Using esterase selectivity to determine the *in vivo* duration of systemic availability and abolish systemic side-effects of topical β-blockers.

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Abstract

For disorders of the skin, eyes, ears and respiratory tract, topical drugs, delivered directly to the target organ, are a therapeutic option. Compared with systemic oral therapy, the benefits of topical treatments include a faster onset of action, circumventing the liver first pass drug metabolism, and reducing systemic side effects. Nevertheless, some systemic absorption still occurs for many topical agents resulting in systemic side-effects. One way to prevent these would be to develop drugs that are instantly degraded upon entry into the bloodstream by serum esterases. Because topical β-blockers are used in glaucoma and infantile haemangioma and cause systemic side-effects, the β-adrenoceptor system was used to test this hypothesis. Purified liver esterase reduced the apparent affinity of esmolol, an ester-containing β-blocker used in clinical emergencies, for the human β-adrenoceptors in a concentration and time-dependent manner. However, purified serum esterase had no effect on esmolol. Novel ester-containing β-blockers were synthesised and several were sensitive to both liver and serum esterases. Despite good in vitro affinity, one such compound, methyl 2-(3-chloro-4-(3-((2-(3-(3-chlorophenyl)ureido)ethyl)amino)-2-hydroxypropoxy)phenyl)acetate (**10**), had no effect on heart rate when injected intravenously into rats, even at 10 times the equipotent dose of esmolol and betaxolol that caused short and sustained reductions in heart rate respectively. Thus ester-based drugs, sensitive to serum esterases, offer a mechanism for developing topical agents that are truly devoid of systemic side-effects. Furthermore, differential susceptibility to liver and serum esterases degradation may also allow the duration of systemic availability for other drugs to be fine-tuned.

Keywords

β-blocker, serum esterase, skin tumour, glaucoma, reduced systemic side-effects

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For disorders of the skin, eyes, ears, respiratory tract and even the gastrointestinal (GI) tract, drugs can be given topically to act locally and thus minimise systemic absorption. Compared with oral therapy, topical drugs also have the benefit of a potentially faster onset of action as they are delivered directly to the target organ. Topical administration also circumvents the issues of first pass drug metabolism by the liver, and, by sparing the rest of the body, minimises systemic side effects. However, for many topical agents, some systemic absorption occurs nevertheless, resulting in systemic side-effects. For example, topical steroids (creams for dermatological disorders and inhaled for asthma and COPD) are commonly used but systemic absorption does occur and over time can result in adrenal insufficiency, osteoporosis, increased risk of pneumonia and stunting of growth in children[1-3]. Furthermore, precise dosing of topical agents is difficult to achieve (e.g. how much cream? how many drops?) resulting in variable daily doses and thus variable systemic absorption.

Topical β-blocker eye-drops (e.g. betaxolol and timolol) are used to treat glaucoma[4], however absorption into the blood stream via the lacrimal duct and GI tract can cause significant cardiovascular and respiratory systemic side effects[5-16]. These can be life-threatening or even fatal[17]. In some cases, the systemic bioavailability is both rapid and high (e.g. 15 minutes and 78% for timolol eye-drops[18]).

More recently, systemic administration of propranolol was found profoundly beneficial for the treatment of infantile haemangiomas[19] and has now become the treatment of choice for this condition, including moves to initiate and maintain treatment in the outpatient setting[20]. However, predictable serious systemic side-effects do occur (bradycardia, hypotension, bronchospasm and hypoglycaemia) as well as more minor issues (sleep disturbance, diarrhoea, cold peripheries and agitation[21-22]). Furthermore, there is concern over the CNS development and growth impairment with systemic medications given to neonates for prolonged periods[23], although two recent studies have suggested no impairment of psychomotor development or growth impairment (at age three years[24-25]). Other β-blockers, have also been shown to be beneficial in infantile haemangioma (e.g. timolol and atenolol[26-28)] and there is increasing interest in using topical β-blockers, and developing improved topical delivery methods, in order to avoid the significant systemic problems associated with oral propranolol[29-34]. β-Blocker treatment for

retinopathy of prematurity has been shown effective (although 20% of infants had serious adverse systemic side-effects[35-36]). β-blocker treatment for other topical vascular tumours are now being investigated and used successfully e.g. pyogenic granuloma[37-38] and Kaposi's sarcoma[39-40] and in reducing epistaxis in hereditary haemorrhagic telangiectasia[41].

Preventing systemic absorption from all the varied potential sites is very difficult, but a method to prevent systemic side-effects would be to ensure that the drugs are instantly degraded upon entry into the blood stream. One answer may be to take advantage of enzymes present in the serum, e.g. the esterases, to enhance the degradation of the drug [42]. Esmolol is a short-acting, methyl ester β-blocker that can be used for intravenous therapy in emergency clinical situations (e.g. during intubation, cardiac arrhythmias, during ECT (electroconvulsive therapy)) and to lower heart rate before cardiac imaging and has been proposed to be safer in those in whom longer-term β-blockade is undesirable[43-44]. Esmolol is hydrolysed into an acid metabolite and methanol by esterase enzymes, primarily thought to be those in the red blood cell (rbc), however the fact that it still has substantial short-term β-blocking action means that its hydrolysis is not instant (half-life in human plasma is 9 minutes[44]).

This study examined whether it was possible to synthesize compounds that retained good *in vitro* properties (similar to topical dosing) but if systemically dosed (by injection directly into the blood stream) would hydrolyse immediately to give no systemic effects. Given the increasing efforts to find topical β-blockers devoid of systemic effects for the treatment of haemangiomas in infants, as well as a potential benefit for glaucoma treatments, and the fact that heart rate gives an immediate measure of systemic β-blockade, we chose to explore β-blockers as an example to test this hypothesis. We examined the esterase sensitivity of esmolol to understand the locations of esmolol hydrolysis in the body. We then synthesized a range of novel ester-β-blockers and measured their susceptibility to liver, serum and rbc esterases. We injected the compounds directly into conscious rats whilst continuously measuring changes in heart rate and finally measured the β-blocking effects of the compounds after exposure to human serum.

Results and discussion

Many drugs are given as topical agents which have the potential benefit of delivering the drug directly to the target whilst minimising any systemic side effects. However, for several of these, systemic absorption does occur and therefore many have the potential to cause significant systemic side effects. This study investigated whether it was possible to generate an active compound that would degrade upon injection into the blood stream. As an example of drugs with clinical dermatological and ophthalmological applicability, we examined whether or not it was possible to synthesise a novel β-blocker with good *in vitro* properties (i.e. potentially similar to a topical treatment) that was degraded so rapidly that even when injected directly into the blood stream, it displayed no *in vivo* activity.

Validation of cell lines

Saturation binding experiments yielded K_D values for ^{3}H-CGP12177 of 0.49nM (CHO-β1) and 0.28nM (CHO-β2[45]). The β1-antagonist CGP20712A inhibited the specific binding of ^{3}H-CGP12177 with high affinity in the CHO-β1 cells (log K_D -8.76 ± 0.04, n=32) and the β2-antagonist ICI118551 with low affinity (log K_D = -6.66 ± 0.02, n=38). In the CHO-β2 cells, ICI118551 was found to have high affinity (log K_D of -9.20 ± 0.02, n=38) and CGP20712A low affinity (log K_D = -5.74 ± 0.02, n=37), similar to previous values[46], thus confirming the presence of the human β1 and β2-adrenoceptors (ARs) in the respective cell lines.

