.63	0.45	150
41	$NH(CH_2)_3SO_2(CH_2)_3O(CH_2)_2Ph$	1.65[a]	B	8.24	0.41	172
42	$NH(CH_2)_2S(CH_2)_3O(CH_2)_2$(Ph-4-$NO_2$)	3.29[a]	B	6.78	0.13	>180
43	$NH(CH_2)_3S(CH_2)_2O(CH_2)_2$-1-naphthyl	3.36	B	5.80	0.54	>180
44	$NH(CH_2)_2SO_2(CH_2)_3O(CH_2)_2$(Ph-4-$NO_2$)	2.32[a]	N	6.73	0.26	77
45	$NH(CH_2)_2S(CH_2)_3O(CH_2)_2$-2-pyridyl	1.75[a]	B	7.51	0.55	>180
46	$NH(CH_2)_3SO_2(CH_2)_2O(CH_2)_2$-1-naphthyl	2.54[a]	B	7.73	0.55	>180
47	$NH(CH_2)_2N(CH_3)CO(CH_2)_2O(CH_2)_2Ph$	1.52[a]	B	8.29	0.75	116
48	$NH(CH_2)_3S(CH_2)_3O(CH_2)_2Ph$	3.01	B	7.32	0.35	>180
49	$NH(CH_2)_3S(CH_2)_2O(CH_2)_2OPh$	2.11[a]	B	7.06	0.15	>180
50	$NH(CH_2)_3SO_2(CH_2)_2O(CH_2)_2OPh$	1.13[a]	B	7.44	0.48	52
51	$NH(CH_2)_2SO_2(CH_2)_3O(CH_2)_2$-2-pyridyl	0.95[a]	N	7.49	0.79	24
52	$NH(CH_2)_2SO_2(CH_2)_3O(CH_2)_2$(Ph-4-Br)	3.36[a]	N	7.40	0.52	>180
53	$NH(CH_2)_2S(CH_2)_3O(CH_2)_2$(Ph-4-$NHSO_2Ph$)	3.52[a]	B	7.57	0.53	>180
54	$NH(CH_2)_2SO_2NH(CH_2)_2O(CH_2)_2Ph$	2.38[a]	B	6.48	0.33	79
55	$NH(CH_2)_3S(CH_2)_2O(CH_2)_3Ph$	2.8[a]	B	7.86	0.30	>180
56	$NH(CH_2)_2S(CH_2)_3O(CH_2)_3Ph$	3.61[a]	B	7.27	0.47	>180
57	$NH(CH_2)_3SO(CH_2)_2O(CH_2)_2Ph$	1.12[a]	B	8.47	0.57	84
58	$NH(CH_2)_3SO_2(CH_2)_2O(CH_2)_3Ph$	2.1[a]	B	8.69	0.50	>180
59	$NH(CH_2)_3O(CH_2)_2S(CH_2)_2Ph$	1.73[a]	B	7.03	0.53	>180
60	$NH(CH_2)_2SO_2(CH_2)_3O(CH_2)_2$(Ph-4-$NHSO_2Ph$)	2.88[a]	N	7.70	0.70	>180
61	$NH(CH_2)_2SO_2(CH_2)_3O(CH_2)_4Ph$	3.62[a]	N	7.76	0.17	>180
62	$NH(CH_2)_2SO_2(CH_2)_3O(CH_2)_3Ph$	3.13[a]	N	7.35	0.36	157
63	$NH(CH_2)_2SO_2(CH_2)_3O(CH_2)_2$(Ph-4-OH)	1.91[a]	N	8.65	0.44	32
64	$NH(CH_2)_3SO_2(CH_2)_2S(CH_2)_2Ph$	2.34[a]	B	7.57	0.41	>180
65	$NH(CH_2)_2SO_2(CH_2)_3O(CH_2)_2$(Ph-4-$CONH_2$)	1.1[a]	N	7.54	0.40	50
66	$NH(CH_2)_2SO_2(CH_2)_3O(CH_2)_2$-2-(5-methylthiazole)	2.87[a]	N	7.61	0.69	126
67	$NH(CH_2)_3NHSO_2(CH_2)_2O(CH_2)_2Ph$	1.22[a]	B	7.51	0.36	69
68	$NH(CH_2)_3SO_2NH(CH_2)_2OCH_2Ph$	1.11[a]	B	7.40	0.59	72
69	$NH(CH)_2)_3SO_2NH(CH_2)_2O(CH_2)_2Ph$	1.46[a]	B	7.54	0.59	>180

[a] Measured logD$_{7.4}$. [b] Intrinsic agonist activity.

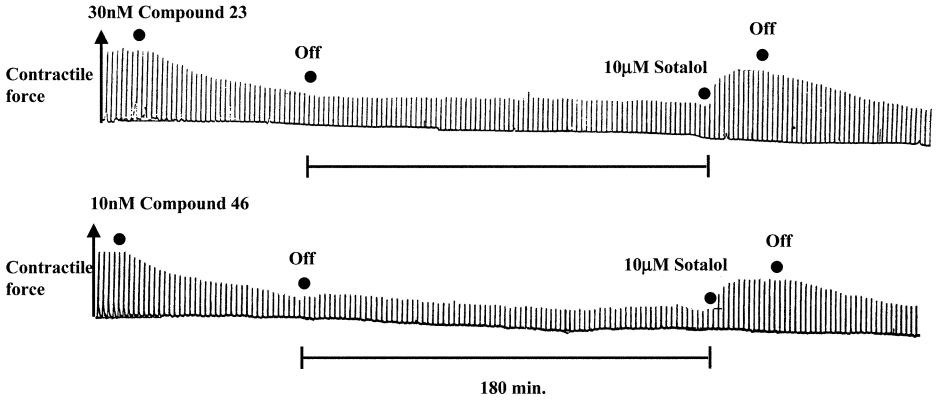


Figure 1. Contractile force vs time for typical long-acting D_2/β_2 agonists in the β_2 superfusion model. Infusion of compound is switched off at time $t = 0$ min, and the duration of tissue relaxation was measured. If no recovery in tissue tone was observed after 3 h, the tissue was recovered with 10 μM sotalol. If the infusion of sotalol is switched off subsequently and washed out of the tissue, reassertion of agonism can be observed.

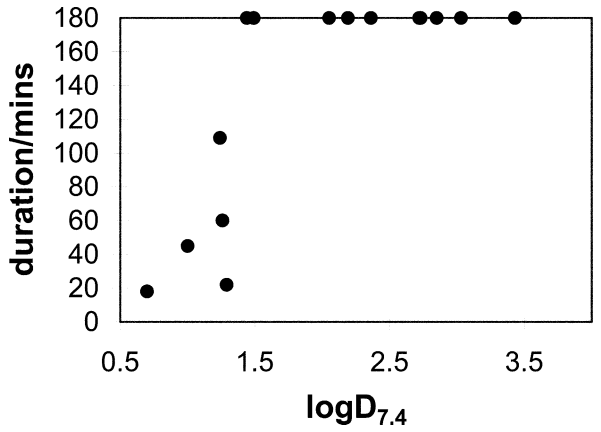


Figure 2. β_2 duration vs $\log D_{7.4}$ for early synthesized compounds **1**−**15**.

tion screen. (The salmeterol analogue of compound **2**, where the ether oxygen is six carbons from the secondary amine and two carbons from the terminal phenyl, has duration in the superfusion screen of only 10 min.[11]) Although we could not exclude the exosite hypothesis, we examined the possibility that lipophilicity alone was the major physical property controlling duration. If this was indeed a more important determinant of functional duration, it might also give us the opportunity to optimize both D_2 and β_2 duration simultaneously. As β_2 duration could be determined from an in vitro screen that was expected to predict in vivo β_2 duration well, QSARs for duration were primarily based on the β_2-adrenoceptor.