Characterisation of existing β-antagonists

Initially, we characterised the properties of two β-blockers commonly used in the treatment of glaucoma and increasingly also used in infantile haemangioma: betaxolol and timolol. Betaxolol was confirmed to have slight β1-selectivity (12.6-fold) whilst timolol had slight β2-selectivity (14.8-fold; Figure 1, Table 1), in keeping with previous findings[46]. Esmolol is a short-acting ester-containing β-blocker that can be given intravenously during certain medical emergencies, for example during anaesthetic induction, cardiac arrhythmias, and ECT[44, 47] or to lower heart rate temporarily to optimise cardiac scans[43, 48]. Esmolol inhibited the specific binding of ^{3}H-CGP12177 to yield log K_D values of -6.72 in the CHO-β1 cells and -5.70 in the CHO-β2-cells and was therefore found to be slightly β1-selective (10.5-fold, Figure 1, Table 1; for structures see Supporting Information Figure S1).

Figure 1

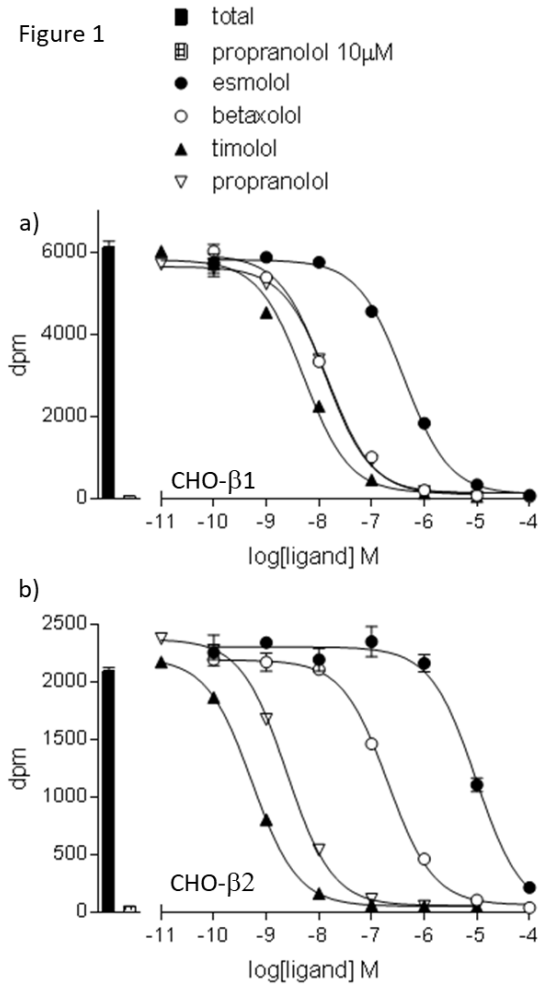

Figure 1

Inhibition of ^{3}H-CGP12177 whole cell binding in a) CHO-β1 and b) CHO-β2 cells by betaxolol, timolol, propranolol and esmolol. The bars represent total binding and non-specific binding as determined in the presence of 10µM propranolol. The concentration of ^{3}H-CGP12177 was 0.84nM. Data points are mean ± s.e.mean of triplicate determinations and these single experiments are representative of a) 9 and b) 5 separate experiments.

Table 1

	CHO-β1					CHO-β2			β1 vs β2 selectivity
	Log K_D	n	Log shift with liver esterase	n		Log K_D	n		
Esmolol 2hr	-6.72 ± 0.03	19	1.95 ± 0.13	7		-5.70 ± 0.05	13		10.5
Esmolol 4hr	-6.61 ± 0.06	6	2.65 ± 0.08	7					
Esmolol 6hr	-6.56 ± 0.03	34	2.81 ± 0.06	19		-5.56 ± 0.04	10		10
Betaxolol 2hr	-8.14 ± 0.04	16	0.03 ± 0.07	7		-7.04 ± 0.05	5		12.6
Betaxolol 4hr	-8.01 ± 0.05	6	-0.02 ± 0.06	6					
Betaxolol 6hr	-8.06 ± 0.03	15	0.01 ± 0.02	11					
Timolol 2hr	-8.69 ± 0.02	16	0.03 ± 0.05	7		-9.86 ± 0.02	5		14.8
Timolol 4hr	-8.52 ± 0.03	6	-0.03 ± 0.03	6					
Timolol 6hr	-8.51 ± 0.04	11	-0.02 ± 0.03	11					
Propranolol 2hr	-8.25 ± 0.01	9				-9.25 ± 0.08	5		10
Propranolol 6hr	-8.33 ± 0.03	7	0.07 ± 0.03	7					
CGP12177 6hr	-9.30 ± 0.06	6	0.00 ± 0.03	6					
CGP20712A	-8.76 ± 0.04	32				-5.74 ± 0.02	37		1047
ICI118551	-6.66 ± 0.02	38				-9.20 ± 0.02	38		347

Affinity (log K_D values) of β-AR ligands for the CHO-β1 and CHO-β2 cells following incubation with ^{3}H-CGP12177 for 2, 4 or 6 hrs. The parallel rightward log shift of the competition binding curve following incubation with porcine liver esterase at 0.85u/ml for 2, 4 or 6 hrs is also given. For example, the value of log 1.95 represents a 89-fold rightward shift in the position of the esmolol competition binding curve following 2 hr incubation with ^{3}H-CGP12177 and esterase. Values are mean ± s.e.mean for n separate experiments. The β1/β2 selectivity of the ligands is also given. Thus esmolol was 10.5-fold β1-selectivve whereas timolol was 14.8-fold β2-selective.

The effects of porcine liver esterase.

Esmolol is hydrolysed by esterase enzymes into ASL-8123 (**31)** and methanol and once hydrolysed, should no longer bind to the β-ARs [44]. As with all enzymes, the hydrolysis of esmolol should depend on both the concentration of the esterase present and the time of incubation. The effects of porcine liver esterase (Sigma E3019) on the binding of esmolol to the β1-AR were therefore investigated. Increasing concentrations of esterase were added to cells along with fixed concentrations of esmolol, timolol or betaxolol. As expected, co-incubation of

the non-ester containing ligands betaxolol and timolol with liver esterase had no effect on the specific binding of betaxolol or timolol, regardless of the concentration of esterase present (Supporting information, Figure S2). However, increasing concentrations of liver esterase caused a reduction in the specific binding of esmolol (Supporting information, Figure S2). Thus. in the presence of 0.85u/ml and above of porcine liver esterase (Sigma E3019), esmolol was hydrolysed, rendering it unable to bind to the β1-AR and thus was not able to inhibit the binding of ^{3}H-CGP12177 (Figure S2).

Next, the time of incubation was investigated. Porcine liver esterase (Sigma E3019 at 0.85u/ml) caused a reduction of esmolol binding such that the competition binding curve for the inhibition of ^{3}H-CGP12177 binding to the β1-AR was shifted to the right compared to that in the absence of esterase (Figure 2, Table 1). Thus after 2 hrs incubation, the inhibition curve to esmolol was shifted 89-fold (log 1.95) to the right. After 4 and 6 hrs, whereas the ability of esmolol alone to inhibit ^{3}H-CGP 12177 binding was not altered, the presence of esterase resulted in further rightward shifts of 447-fold (log 2.65) at 4 hrs and 646-fold (log 2.81) at 6 hrs (Table 1). Thus, whereas the binding of betaxolol and timolol were not affected by the presence of esterase at any time point, longer incubations resulted in greater rightward shifts of the esmolol competition binding curve for the inhibition of ^{3}H-CGP12177 binding (Figure 2). Similar experiments demonstrated no effect of liver esterase at 0.85u/ml with propranolol (used to measure non-specific binding, n=7) or CGP12177 (n= 6) after 6 hrs incubation. Also, the maximum ^{3}H-CGP12177 binding achieved was not substantially reduced following 6 hr incubation with 0.85u/ml porcine liver esterase suggesting that neither ^{3}H-CGP12177, CGP12177 nor propranolol were affected by the esterase.

Figure 2

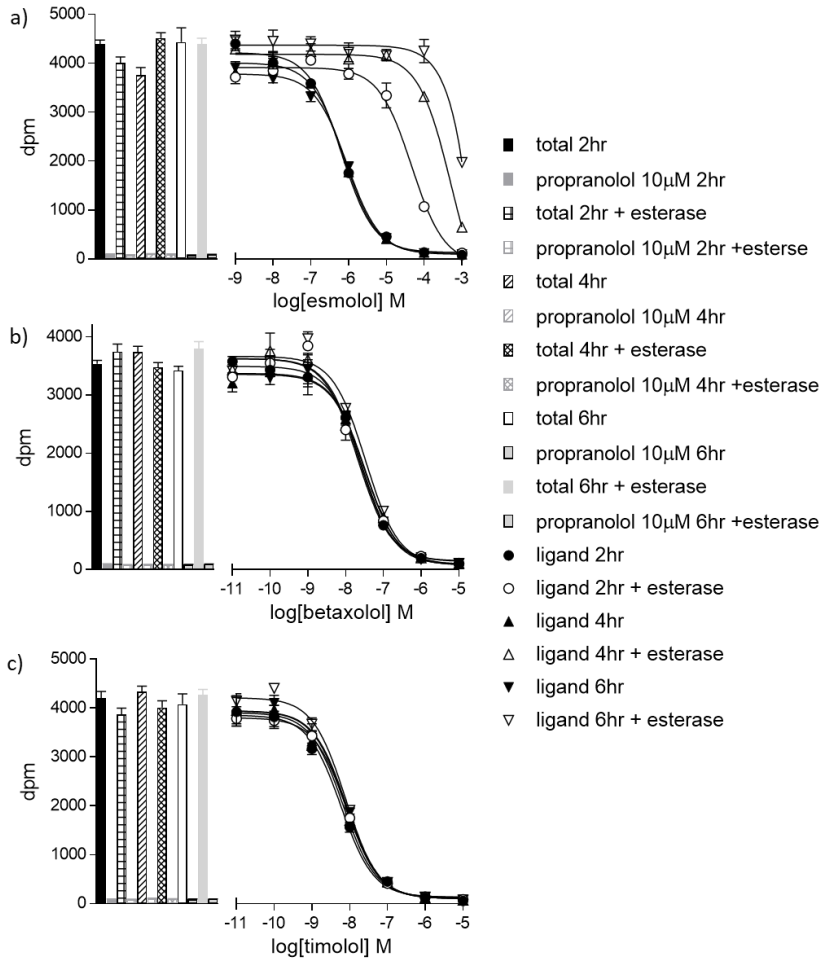

Figure 2

Inhibition of ^{3}H-CGP12177 whole cell binding in CHO-β1 cells by a) esmolol, b) betaxolol, and c) timolol. Cells were incubated for 2 hr, 4hr or 6hr with ligand and ^{3}H-CGP12177 in the absence and presence of 0.85u/ml liver esterase (Sigma E3019). Bars represent total and non-specific binding (determined by 10μM propranolol) at each time point in the absence and presence of 0.85u/ml esterase. Longer incubation caused more hydrolysis of esmolol and thus increasing rightward shifts of the esmolol competition-binding curve whereas the response to betaxolol and timolol were unaffected. Data points obtained in the presence or absence of esterase treatment are plotted as the original concentration of competing ligand in the well. The concentrations of ^{3}H-CGP12177 were a) 1.05nM, b) 0.73nM and c) 0.84nM. Data points are mean ± s.e.mean of triplicate determinations and these single experiments are representative of 6 separate experiments in each case.

Thus, the presence of porcine liver esterase reduced the binding of esmolol to CHO-β1 cells in a manner that was both concentration dependent and time dependent but had no effect on the binding of betaxolol, timolol, propranolol or CGP12177. Experiments with a different preparation of porcine liver esterase (Sigma E2884) yielded a similar pattern of results (Supporting Information Figures S3 and S4). It is worth noting, however, that there may be some species differences in the activity of liver esterases[49]. For example, clopidogrel (an anti-platelet agent) is metabolised by liver microsomes derived from a wide variety of species, but there are differences in hydrolytic rates[49].

The effects of different esterase enzymes.

Although there are several different esterases in the body, for rapid hydrolysis of any ester-containing drug that entered the blood stream, the enzymes present in the blood would be most important. The effect of human serum butyrylcholinesterase (at the maximal concentration possible of 0.79u/ml) and human red blood cell (rbc) acetylcholinesterase (at maximal concentration possible of 0.47u/ml) were therefore examined. As can be seen in Figure 3, as expected, neither the serum nor the rbc esterase had any effect on the binding of betaxolol or timolol. However, neither esterase had any effect of the binding of esmolol, suggesting that esmolol was not being hydrolysed by these serum esterases. This is in keeping with human studies where, following esmolol i.v. injection, full β-blockade occurs within 2 mins and full recovery from esmolol takes 18-30 mins[44]. Thus esmolol, being insensitive to serum esterases, is intact in the blood and thus causes a systemic effect until it is metabolised by the liver esterase. Therefore, by being sensitive to liver esterase but insensitive to serum esterase, esmolol survives long enough in the blood circulation to have a useful systemic cardiovascular effect in clinical practice.