Early indications suggested that lipophilicity alone was enough to allow us to rationally control β_2 duration, Figure 2, Table 1. It appeared that high $\log D_{7.4}$ compounds showed very long duration of action, with reassertion of agonism upon washout. With $\log D_{7.4}$'s < 2.0, we observed a rapid decrease in duration and $\log D_{7.4}$ < 0.5; all compounds were short duration, essentially salbutamol-like in their behavior. Our rational design rule was to synthesize compounds with $\log D_{7.4} > 2$ to obtain very long duration compounds, at least at the β_2-adrenoceptor. The small amount of D_2 duration data we had surprisingly appeared to follow the β_2 duration data. This lipophilicity-based duration "rule" allowed us significant freedom in investigating optimization of D_2-, β_2-, and α_1-adrenoceptor potency (as will be described in further papers in this series), with confidence of maintaining long functional β_2 (and D_2) duration.

Table 2. pK_a Values for Selected D_2/β_2 Agonists with σ^* Values Calculated for Amino Chain Substituent on Aminoethyl-benzthiazolone Headgroup

compd no.	σ^*	pK_{a1}	pK_{a2}
17	0.1		10.19
21	0.24	7.35	9.07
22	0.12	7.65	9.23
23	0.29	7.55	8.68
26	0.22	7.62	8.75
27	0.59	8.55	6.58
31	0.23	7.50	8.58

A prerequisite for using this model was the ability to accurately estimate $\log D_{7.4}$ for compounds yet to be synthesized. This required good estimates of both calculated pK_as and logP to be made for compounds yet to be synthesized. Solubility limited the measurement of pK_as to only a small number of key compounds synthesized, Table 2. The amine pK_a was estimated using a Taft relationship derived from these measurements, eq 1

$$\text{amine } pK_a = 10.362 - 6.436 \times \sigma^* \qquad (1)$$

$$n = 7,\ r^2 = 0.917,\ F = 55.38,\ p = 0.0007$$

Introduction of polar substituents into the alkyl chain within three carbons of the basic amine, introduces new polar fragments, 2−3 carbon proximity corrections, as well as effects on the amine pK_a. These effects change $\log D_{7.4}$ directly as well as changing the neutral:zwitterion ratio. This made estimation of $\log D_{7.4}$ for many compounds rather difficult. But careful examination of $\log D_{7.4}$ changes of close analogues with already measured logD's enabled good estimates for synthetic targets to be made. Figure 3 shows the validity of this approach. Figure 3 shows the relationship between ClogP and $\log D_{7.4}$ retrospectively for the whole dataset where measured $\log D_{7.4}$ data is available. While the overall correlation is not strong, the correlation is composed of series of parallel correlations of close structural analogues, as has been illustrated for three compound series, $-NH(CH_2)_2SO_2R$, $-NH(CH_2)_3SO_2R$, and analogues without polar substituents close to the basic amine, Figure 3.

Although this simple model for duration appeared initially successful, we found a significant number of

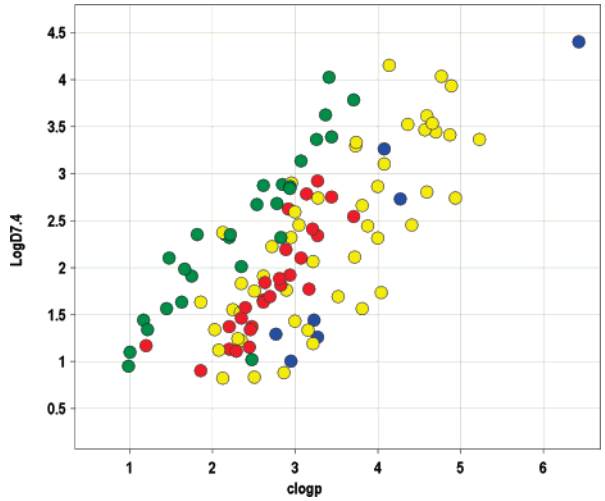


Figure 3. Plot of ClogP vs $logD_{7.4}$ for all measured D_2/β_2 compounds. Plot is color coded by substructure features; green circles = $NH(CH_2)_2SO_2R$, red circles = $NH(CH_2)_3SO_2R$, blue circles = $NH(CH_2)_4R$, yellow circles = all other D_2/β_2 compounds.

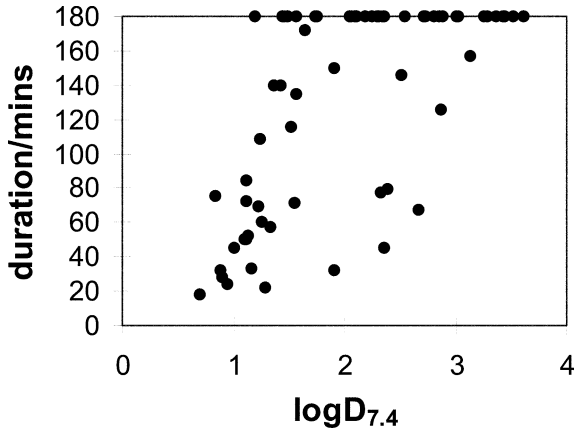


Figure 4. β_2 duration vs $logD_{7.4}$ for compounds **1**−**69**.

compounds that failed this model, Figure 4, Table 1. Viewed overall, our initial design rule was not valid.

Our hypothesis was that a new structure−activity relationship had been overlaid on the simple dependence of duration upon lipophilicity that was valid for early compounds. GRID-GOLPE and CoMFA had been successfully applied to complex SAR relationships involving bulk hydrophobicity and directional shape and hydrogen bonding interactions.[13,14] GRID-GOLPE analysis using an alkyl hydroxyl probe together with $logD_{7.4}$ suggested lipophilicity together with hydrogen bonding groups two and three carbons from the basic amine group appeared important in determining β_2 duration, Figure 5. Although this model appeared robust, it did not do well in predicting subsequent compounds. But the model prompted a closer analysis of the data-set.