Figure 3

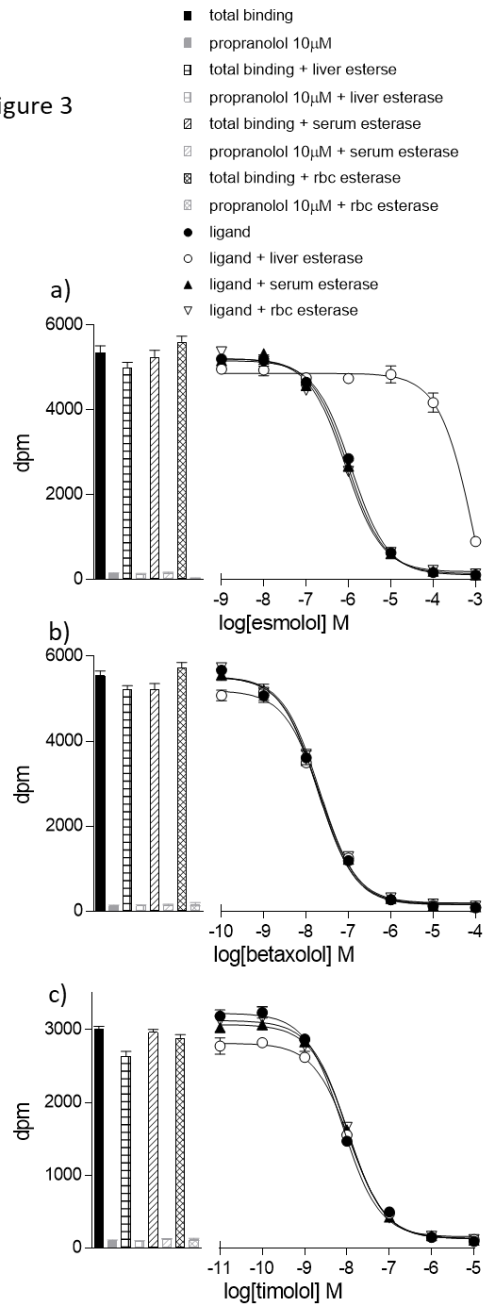

Figure 3

Inhibition of ^{3}H-CGP12177 whole cell binding in CHO-β1 cells by esmolol, betaxolol and timolol in the absence and presence of porcine liver esterase (0.85u/ml), human serum butyrylcholinesterase (0.79u/ml) and human red blood cell (rbc) acetylcholinesterase (0.47u/ml) following 6 hrs incubation. Bars represent total and non-specific binding (determined by 10μM propranolol) in the absence and presence of each esterase. Data points obtained in the presence or absence of esterase treatment are plotted as the original concentration of competing ligand in

the well. Only the liver esterase was able to hydrolyse esmolol. The concentrations of ^{3}H-CGP12177 were a) 0.44nM, b) 0.73nM and c) 0.86nM. Data points are mean ± s.e.mean of triplicate determinations and these single experiments are representative of a) 7, b) 4 and c) 4 separate experiments.

Sensitivity of novel β-blocking ester to liver, serum and rbc esterase.

In order to have a topical agent truly devoid of systemic side effects, sensitivity to serum esterases is therefore desirable, as is a higher affinity. A series of novel β-blocking ester compounds were synthesised (see Supplementary Data for structures and all chemical analytical data). These compounds had a range of β-AR affinities and a range of β1/β2 selectivities. Importantly however, the novel compounds did show a range of sensitivities to the different esterases. Some of the compounds were similar to esmolol, in that they were only sensitive to the liver esterase, whilst others were sensitive to both liver and serum esterase (e.g. **10**; Figure 4, Table 2). Finally, a few were sensitive to liver, serum and red blood cell (rbc) esterase. Interestingly, none of these novel compounds were sensitive to either serum or rbc esterase alone. It may therefore be that the serum esterases are more specific in their nature and the liver esterase is a more promiscuous enzyme with regard to the molecules it is capable of hydrolysing.

There are clear structural parameters that are evident with regard to compound sensitivity. Co-incubation with liver esterase produced greater than 10-fold shifts in apparent β1-affinity with 18 compounds and of this sub-set just under 90% possessed simple aliphatic methyl esters (**9**-**18**) including esmolol itself. The benzoic acid ester containing compounds (**3**, **5**-**8**, **20**, **22** and **23**) displayed an overall greater stability to liver esterase. Serum esterase activity also mirrored this observation, with aliphatic methyl esters again displaying higher serum esterase sensitivity (10-fold shifts in apparent affinity) when compared to the benzoic acid esters. Intriguingly however, esmolol was insensitive to serum esterase. Rbc esterase displayed significantly reduced activity across the entire compound set with almost no compounds showing a 10-fold shift in their β1-affinity when incubated with this enzyme class.

Figure 4

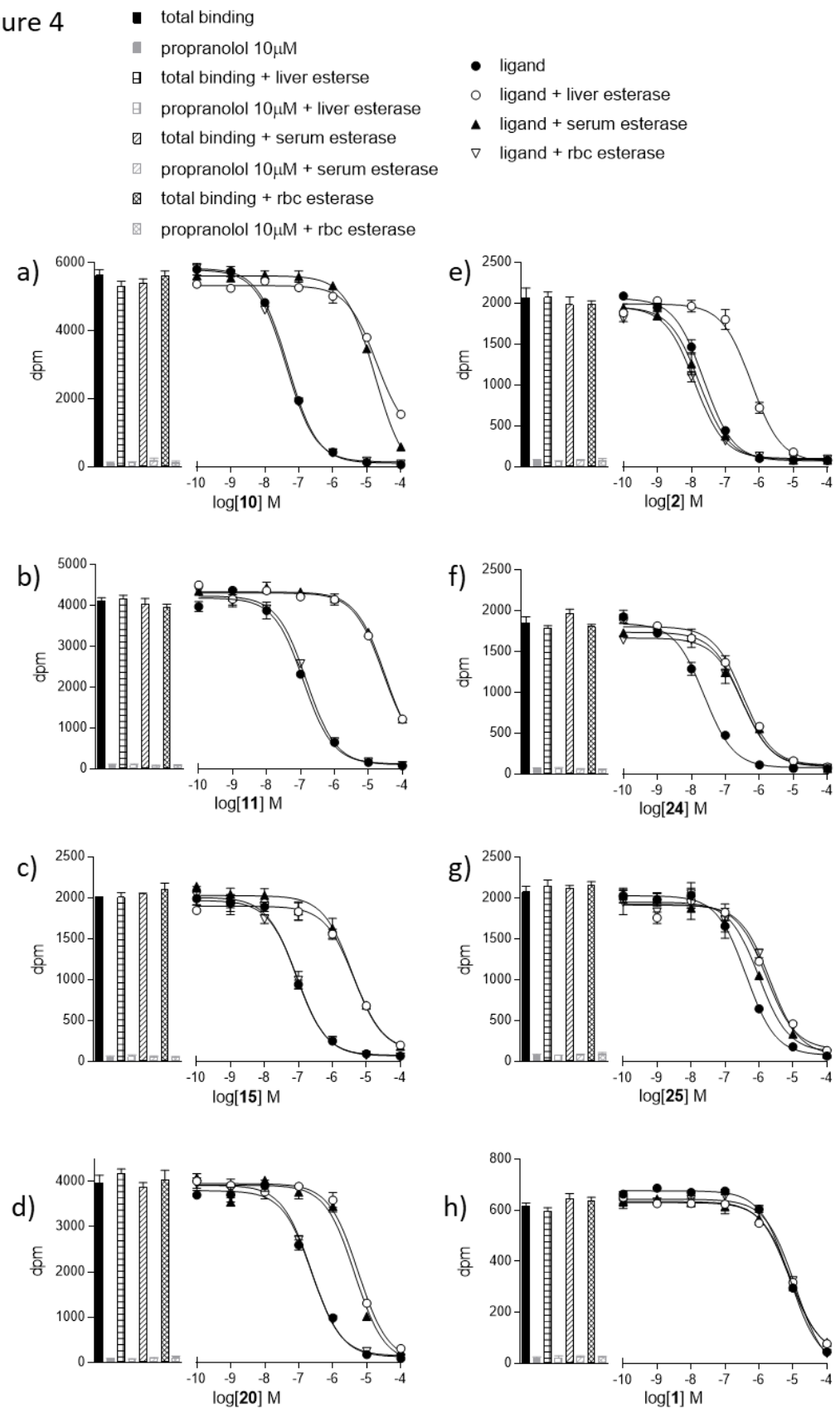
total binding
propranolol 10μM
total binding + liver esterse
propranolol 10μM + liver esterase
total binding + serum esterase
propranolol 10μM + serum esterase
total binding + rbc esterase
propranolol 10μM + rbc esterase
ligand
ligand + liver esterase
ligand + serum esterase
ligand + rbc esterase
a)
dpm
log[10] M
b)
log[11] M
c)
log[15] M
d)
log[20] M
e)
log[2] M
f)
log[24] M
g)
log[25] M
h)
log[1] M