Examining those compounds in Table 1 of duration < 180 min whose $logD_{7.4}$'s > 2 highlighted a common structural feature. All this series had a sulfonyl group two carbons from the secondary amine. One major effect this substitution has on the physicochemical properties of the compounds is to drastically reduce the pK_a of the basic secondary amine group. Introduction of SO_2 group two carbons from the secondary amine reduces the pK_a from >10 to 6.8. While other substitutions into this chain modulate the amine pK_a, only the $(CH_2)_2SO_2$

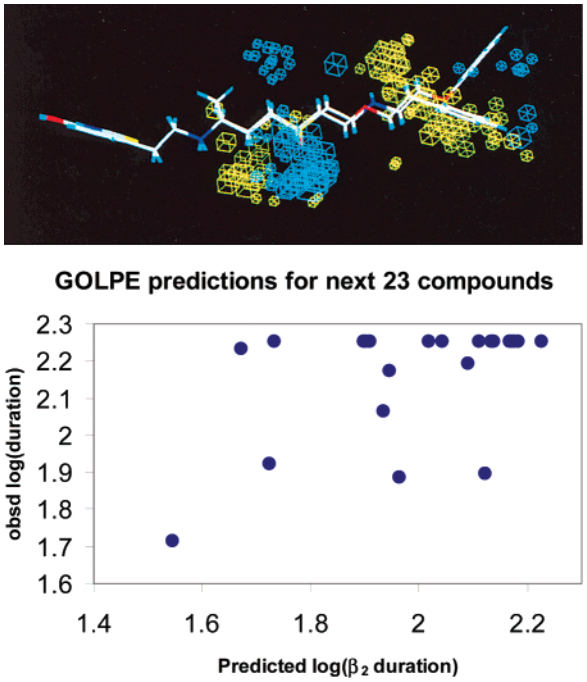


Figure 5. (a) GOLPE model, using compounds **1**−**24**, alkyl hydroxyl GRID probe, block-scaled with $logD_{7.4}$, variable reduction from 12168 variables to 188 using D-optimal selection followed by Full Factorial Design selection. Blue fields represent negative coefficients, good positions to place hydrogen binding groups, poor places to place steric bulk; yellow fields, good places for steric bulk, poor places for hydrogen binding interactions. (b) Duration predictions from the GOLPE model for 23 subsequently synthesized compounds.

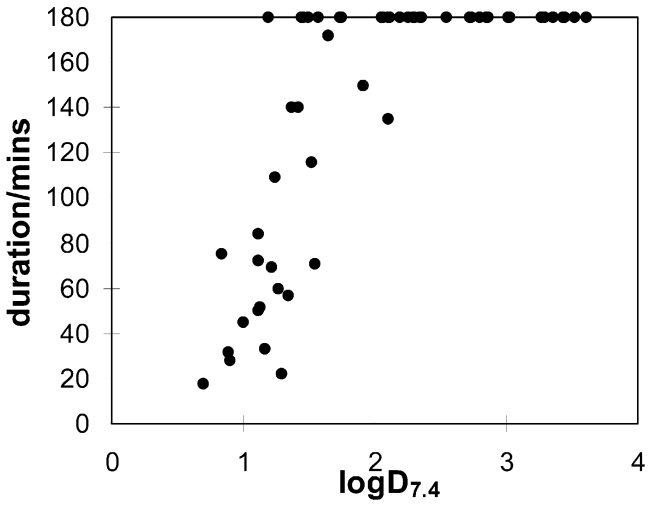
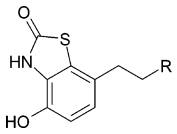


Figure 6. β_2 duration vs $logD_{7.4}$ for compounds with amine pK_a >7.4, basic amine series.

substitution reduced the amine pK_a so low, that it is predominantly uncharged at physiological pH. The dependence of duration upon $logD_{7.4}$ for the basic amine class is shown in Figure 6, while the dependence of the neutral amine series is shown in Figure 7. It is clear from Figures 6 and 7 that β_2 duration for compounds containing a weakly basic secondary amine and basic amine compounds follow qualitatively similar, but displaced, dependencies upon *n*-octanol−water $logD_{7.4}$ values. Neutral compounds needed to be at least 1−1.5 log units higher $logD_{7.4}$ to achieve a similar duration to basic compounds.

Table 3. Physicochemical Properties, Predicted β_2 Duration, and Observed β_2 Duration Data for Compounds Subsequently Synthesized



compd no.	side chain	log $D_{7.4}$	B = pK_a > 8 N = pK_a < 8	predicted β_2 duration, min	measured β_2 duration, min
71	$NH(CH_2)_2NHSO_2(CH_2)_3O(CH_2)_2Ph$	1.83[a]	B	180	151
71	$NH(CH_2)_3SO_2(CH_2)_3O(CH_2)_2Ph$	1.34[a]	B	80	45
73	$NH(CH_2)_3SO_2(CH_2)_2OCH_2Ph$	1.15[a]	B	60	74
74	$NH(CH_2)_2SO_2(CH_2)_3O(CH_2)_2$(1-isoquinolyl)	2.35[a]	N	50	60
75	$NH(CH_2)_2NHSO_2(CH_2)_2O(CH_2)_3Ph$	2.22[a]	B	>180	>180
76	$NH(CH_2)_3SO_2(CH_2)_2O(CH_2)_2$(Ph-5-$SCH_3$)	1.63[a]	B	150	>180
77	$N(CH_2)_3N(CH_3)SO_2(CH_2)_2O(CH_2)_2Ph$	1.24[a]	B	60	48
78	$NH(CH_2)_2SO_2(CH_2)_3O(CH_2)_2$(5-(4-methyl-1−3-thiazolyl)	1.44[a]	N	40	31
79	$NH(CH_2)_2SO_2(CH_2)_3O(CH_2)_2NHPh$	1.98[a]	N	40	32
80	$NH(CH_2)_2SO_2(CH_2)_3O(CH_2)_2$(2-(5-methylpyridyl)	1.56[a]	N	40	37
81	$NH(CH_2)_2SO_2(CH_2)_3O(CH_2)_2$(Ph-4-$OCH_3$)	2.42	N	60	56
82	$NH(CH_2)_2SO_2(CH_2)_3O(CH_2)_2$(Ph-4-$NHSO_2Me$)	1.34[a]	N	40	30
83	$NH(CH_2)_2SO_2(CH_2)_3O(CH_2)_2$-1-benzofuran	2.86[a]	N	160	>180
84	$NH(CH_2)_2SO_2(CH_2)_3O(CH_2)_2$(Ph-4-CN)	2.00[a]	N	40	40
85	$NH(CH_2)_3S(CH_2)_2O(CH_2)_2Ph$	3.53[a]	B	>180	>180
86	$NH(CH_2)_3SO_2NH(CH_2)_2S(CH_2)_2Ph$	1.77[a]	B	180	>180
87	$NH(CH_2)_3NHSO_2(CH_2)_2OCH_2$(1-naphthalene)	1.69[a]	B	180	>180
88	$NH(CH_2)_3SO_2NH(CH_2)_2O(CH_2)_2$(Ph-4-F)	1.57[a]	B	150	164
89	$NH(CH_2)_2SO_2(CH_2)_3)O(CH_2)_2$(Ph-2-$CH_3$)	2.84[a]	N	160	144
90	$NH(CH_2)_2SO_2(CH_2)_3O(CH_2)_2SPh$	2.68[a]	N	120	155
91	$NH(CH_2)_2SO_2(CH_2)_3O$-[(2*R*)-2-phenylpropyl]	2.32[a]	N	60	135
92	$N(CH_3)(CH_2)_3O(CH_2)_2O(CH_2)_2Ph$	2.44[a]	B	>180	>180
93	$NH(CH_2)_3SO_2(CH_2)_2O(CH_2)_3$(5-methylthiophene)	2.41[a]	B	>180	>180
94	$N(CH_2)_2CH_3)(CH_2)_3O(CH_2)_2O(CH_2)_2Ph$	2.74[a]	B	>180	>180
95	$NH(CH_2)_3SO_2NH(CH_2)_2O(CH_2)_2$(Ph-3-$CF_3$)	2.62[a]	B	>180	>180
96	$NH(CH_2)_2NHSO_2(CH_2)_2O(CH_2)_2$(Ph-3-$CH_3$,5-$CH_3$)	2.59[a]	B	>180	>180
97	$N(CH_3)(CH_2)_2N(CH_3)CO(CH_2)_2O(CH_2)_2Ph$	2.59[a]	B	>180	>180
98	$N(CH_2CH_3)(CH_2)_3O(CH_2)_2O(CH_2)_2Ph$	2.45	B	>180	>180
99	$NH(CH_2)_3SO_2NH(CH_2)O(CH_2)_2$(Ph-3-$CH_3$,5-$CH_3$)	2.92	B	>180	>180
100	$NH(CH_2)_2SO_2(CH_2)_3O$-[(1*R*)-1-methyl-2-phenylethyl]	1.81[a]	B	180	>180
101	$NH(CH_2)_3SO_2NH(CH_2)_2O(CH_2)_2$(Ph-3-Cl)	1.92[a]	B	180	>180
102	$NH(CH_2)_3SO_2NH(CH_2)_2O(CH_2)_2SPh$	1.84[a]	B	180	>180
103	$NH(CH_2)_3SO_2NH(CH_2)_2O(CH_2)_2$(Ph-4-$CH_3$)	2.07[a]	B	>180	>180