Figure 4

Inhibition of ^{3}H-CGP12177 whole cell binding in CHO-β1 cells by several novel ligands in the absence and presence of porcine liver esterase (0.85u/ml), human serum butyrylcholinesterase (0.79u/ml) and human red blood cell (rbc) acetylcholinesterase (0.47u/ml) following 6 hrs incubation. Bars represent total and non-specific binding (determined by 10μM propranolol) in the absence and presence of each esterase. Data points obtained in the presence or absence of esterase treatment are plotted as the original concentration of competing ligand in the well. As for esmolol, some ligands were sensitive to liver esterase only (e.g. ***2****) whilst other were sensitive to liver and serum esterase (e.g.* ***10****,* ***11****,* ***15*** *and* ***20****) although to different degrees. Some ligands were sensitive to all three esterases (e.g.* ***24****) whilst other not sensitive to any (e.g.* ***1****). The concentrations of ^{3}H-CGP12177 were a) 0.86nM, b) 0.77nM, c) 0.44nM, d) 0.77nM, e) 0.52nM, f) 0.44nM, g) 0.52nM and h) 0.71nM. Data points are mean ± s.e.mean of triplicate determinations and these single experiments are representative of a) 4, b) 8, c) 4, d) 4, e) 4, f) 4, g) 4 and h) 4 separate experiments.*

Table 2

	CHO-β1			CHO-β2					Log shift of CHO-β1 response with esterase					
	Log K_D at β1	n		Log K_D at β2	n		β1 vs β2 selectivity		liver esterase	n	serum esterase	n	rbc esterase	n
Ligands not sensitive to esterases														
betaxolol	-8.06 ± 0.03	15							0.01 ± 0.02	11	0.01 ± 0.01	4	0.00 ± 0.00	4
timolol	-8.51 ± 0.04	11							-0.02 ± 0.03	11	0.02 ± 0.02	4	0.07 ± 0.07	4
1	-5.66 ± 0.09	8		-5.37 ± 0.03	4		2		0.00 ± 0.00	4	0.03 ± 0.03	4	-0.03 ± 0.03	4
Ligands sensitive to liver esterase only														
Esmolol	-6.56 ± 0.03	34		-5.56 ± 0.04	10		10		2.81 ± 0.06	19	0.01 ± 0.01	7	-0.01 ± 0.03	7
2	-8.01 ± 0.05	8		-7.63 ± 0.06	4		2		1.41 ± 0.01	4	-0.10 ± 0.05	4	-0.21 ± 0.02	4
3	-7.81 ± 0.07	8		-7.48 ± 0.03	8		2.1		1.23 ± 0.08	4	-0.09 ± 0.06	4	-0.29 ± 0.07	4
4	-7.78 ± 0.03	8		-7.42 ± 0.07	4		2		0.91 ± 0.04	4	0.00 ± 0.00	4	-0.17 ± 0.04	4
5	-7.85 ± 0.04	8		-7.74 ± 0.03	4		1.3		0.75 ± 0.03	4	0.13 ± 0.08	4	-0.11 ± 0.04	4
6	-7.52 ± 0.06	7		-7.71 ± 0.07	4		1.5		0.52 ± 0.01	4	-0.06 ± 0.05	4	-0.39 ± 0.06	4
7	-8.83 ± 0.09	9		-9.41 ± 0.14	5		3.8		0.30 ± 0.04	4	0.02 ± 0.02	4	-0.29 ± 0.19	4
8	-7.13 ± 0.03	14		-5.89 ± 0.02	4		17		0.28 ± 0.04	8	0.02 ± 0.02	4	0.00 ± 0.03	4
Ligands sensitive to liver and serum esterase														
9	-6.68 ± 0.02	12		-4.80 ± 0.04*	4		76		>3**	8	~3**	4	0.04 ± 0.03	4
10	-7.71 ± 0.03	35		-5.42 ± 0.03	18		209		2.79 ± 0.07	8	2.45 ± 0.10	4	-0.01 ± 0.03	4
11	-7.37 ± 0.02	24		-6.66 ± 0.03	12		5		2.38 ± 0.07	12	2.35 ± 0.03	8	0.02 ± 0.01	8
12	-6.43 ± 0.02	12		>-4	4		>269		2.21 ± 0.07**	8	2.25 ± 0.05**	4	0.00 ± 0.00	4
13	-6.47 ± 0.02	14		-5.40 ± 0.03	4		12		1.86 ± 0.08	8	1.83 ± 0.06	4	-0.01 ± 0.02	4
14	-7.30 ± 0.04	7		-7.73 ± 0.06	4		2.7		1.82 ± 0.06	4	1.78 ± 0.05	4	0.00 ± 0.00	4
15	-7.34 ± 0.01	8		-7.93 ± 0.06	4		3.9		1.65 ± 0.03	4	1.64 ± 0.02	4	0.01 ± 0.01	4
16	-7.67 ± 0.03	8		-7.89 ± 0.05	4		1.7		1.64 ± 0.02	4	1.66 ± 0.03	4	-0.03 ± 0.02	4
17	-7.67 ± 0.04	8		-7.98 ± 0.06	4		2.0		1.48 ± 0.05	4	1.45 ± 0.06	4	-0.07 ± 0.03	4
18	-6.49 ± 0.03	8		-7.41 ± 0.05	4		8.3		1.41 ± 0.04	4	1.38 ± 0.06	4	0.06 ± 0.04	4
19	-7.07 ± 0.03	12		-5.68 ± 0.02	4		25		1.35 ± 0.08	8	1.40 ± 0.04	4	0.00 ± 0.00	4