[a] Measured $logD_{7.4}$.

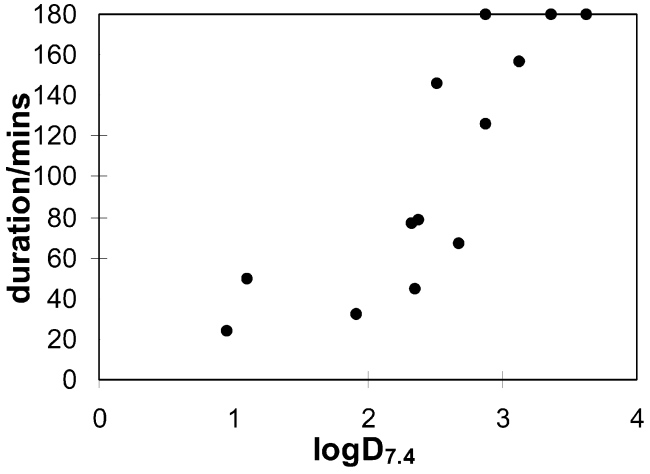


Figure 7. β_2 duration vs $logD_{7.4}$ for compounds with amine pK_a < 7.4, neutral amine series.

We were able to amend our initial design "rule" for long β_2 duration:

amine pK_a > 8.0:

$logD_{7.4}$ > 2 to obtain very long duration compounds, at the β_2 receptor

amine pK_a < 8.0:

$logD_{7.4}$ > 3.5 to obtain very long duration compounds, at the β_2 receptor.

These two rules were adequate to predict duration of subsequent compounds synthesized in this project. For example, Table 3 shows the excellent predictions for a subsequent group compounds synthesized by the project. Quantifying the predictivity is not straightforward. As we were attempting to predict compounds whose duration exceeded 180 min, many of the predictions are qualitative (>180 min). If we assume >180 min is a numerical prediction of 180 min, the predictivity can be assessed using the Q^2 statistic

$$Q^2 = 1 - \Sigma(y_{pred} - y_{obsd})^2/\Sigma(y_{obsd} - y_{mean})^2 = 0.91$$

This measure of predictivity could be criticized as being biased, generating an overoptimistic Q^2. As 18 compounds are predicted to be >180 min duration, this would lead to a predicted error of 0 for these compounds, artificially depressing the prediction error sum of squares, $(y_{pred} - y_{obsd})^2$. Excluding compounds whose observed duration was >180 min would remove this bias, and calculating prediction Q^2 on the remaining 15 compounds generates $Q^2 = 0.88$. This is a truly predictive QSAR model.

The Mechanism Controlling Duration. The observation of the critical importance of lipophilicity *and* ionization upon β_2 duration could be in accord with

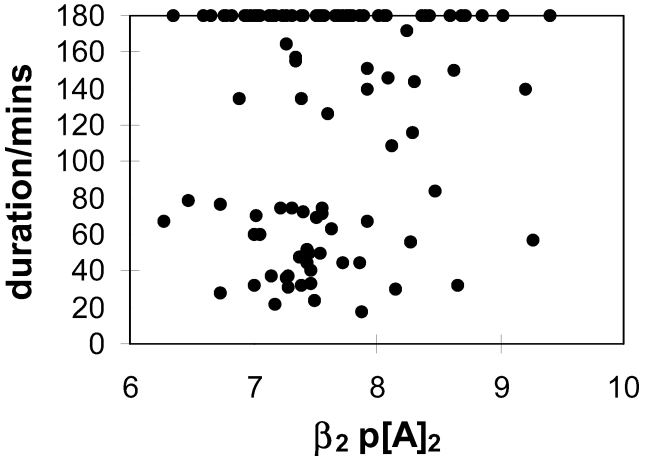


Figure 8. β_2 duration vs β_2 p$[A]_{50}$ for partial agonists with intrinsic activity < 0.5.

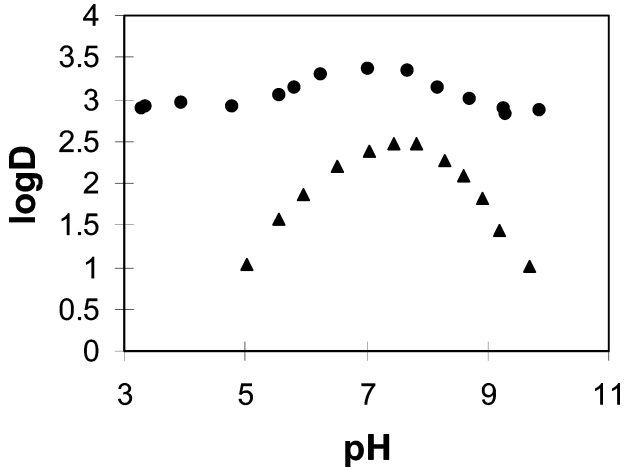


Figure 9. $logD_{1\text{-octanol/water}}$ and $logD_{membrane/water}$ distribution coefficients vs pH for compound **27** representative neutral compound. Circles, distribution coefficients measured in DMPC multilamellar vesicles; triangles, *n*-octanol−water distribution coefficients.

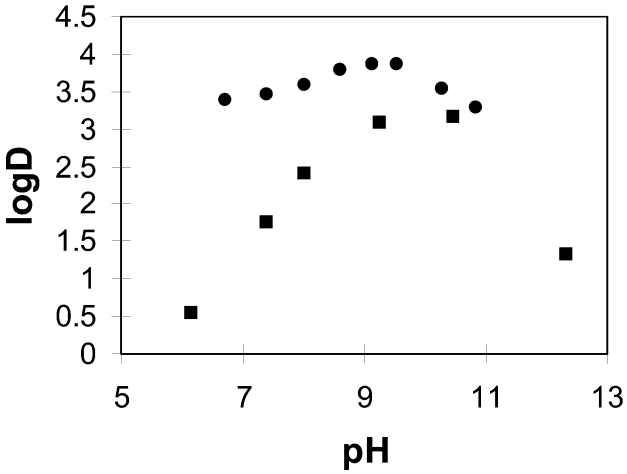


Figure 10. $logD_{1\text{-octanol/water}}$ and $logD_{membrane/water}$ distribution coefficients vs pH for salmeterol. Circles, distribution coefficients measured in DMPC multilamellar vesicles; squares, *n*-octanol−water distribution coefficients.

either the exosite or the plasmalemma diffusion microkinetic hypotheses.