20	-7.04 ± 0.04	14		-5.25 ± 0.09	4		62		1.20 ± 0.05	8	1.28 ± 0.03	4	-0.06 ± 0.06	4
21	-7.33 ± 0.06	8		-8.14 ± 0.07	4		6.5		1.03 ± 0.03	4	1.03 ± 0.03	4	-0.09 ± 0.02	4
22	-9.04 ± 0.05	10		-10.12 ± 0.06	6		12		0.95 ± 0.03	4	0.98 ± 0.03	4	-0.12 ± 0.06	4
23	-6.38 ± 0.08	8		-8.09 ± 0.06	4		51		0.51 ± 0.07	4	0.52 ± 0.07	4	-0.09 ± 0.04	4
Ligands with rbc esterase activity														
24	-7.83 ± 0.04	8		-5.56 ± 0.06	4		186		1.10 ± 0.04	4	1.08 ± 0.03	4	1.03 ± 0.08	4
25	-6.81 ± 0.07	8		-4.84 ± 0.06*	4		93		0.69 ± 0.05	4	0.59 ± 0.07	4	0.75 ± 0.06	4
26	-7.37 ± 0.03	7		-5.69 ± 0.08	4		48		0.64 ± 0.04	4	0.65 ± 0.04	4	0.73 ± 0.04	4
27	-7.09 ± 0.10	8		>-4.5	4		>123		0.80 ± 0.08	4	0.79 ± 0.08	4	0.49 ± 0.08	4
28	-7.87 ± 0.10	8		>-4.5	4		>741		0.84 ± 0.05	4	0.87 ± 0.05	4	0.32 ± 0.06	4
29	-6.15 ± 0.05	12		-5.10 ± 0.09	4		11		1.00 ± 0.08	8	0.04 ± 0.02	4	0.27 ± 0.09	4
30	-6.57 ± 0.04	8		-5.97 ± 0.07	4		4		0.68 ± 0.03	4	0.33 ± 0.03	4	0.23 ± 0.05	4

*Affinity (log K_D values) and β1/β2 selectivity for novel of β-AR ligands for the CHO-β1 and CHO-β2 cells following incubation with ^{3}H-CGP12177 for 6 hrs. The parallel rightward shift of the competition binding curves (measured in the CHO-β1 cells) are also given in log units following 6 hrs co-incubation of the ligand with ^{3}H-CGP12177 and either porcine carboxyl liver esterase (0.85u/ml), human serum butyrylcholinesterase (0.79u/ml) or human red blood cell (rbc) acetylcholinesterase (0.47u/ml). Thus for **10**, a value of 2.79 represents a 617-fold rightward shift of the **10** response following 6hr co-incubation with liver esterase. Values are mean ± s.e.mean for n separate experiments.*

**apparent K_D – the ligand affinity was so low that even the maximum concentration of 10μM only inhibited about 70% of the specific binding and thus the binding curve did not reach the level of non-specific binding. The value given is therefore an apparent K_D*

***in the presence of esterase, the right-shifted binding curve did not completely reach non-specific binding. The rightward shift is therefore taken as the parallel shift of the top part of the curve.*

Confirmation of the reduction in affinity of the ester hydrolysis products

The reasons for the variation in rightward shift for different ligands in the presence of esterase is likely to be multifactorial. Thus, as well as different sensitives of the parent compounds to the various esterase treatments, the products of ester hydrolysis may also have significant β-AR affinity which would limit the rightward shift of the binding curve obtained. It is reasonable to hypothesise that the hydrolysis products of esmolol (following liver esterase) and **10** (following liver and serum esterase) did not have high affinity for the β1-AR, or the large shift in the binding curve would not have been observed. However, to confirm these observations, the binding affinity of the hydrolysis products for a selection of the ester ligands were synthesised and studied (Supporting Information Table S1). Whilst esmolol had an affinity (log K_D value) of -6.56 for the β1-AR, the esmolol hydrolysis product **31** had a β1-affinity of -4.06 ± 0.02 (n=4). The affinity of **31** for the β2-AR was so low that specific binding was hardly inhibited (at 100μM, less that half of specific binding was inhibited, thus an IC_{50}, and therefore K_D value could not be determined). Similar reductions in affinity were seen for the other ester hydrolysis products studied (esmolol, **2**, **3**, **10**, **18**, **24**, **26**, **28**; Supporting Information Table S1).

Agonist effects of esterase ligands – stimulation of cAMP.

As we wished to examine the effect of these esterase-sensitive ligands in our conscious rat cardiovascular model, using changes in heart rate as a readout of β1-blockade, it was important to determine whether any of the ligands to be tested had β-agonist activity. It has been previously shown that ligands with partial agonist activity in these cell lines correlate very well with partial agonist stimulation of heart rate in this rat model[50]. It was therefore very important to determine the intrinsic efficacy of the ligands. In CHO-β1 cells, esmolol stimulated a small increase in cAMP accumulation (log EC_{50} -7.12 ± 0.07) that was 3.3 ± 0.4% that of isoprenaline (10μM), thus a long way below the 20% cAMP response in these cells that is required to have a physiological stimulation in heart rate in the rat model[50]. Neither betaxolol nor **10** stimulated a measurable response (Figure 5). Likewise, no responses to esmolol, betaxolol or **10** were observed in CHO-β2 cells.

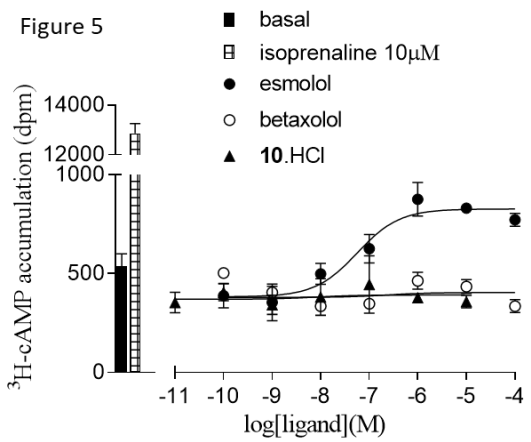

Figure 5

*^{3}H-cAMP accumulation in CHO-β1 cells in response to betaxolol, esmolol and **10**. Bars represent basal ^{3}H-cAMP accumulation, that in response to 10 μM isoprenaline alone. Data points are mean ± s.e.mean of triplicate determinations and this single experiments is representative of 4 separate experiments.*

Rat in vivo activity

To determine whether the additional property of serum and liver esterase sensitivity resulted in less systemic effects than liver esterase sensitive alone, the effects of betaxolol, esmolol and **10** (the compound with the greatest sensitivity to serum esterase) were compared. Experiments were conducted in our rat model which measures heart rate remotely in awake, freely moving rats. Previous studies (e.g. inhibition of both basal and isoprenaline-stimulated heart rate responses by the β1-antagonist CGP20712A and the β2-antagonist ICI118551) have demonstrated that the heart rate response is a β1-mediated effect in this rat model[50]. Thus, regardless of whether β1 or β2-selective ligands are optimal for the treatment of glaucoma or infantile haemangiomas (current treatments are non-selective, Table 1[46]), good β1-affinity (be that selective or non-selective) was required for this model.

For solubility reasons, the HCl salt form of **10** (**10**.HCl) was used. This had the same affinity for the human β1 and β2-AR receptor as the free base (log K_D values for **10**.HCl were -7.66 ± 0.02, n=8 at β1 and -5.36 ± 0.04, n=8 at β2; β1/β2 selectivity of 200; compared with **10**, Table 2).