How could the importance of amine pK_a and lipophilicity be rationalized with the exosite hypothesis? First, if the exosite were a lipophilic pocket, it would be expected that more lipophilic compounds could give greater affinity for the exosite and longer duration. Modeling studies suggest the putative exosite is indeed lipophilic.[7] Compounds with uncharged secondary amine nitrogen might be expected to form a much weaker interaction with the β_2-adrenoceptor and therefore show lower affinity and shorter duration. Duration of the neutral amine class might be regained, by making them more lipophilic to balance the loss in free energy from the charge interaction.

If the exosite is occupied simultaneously with the agonist-binding site, the free energy of binding to an exosite must manifest itself in the overall measured potency of these compounds to be thermodynamically consistent. As all compounds studied were previously shown to be partial β_2-adrenoceptor agonists with roughly similar efficacies, the measured potency should be a measure of receptor binding.[15,21] In which case, we should expect the overall duration to be correlated with potency. But not only did we find no overall correlation between β_2 potency and β_2 duration, Figure 8, some of the most potent compounds were some of the shortest duration compounds synthesized. We could only conclude that the secondary amine pK_a and lipophilicity were controlling duration through some other mechanism.

Another possible explanation is that the phospholipid membrane is itself the "exosite", and it is the partition into the bilayer that controls the duration of action. This is the basis for the plasmalemma diffusion microkinetic theory rationalizing β_2 duration. Studies have already shown that basic amines show a much greater affinity for phospholipid bilayers than would be predicted from their *n*-octanol−water distribution coefficients.[22,23,28,29] This is because lipophilic basic amines can partition into a phospholipid bilayer in an ordered way. They can form both an ion-pair interaction with the negative charged phosphate group of membrane phospholipids, while orientating their lipophilic groups down into the hydrophobic core of the bilayer. The closer in structure the base is to the geometry of the phospholipid membrane, the more favorable the distribution of the compound in this interfacial way would be. The importance of this interaction can be demonstrated by comparing the distribution of a basic compound between *n*-octanol−water and membrane−water with pH. The distribution−pH profiles of salmeterol and compound **27**, a representative of a neutral D_2/β_2 compound, between 1-octanol/water and DMPC phospholipid vesicles/water are shown in Figures 9 and 10. While at pH 7.4 compound **27** shows a 7-fold favorability for the phospholipid, salmeterol shows a >50-fold favorability for the membrane than for *n*-octanol. The relative increase in partition of salmeterol in phospholipid membranes is largely due to the efficient partition of the protonated form.

If membrane affinity was the primary factor controlling the observed β_2 duration, then using membrane−water instead of *n*-octanol−water distribution coefficients would cause the two separate dependencies of duration upon $logD_{n\text{-octanol/water}}$, for low/high p$K_a$ bases, Figures 6 and 7, to coalesce into a single dependence. Membrane affinities of a selection of basic and neutral

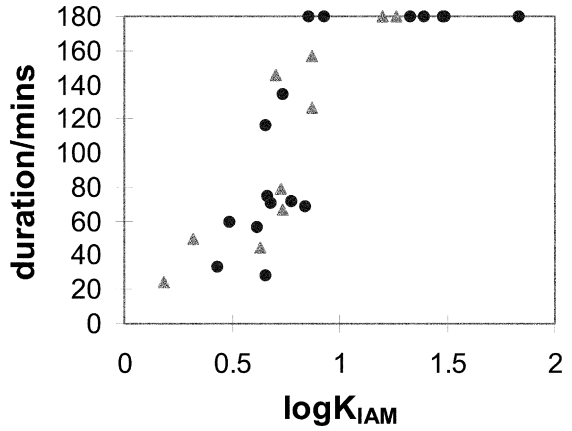


Figure 11. β_2 duration vs log K_{IAM} representative neutral and basic amine series compounds. Circles, compounds with amine pK_a > 8.0; triangles, compounds with amine pK_a < 8.0.

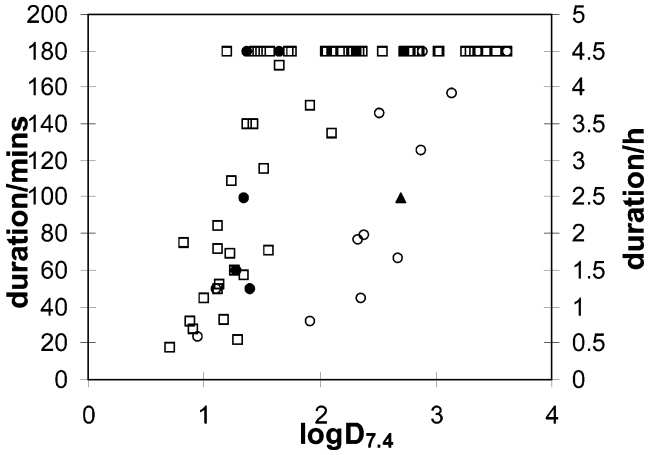


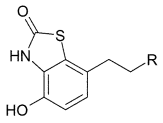
Figure 12. β_2 duration (Y1 axis), D_2 duration (Y2 axis) vs log K_{IAM} for representative neutral and basic amine series compounds. $y - 1$ axis β_2 duration; $y - 2$ axis D_2 duration Open squares, compounds with amine pK_a > 8.0 β_2 duration. Open circles, compounds with amine pK_a < 8.0 β_2 duration. Filled circles, compounds with amine pK_a > 8.0 D_2 duration. Filled squares, compounds with amine pK_a < 8.0 D_2 duration.

amine D_2/β_2 compounds were measured on a Regis membrane HPLC column. A graph of β_2 duration vs log(K_{IAM} column capacity factors) shows a single dependence for both neutrals and bases, Figure 11. This is very strong evidence that the primary mechanism controlling the observed changes in duration is in fact the compounds membrane affinity.

This would suggest that duration might be independent of the nature of the receptor. This is supported by the fact that the limited D_2 duration data quantitatively follows the dependence of the β_2 duration data upon log$D_{7.4}$ in all the compounds for which we have measured both activities, Figure 12, Table 4. While the bases change from short D_2 duration to long D_2 duration over the same log$D_{7.4}$ range as the β_2 duration data, it is particularly instructive that the only weakly basic amine measured in the D_2 duration assay is an outlier from the trend defined by the bases. It shows intermediate duration, as expected for its logD based on the in vitro β_2 duration model.

It is clear from this work that the major factor controlling duration of action at the β_2 receptor is the membrane affinity of these compounds. This does not abrogate necessarily the role of the receptor itself from

Table 4. Physicochemical Properties and in Vivo D_2 Duration Data for Selected Compounds



compd no.	side chain	log$D_{7.4}$	D_2 duration, min
2	$NH(CH_2)_6O(CH_2)_2Ph$	2.73	270
8	$NH(CH_2)_5CONH(CH_2)_2Ph$	1.29	90
17	$NH(CH_2)_3S(CH_2)_2O(CH_2)_2Ph$	2.31	270
21	$NH(CH_2)_3SO_2(CH_2)_2O(CH_2)_2Ph$	1.37	270
26	$NH(CH_2)_2NHCO(CH_2)_2O(CH_2)_2Ph$	1.34	150
41	$NH(CH_2)_3SO_2(CH_2)_3O(CH_2)_2Ph$	1.65	270
109	$N(CH_3)(CH_2)_3SO_2(CH_2)_2O(CH_2)_2Ph$	2.78	150

also playing a role in controlling duration. There are at least four main pieces of evidence supporting the duration control through exosite hypothesis:

1. In the superfusion assay, agonism of salmeterol can reassert itself after competitive displacement by a hydrophilic antagonist sotalol, followed by tissue washout. This reassertion can be repeated many times.[11]

2. Duration of salmeterol is specific to the β_2-adrenoceptor, as it is a short-acting agonist on the closely related β_1-adrenoceptor.[16,17]

3. Mutagenesis experiments with the β_2- and β_1-adrenoceptor and chimeric proteins appear to have identified the physical location of the exosite to a region on TM4 (aa 149−159).[8,26,18]

4. Although salmeterol is very lipophilic, its duration in biological membranes following washout is only 25 min, whereas its functional duration is in excess of 12 h.[19]

It is interesting though to consider how far considerations of membrane affinity can rationalize the published experimental data surrounding salmeterol and the exosite hypothesis. We will consider each of the four points above in turn:

1. In the superfusion assay, agonism of salmeterol can reassert itself after competitive displacement by a hydrophilic antagonist sotalol, followed by tissue washout. This reassertion can be repeated many times.

It is already well documented that reassertion of agonism is not unique to salmeterol and that similar behavior has also been demonstrated for formoterol, clenbuterol, and salmefamol.[9,10] But salmeterol is unique in that, unlike formoterol, it apparently cannot be washed out of the tissue—even after 12 h. This is mainly, though not exclusively, because of the increased lipophilicity of salmeterol relative to formoterol. The reassertion experiment may best be explained in terms of an exosite, but the exosite need not be part of the receptor, and considering the literature data and that presented in this paper, the biological membrane itself may be the exosite. The drug may enter and leave the receptor directly from the membrane, and hence the hydrophilic antagonist sotalol may displace salmeterol from the receptor. Upon washout, sotalol, having little membrane affinity, is easily washed out of the tissue whereas little salmeterol is lost and, on removal of sotalol, can reoccupy the receptor and hence show reassertion of agonism.

2. Duration of salmeterol is specific to the β_2-adrenoceptor, as it is a short acting agonist on the closely related β_1-adrenoceptor.[16]

This observation is contrary to the data presented in this paper. We find that the changes in β_2 duration observed in vitro and in vivo correlate closely with in vivo D_2 duration and $\log D_{7.4}$. As the D_2 receptor is in phylogenetic terms far more distant from β_2-adrenoceptor than is the β_1-adrenoceptor from the β_2-adrenoceptor, this is even more surprising. But the interpretation of the absolute measures of duration in differing in vitro screens may be equivocal in the absence of considerations of the lipid:water ratio as discussed in the following sections.

3. Mutagenesis experiments with the β_2- and β_1-adrenoceptor and chimeric proteins appear to have identified the physical location of the exosite to a region on TM4 (aa 149−159)[8,18]

This is some of the most intriguing data so far presented supporting the concept of the exosite in control of β_2 duration of salmeterol. If the duration of action of salmeterol were solely dependent upon the exosite, similar durations would be expected in cellular, in vitro tissue, and in vivo models. Even for the cloned wild-type β_2 receptor, salmeterol only shows a residence time in the receptor of 10−40 min, not the 12 h one observes in the superfusion screen. These differences in duration though may be partially understood from the mathematical model described in the following sections. But receptor mutations do appear to have a modulatory role upon duration, which cannot be explained by the plasmalemma diffusion microkinetic hypothesis. These residues may be important in controlling access of the drug in the lipid reservoir directly into the receptor, as required by the plasmalemma diffusion microkinetic hypothesis. If the bound drug cannot be displaced by antagonist to the plasma membrane or cannot reoccupy the receptor upon washout of the hydrophilic antagonist, duration will be compromised, as will its ability to show reassertion of agonism.

4. Although salmeterol is very lipophilic, its duration in biological membranes following washout is only 25 min, whereas its functional duration is in excess of 12 h.[19,27]

Published washout experiments from membrane vesicles suggest under washout salmeterol only has a tissue residence half-life of 25 min. These data were used by the authors to invalidate the membrane hypothesis, but this interpretation is in error, as the duration of the drug in the tissue is a function of its distribution coefficient, the rate constants describing transport of the compound between tissue and water phases, and the volumes and mutual surface area of the phases. It is possible to radically alter the duration of a compound in a piece of tissue within a tissue/water system simply by varying the phase volumes and mutual surface area even though the actual rate constants remain identical. This has implications not only to the interpretation of the already published washin and washout data, but also functional measurements made in cellular, in vitro tissue, and in vivo screens.

A crucial problem with the use of liposomes in the context of modeling tissue duration is the enormous disparity in surface area-to-volume ratio shown by liposomes and by pieces of tissue. The very large surface area-to-volume ratio characteristic of liposomes will tend to greatly increase the observed rate of compound equilibration compared to what would be observed in tissue. In an attempt to clarify the role of membrane affinity in the observed duration of salmeterol and the compounds herein described in this paper, the washin and washout kinetics of salmeterol were investigated in a more appropriate system than that studied by Herbette, the tracheal strip used in the superfusion screen itself. The experiment involved adding a tracheal strip prepared for the superfusion experiment to a stirred 15 μM solution of salmeterol followed by analysis of the solution with time using HPLC. A plot of the washin kinetics of salmeterol is shown in Figure 13. To extract rate constants from these data and to further understand the observed behavior, a mathematical model of washin and washout was constructed (for details see Supporting Information). The model assumes a simple situation involving an aqueous phase of volume V_a and a piece of tissue, which is assumed to be a homogeneous phase, of volume V_t and surface area A_t. The model also has two rate constants; k_{at} describes the transfer of the compound from the aqueous phase to the tissue, and k_{ta} describes the transfer of the compound from the tissue to the aqueous phase. Concentration terms in the model are C_a (aqueous concentration at time t) and $C_{a,0}$ (initial aqueous concentration) and $C_{a,e}$ (equilibrium aqueous concentration). According to this simple model, the aqueous concentration of a compound when transporting into a piece of tissue should follow eq 2.