However, to check that the ligands bound to the rat β1-AR with similar affinities to that of the human receptor, whole cell binding studies were undertaken with transiently transfected rat β1-AR. These studies yielded log K_D values at the rat β1-AR very similar to that of the human β1-AR (log K_D values for the rat β1-AR were: betaxolol -8.29 ± 0.03, n=6, esmolol -6.68 ± 0.04 (n=6) and **10** -7.79 ± 0.06, n=6).

Doses of ligand chosen for injection into rats were equipotent with regards to their affinity for the β1-AR (i.e. the affinity of esmolol (275nM) was 30 fold less than that for betaxolol (8.7nM) at the β1-AR so a 30 times higher dose was given; the affinity for **10** (19.5nM) was twice that of betaxolol, but to ensure at least equipotency, a 3 times higher dose was given).

In the presence of fenoterol, baseline heart rate was increased from 352 ± 5 to 496 ± 10 beats / min (n=20) and remained stable following administration of saline (Figure 6). Bolus i.v. injections of betaxolol resulted in a decrease in heart rate that was observed at the first time point (after 1 min) and remained low for the following 2 hrs (Figure 6), in keeping with ongoing cardiac β-blockade. A bolus injection of esmolol (at an equipotent dose for β1-affinity) resulted in a similar initial reduction in heart rate which remained low for 10 minutes and gradually increased after 40 minutes. This is in keeping with the human studies[44] and the findings above where esmolol is only sensitive to hydrolysis to liver esterase. Thus, due to the lack of serum esterase sensitivity, esmolol remained present in the blood stream allowing the measurable systemic response of a decrease in heart rate. However, as the blood passed through the liver over time, it is likely that esmolol was hydrolysed by the liver enzyme resulting in the decrease in response over 40 minutes. Thus the data obtained here, with in vitro studies with porcine liver esterase and rat in vivo studies correlates very well with the human studies[44] with esmolol suggesting similar liver esterase activity and also that the rat in vivo model used here may be a good predictor of the effects in humans.

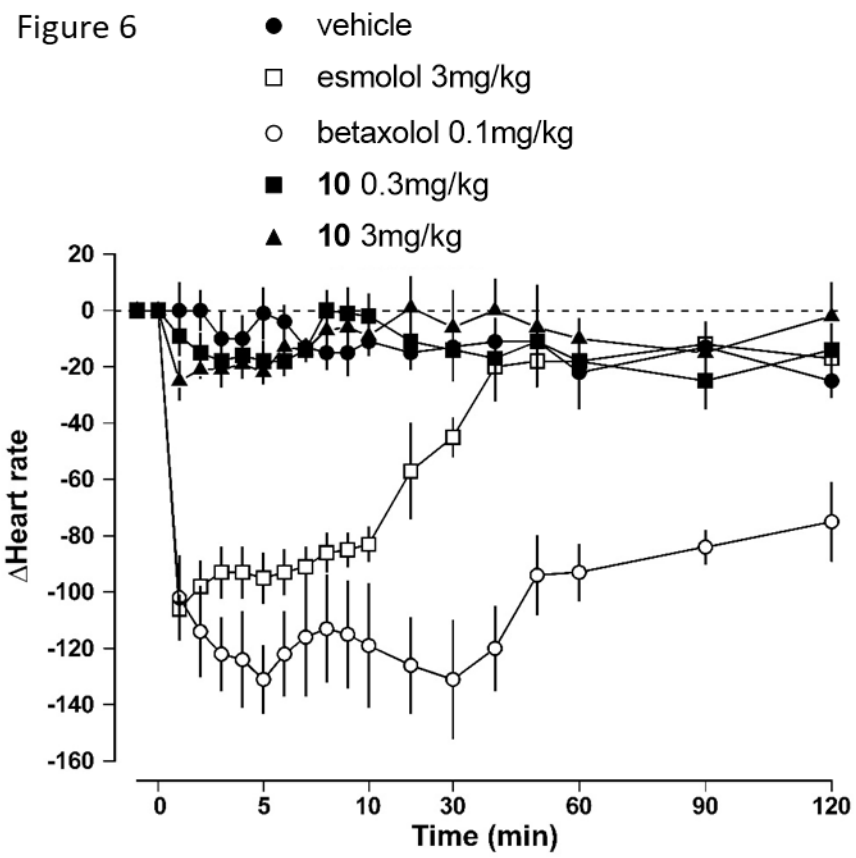

Figure 6

Change in heart rate measured in conscious rats following i.v. injection of esmolol (3mg/kg, n=4 rats), betaxolol (0.1mg/kg, n=3 rats), ***10****.HCl (0.3mg/kg, n=5 rats),* ***10****.HCl (3mg/kg, n=5 rats) and vehicle (n=3 rats) and followed for 2 hrs. 0.1mg betaxolol, 3mg esmolol and 0.3mg* ***10****.HCl are equipotent with respect affinity at the β1-receptor. 3mg* ***10****.HCl is therefore at least 10 times the equipotent dose, and despite being directly injected into the blood stream it does not cause a decrease in heart rate. Data points are mean ± s.e.mean at each time point.*

When injected at equipotent dose to betaxolol and esmolol, **10**.HCl (0.3mg/kg) did not cause any observable decrease in heart rate in rats. In further experiments, larger doses of **10**.HCl were therefore given (3mg/kg, i.e. at least 10 times the equipotent dose for betaxolol and esmolol) and similarly resulted in little decrease in heart rate from baseline. Thus it appears that despite direct injection into the blood stream, serum esterases were able to hydrolyse the compound sufficiently quickly that no systemic effect on heart rate was observed. If **10** was therefore used

as a topical agent, any systemically-absorbed compound should be immediately inactivated in the blood stream by serum esterase and thus indeed be truly devoid of systemic side effects.

Effect of human serum

Finally, the effect of human serum (as opposed to purified human enzymes used at the highest concentration possible) on the binding of esmolol and **10** was investigated. The presence of serum during the 6 hr binding experiments caused an overall increase in the total binding measured, most probably as a result of serum-induced cell growth within the time of the assay (Figure 7). Incubation of esmolol with the human serum had no effect on the ability of esmolol to bind to the β1-AR (Figure 7, Table 3). However, the presence of 10% human serum caused a 380-fold (log 2.58) rightward shift of the competition binding curve of **10.** Interestingly, this was similar whether or not the serum had been heat inactivated (30 mins 56ºC) a procedure often used in handling of laboratory serum, suggesting that the human enzyme has some heat stability (Figure 7, Table 3). 50% human serum caused a further rightward shift suggesting that this was a concentration dependent phenomenon. Interestingly, foetal calf serum (at 10% or 50% whether heat inactivated or not) did not cause the same right-ward shift in the concentration response of **10** compared to human serum suggesting some potential species differences in the individual esterase enzymes. However, foetal calf serum did show the same increase in total binding, consistent with an effect on cell growth. As the inhibition of ^{3}H-CGP12177 binding by esmolol or **10** was unchanged by co-incubation with foetal calf serum (which would have contained albumin and other serum proteins), the reduction in binding seen when co-incubated with human serum is unlikely to be due to loss of free ligand binding to serum proteins/albumin.