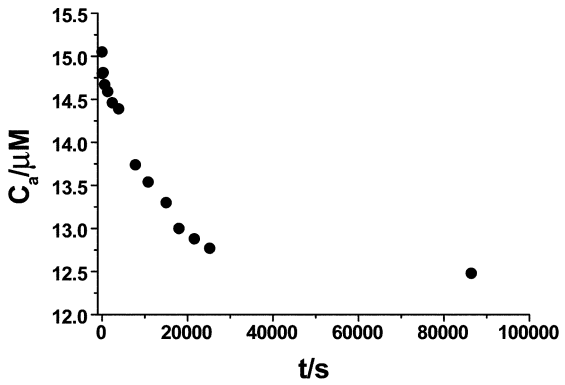


Figure 13. Plot of aqueous concentration against time for transport of salmeterol into tracheal tissue.

$$\ln(C_a - C_{a,e}) = -A_t\left(\frac{k_{at}}{V_a} + \frac{k_{ta}}{V_t}\right)t + \ln(C_{a,0} - C_{a,e}) \quad (2)$$

According to this equation a plot of $\ln(C_a - C_{a,e})$ against time should give a straight line of slope equal to $-A_t(k_{at}/V_a + k_{ta}/V_t)$. This plot is shown in Figure 13. The plot shows good linearity, and the half-life is 145 min. The equilibrium concentrations from the washin experiment allow the calculation of the tissue/water distribution coefficient, which is also the ratio k_{at}/k_{ta} and is equal to 274. Knowledge of the volume and surface area terms in eq 1 along with the slope of the line in Figure 14 then allows the calculation of the sum of the rate constants. The individual values of the rate con-

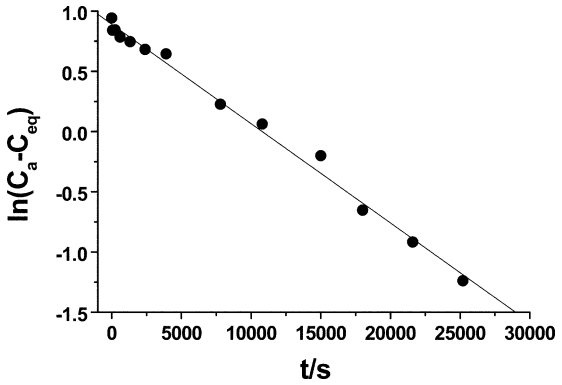


Figure 14. Plot of $\ln(C_a - C_{eq})$ against time for transport of salmeterol into tracheal tissue.

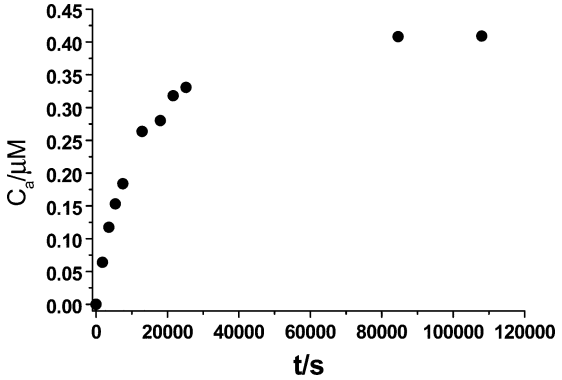


Figure 15. Plot of C_a against time for transport of salmeterol out of tracheal tissue.

stants are then found to be $k_{at} = 2.5 \times 10^{-4}$ cm s^{-1} and $k_{ta} = 9.2 \times 10^{-7}$ cm s^{-1}. These values are used later in a simulation of the in vitro superfusion screen.

A second washin experiment was performed using a different piece of tissue and a different aqueous volume. The data again showed good first-order behavior, and the half-life was rather longer at 182 min. However, this longer half-life is due to the different aqueous volume, tissue volume, and tissue surface area. The rate constants extracted from the experimental data, $k_{at} = 2.2 \times 10^{-4}$ cm s^{-1} and $k_{ta} = 7.7 \times 10^{-7}$ cm s^{-1}, are very close to those from the first experiment, which goes some way to validate the kinetic model. The tissue/water distribution coefficient k_{at}/k_{ta} was found in this experiment to be equal to 285. The model also suggests that the half-life for washing the compound back out of the tissue when it is added to fresh buffer should be identical to that for the washin experiment if the aqueous volumes are the same in each experiment. Figure 15 shows the data for just such an experiment when the tissue from the first washin experiment was added to fresh buffer. The data again followed good first-order kinetics, and the half-life for washout was 186 min compared to 182 min for washin with the same aqueous volume. This observation again helps validate the model, which is very important since further steps are now needed to go from a measured half-life of 3 h under semisink conditions (8 mg of tissue in 25 mL of buffer) to an estimate of the half-life under superfusion conditions (8 mg tissue under aqueous perfusion of 2 mL min^{-1}). It is not immediately obvious if the compound will persist longer in the tissue under semisink conditions or under perfusion. However, the duration under perfusion can be modeled using the following steps. The perfusion conditions are described by a flow rate F (mL s^{-1}), and as this solution passes over the tissue it collects on the tissue in droplets of volume V_a (mL). The time that the tissue is in contact with each droplet of volume V_a is given by V_a/F s. Knowledge of the parameters V_a, V_t, k_{at}, k_{ta}, and A_t along with a suitable equation (see Supporting Information) allows calculation of the tissue concentration after the time for which the tissue is in contact with the first droplet. This tissue concentration now becomes the new initial concentration, and the transfer of compound into the second droplet is then simulated as for the first. This process is repeated for each droplet using the recurrence relation shown in eq 3, which gives the tissue concentration after the passage of n droplets $C_{t,n}$ at time $t = nV_a/F$ in terms of the tissue concentration after $n - 1$ droplets $C_{t,n-1}$.

$$C_{t,n} = \frac{C_{t,n-1}}{r + D_t}\left[r \exp\left(-\frac{A_t V_a}{F}\left(\frac{k_{at}}{V_a} + \frac{k_{ta}}{V_t}\right)\right) + D_t\right] \quad (3)$$

In eq 3, r is the phase volume ratio equal to V_a/V_t, and D_t is the tissue/water distribution coefficient equal to k_{at}/k_{ta}. The droplet volume V_a was estimated to be that of a typical water droplet at 0.05 mL. However, variation of this parameter changes the simulated kinetics little, because bigger droplets lead to longer equilibration times since the perfusion rate is constant. Using this description of the perfusion, the half-life of the tissue concentration of salmeterol is estimated to be rather longer than under the semisink conditions and is equal to 4 h. This model assumes that every drop is in contact with the tissue until the next drop is released. For a perfusion rate of 2 cm^3/min and a drop-size of 0.05 cm^3, the equilibration time would be $V_a/F = 1.5$ s. But observing the behavior of drops in the screen demonstrated the drop would roll off the tissue more quickly than this, leading to shorter equilibration times per drop at constant infusion. Assuming a 0.05 cm^3 drop every 1.5 s that equilibrates for 0.25 s would drastically change the half-life from 4 h to 20 h. Considerations of membrane affinity and washin and washout kinetics alone quite adequately describe the observed duration of salmeterol in the superfusion screen.

The main evidence supporting the exosite theory appears equivocal. The evidence presented in this paper strongly suggests membrane affinity being the major controlling factor of β_2 duration. There are a number of key pieces of evidence that argue against the exosite being part of the β_2 receptor:

1. The potency of this large series of β_2 partial agonists does not correlate with duration.

2. Duration in a distantly related GPCR receptor D_2 receptor exactly follows that of the β_2 data.

3. Duration of salmeterol is not constant in cellular, in vitro, and in vivo screens, suggesting the exosite cannot be part of the β_2 receptor directly.

4. A recent publication demonstrates that while salmeterol is long duration following an inhaled dose, it is short duration following an equieffective iv dose.[20]

5. Simulations presented in this paper suggest it is reasonable for the washout of salmeterol in the in vitro superfusion experiment to show long duration >12 h and appear to show dose independent duration.

Conclusions

A QSAR model is presented that was used to guide the synthesis of ultra-long-acting and long-acting dual D_2/β_2 agonists. Duration was found to be controlled both by the bulk lipophilicity of the molecules, as measured by *n*-octanol−water distribution coefficients, *and* the basicity of the secondary amine. It was found that basic and weakly basic secondary amine compounds showed similar but separate duration−log$D_{7.4}$ profiles. The fact that these two separate dependencies could be collapsed to a single profile by describing duration using membrane−water distribution coefficients (measured using an IAM membrane column) instead of *n*-octanol−water distribution coefficients strongly supports the primary importance of membrane affinity controlling D_2/β_2 duration. It has recently been suggested that the high volumes of distribution and the differing sites of distribution shown by bases in vivo is a function of their high membrane affinity.[21,22] Data presented in this paper further highlight the problems in the *n*-octanol−water partitioning system in predicting the in vivo properties of drugs and supports the importance of considering membrane distribution as a complementary physicochemical descriptor of the in vivo behavior of drugs.

Experimental Section

The synthesis of all compounds has been described previously.[23,24] The *n*-octanol−water distribution coefficients were determined by the well-known shake-flask method.[25] Where simple analogues were being prepared, estimated logDs were used based on measured values of close analogues and ClogP's from MEDCHEM.[26] Ionization constants were determined for selected compounds by potentiometric titration at 25 °C in 0.1 M KCl using a Radiometer Titralab 90 autotitrator and the program BEST for estimating pK_a's from the potentiometric data.[27] Because of the very poor solubility of many of the compounds at pHs between the phenol and amine pK_as, pK_a values were estimated based on a Taft plot derived from the more soluble compounds in the series. Membrane distribution coefficients into dimyristoylphosphatidylcholine (DMPC) multilamellar vesicles were measured at pH 7.4 as previously described.[28,29]

Determination of the Capacity Factor on the Immobilized Artificial Membrane Column (IAM). Membrane affinities were determined from capacity factors on the Regis immobilized artificial membrane high performance liquid chromatography column.

Capacity factors (K_{IAM}) determined on the IAM column are well correlated with the partitioning into phospholipid vesicles.[30]

All measurements were carried out at pH 7.4 using disodium orthophosphate/monosodium orthophosphate (Sorensons buffer) at 0.03 M. All buffers were filtered prior to use, as this was found to be critical to the lifetime of the column. The column used was the 3 cm Regis IAM.PC.DD column fitted with a 1.0 cm guard column. The flow rate used for all compounds was 1 mL/min, and detection was carried out at the λ_{max} of the compound. The concentration of the compound was around 0.25 mg/mL, and the injection volume used was 20 μL. The column temperature was maintained at 40 °C and was found to be important for the reproducibility of capacity factors. The effect of temperature on capacity factors determined on the IAM column has been well described previously.[31] The column performance was monitored using benzoic acid and *p*-toluidine as recommended by the manufacturer. The reproducibility on a day-to-day basis was very good with repeat log K_{IAM} values being determined to within ±0.1 units. Small changes in pH and ionic strength were observed to have marked effects on the capacity factor and care was needed in the preparation of the mobile phases. The column-to-column variability was found to be acceptable and was around that which was found on the day-to-day variability of 0.1 log unit. In all cases the void volume was determined using citric acid as set out in the manufacturers recommendations.

Where possible the capacity factor was determined using only an aqueous mobile phase. However, when the affinity of the compound was too high to be eluted under these conditions, an organic modifier was added to the mobile phase. Acetonitrile HPLC grade, for UV, was found to be the optimum solvent. A calibration graph of capacity factor versus percentage organic modifier was then constructed and the retention time at 0% modifier determined by extrapolation. For neutral compounds the plot of capacity factor versus % organic modifier was linear with $r^2 > 0.9$. For basic compounds, the calibration was not linear, and the retention time had to be determined using a purely aqueous mobile phase.

The retention time (t_r) of the compound, together with the retention time of the void volume marker (t_0) was used to calculate the capacity factor from the following equation:

$$K' = \frac{t_r - t_0}{t_0}$$

HPLC System. A Waters modular system consisting of a 600s controller unit, a 996 photodiode array detector, a 616 pump, and a 717 autosampler was used for the whole of this work. The data analysis was performed using Millennium Software.

Pharmacology. β_2 duration was determined using the electrically stimulated, guinea-pig isolated trachea superfusion system, described previously.[32] Before being tested in this system, the β_2-adrenoceptor potencies (pA_{50}) and intrinsic activities (α) of all the compounds were measured in rings of guinea-pig isolated trachea under standard organ bath conditions.[33] Using this potency information, the β_2 effects of the agonists (submaximal concentrations) were subsequently studied in the superfusion system. The effects of the compounds were routinely studied for a period of 180 min at which time the agonist effects were reversed by the addition of the β_2-adrenoceptor antagonist, sotalol (10 μM), Figure 1. Earlier experience with a range of compounds demonstrated that compounds which showed no recovery in 180 min would show no recovery after many subsequent hours. Hence, they were classified as salmeterol-like in their duration. Therefore, our target was to discover compounds that showed no recovery in the β_2 superfusion assay after 180 min.

An equivalent in vitro model of D_2-receptor duration was not available, and therefore we were unable to carry out similar experiments to assess the behavior of our dual D_2/β_2-adrenoceptor agonists. Consequently, all structure−activity work was focused on optimizing β_2 duration. A small number of compounds were assessed for D_2 duration in vivo, the procedure for which has been described elsewhere.[34]

Kinetic Studies. The kinetics of salmeterol washin and washout using guinea pig tracheal strips was measured in duplicate, and followed at 25 °C using HPLC with UV detection. Guinea pig tracheal strips were weighed and dimensions were also measured in order to calculate the surface area of the tissue. For the washin experiments, the strip was suspended in a beaker of pH 7.4 Krebs buffer (experiment 1 volume = 10 mL, experiment 2 volume = 25 mL) containing salmeterol at an initial concentration of 15 μM. The contents of the beaker were then stirred with a magnetic stirrer, 50 μL aliquots of the solution were removed at regular time intervals for HPLC analysis, and the peak areas were compared with a standard curve previously measured for salmeterol. The washout experiment was carried out by removing a piece of tissue that had come to equilibrium in a washin experiment and adding it to 25 mL of fresh buffer followed by measurement of the salmeterol concentration in the buffer as a function of time using HPLC with UV detection as before. The tissue/water distribution coefficients were calculated from the measured aqueous concentrations at equilibrium in the washin experiments and the volume of the aqueous phase and tissue (tissue volume calculated from tissue mass assuming density of 1 g/mL).

Statistical Analysis. Statistical analysis was performed using RS/1.[35] 3-D QSAR was performed using 3-D interaction fields calculated using GRID,[36] block-scaled with $\log D_{7.4}$, and PLS analysis with variable selection using GOLPE.[37] GOLPE was run using default parameters, with D-optimal selection followed by full-factorial design selection.

Supporting Information Available: Derivation of the kinetic model for the transfer of a compound between an aqueous and a tissue phase. This material is available free of charge via the Internet at http://pubs.acs.org.

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