Figure 7

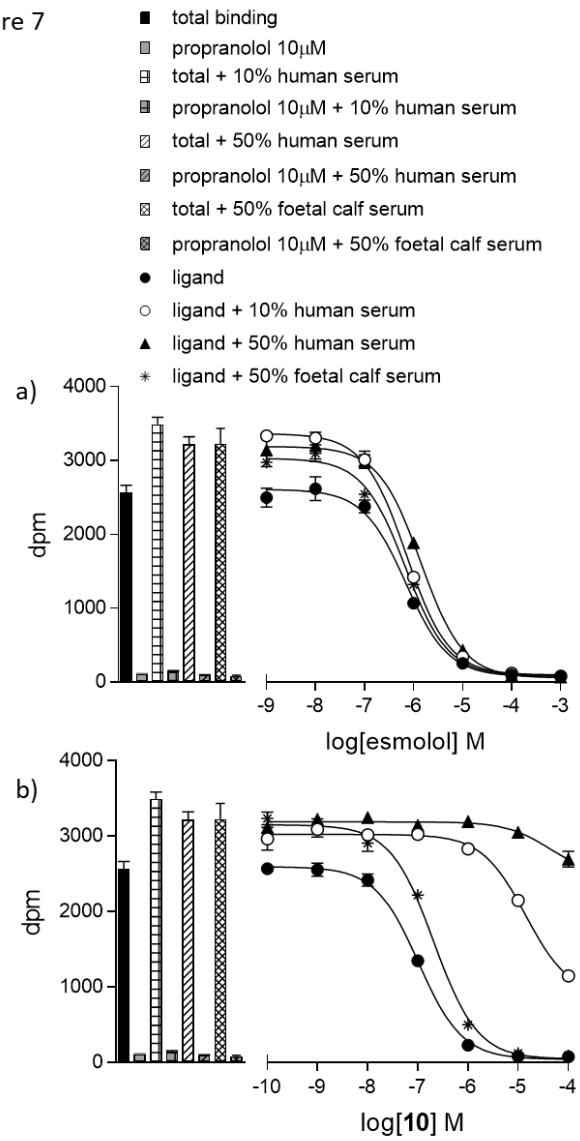

Figure 7

Inhibition of ^{3}H-CGP12177 whole cell binding in CHO-β1 cells by a) esmolol and b) ***10*** *in the absence and presence of human serum and foetal calf serum following 6 hrs incubation. Bars represent total and non-specific binding (determined by 10μM propranolol) in the absence and presence of each serum. Data points obtained in the presence or absence of esterase treatment are plotted as the original concentration of competing ligand in the well. Total binding was increased in the presence of serum, presumably resulting from serum-induced cell growth. Esmolol was not affected by the serums, however the human serum resulted in a reduction in* ***10*** *binding. The concentration of ^{3}H-CGP12177 was 0.75nM. Data points are mean ± s.e.mean of triplicate determinations and this single experiment is representative of 3 separate experiments.*

Table 3

	Log shift of:				
	esmolol	n		**10**	n
Human serum					
10%	0.04 ± 0.07	6		2.60 ± 0.10	6
10% HI	0.01 ± 0.01	3		2.51 ± 0.06	3
50%	0.33 ± 0.06	3		>3	3
50% HI	0.16 ± 0.01	3		>3	3
Foetal calf serum					
10%	-0.05 ± 0.01	3		0.16 ± 0.04	3
10% HI	0.01 ± 0.04	4		0.08 ± 0.05	4
50%	-0.02 ± 0.05	3		0.24 ± 0.02	3
50% HI	-0.15 ± 0.06	4		0.37 ± 0.05	4

*Log values for the rightward shift of the esmolol and **10** competition binding curves in CHO-β1 cells following co-incubation with ^{3}H-CGP12177 and 10% or 50% human and foetal calf serum for 6 hrs. The serum was examined both in its native form and once heat inactivated (HI; 30 minutes at 56ºC). Values are mean ± s.e.mean for n separate experiments.*

Given that there may be important species differences in serum esterase activity, it is important to emphasise that this study has not totally been based on human enzymes. Although purified human serum and rbc esterase were used, the liver esterase was porcine in origin as we were unable to obtain purified human liver esterase. Nevertheless, this study shows that it is possible to make ester compounds that firstly retain pharmacological activity during *in vitro* assays (an appropriate model for a topical effect), that secondly have different sensitivities to different esterase enzymes, and that thirdly are devoid of systemic effects in an *in vivo* model. Whilst it would be interesting to know whether **10** was effective in an animal model of glaucoma, haemangioma and other vascular tumours, further determination of the best β1/β2 subtype selectivity for optimum treatment of each of these disorders would be ideal and formulation for eye installation and skin preparations would be required. It is also important to note that genetic variants of human serum esterases are known to influence drug metabolism[51-52] . For example, the variant most frequently found in patients who respond abnormally to the muscle relaxant succinylcholine is atypical cholinesterase which has a single nucleotide polymorphism[52]. This will need to be investigated further for the ester-based β-blockers reported here. Furthermore, a

number of existing antihypertensive drugs are substrates for serum esterases at high concentrations[53], so potential drug interactions will need to be evaluated for patients with comorbidities.

In conclusion, we have demonstrated that it is possible to make compounds that have different sensitivities to different esterases and from this draw two important conclusions. Firstly, it is possible to affect the duration of systemic availability of a drug based upon its pattern of esterase sensitivity. For a drug to achieve a short–term presence in the blood stream, sensitivity to liver esterase, without any serum esterase sensitivity, appears ideal. However, in order to have a ligand that is truly devoid of systemic side effects, sensitivity to serum esterase is required in order to achieve immediate and complete hydrolysis upon entry into the blood stream. Thus, altering a ligand's susceptibility to liver vs serum esterase allows the duration of systemic availability to be titrated and fine-tuned. Secondly, having ester based topical agents that are sensitive to serum esterases offers a mechanism for delivering topical agents that are truly devoid of systemic side effects because if systemic absorption occurs, they would immediately be hydrolysed and thus inactivated. This may offer a safer mechanism for many future topical treatments, including β-blocker treatments of skin tumours e.g. haemangiomas and other vascular tumours, and eye disorders e.g. retinopathy of prematurity and glaucoma.

Methods

Materials

^{3}H-CGP12177, ^{3}H-adenine, ^{14}C-cAMP and Microscint 20 were from PerkinElmer (Buckinghamshire, UK). Rat β1-AR DNA was from cDNA Resource Centre: www.cdna.org. The esterases used were porcine liver carboxyl esterase (Sigma E3019), porcine liver carboxyl esterase (Sigma E2884), human serum butyrylcholinesterase (Sigma B4186) and human red blood cell (rbc) acetylcholinesterase (Sigma C0663). All were initially used at the highest concentrations possible, even though this meant that they were not quite equipotent with respect to u/ml. Human serum was obtained from Sigma and foetal calf serum was from PAA Laboratories (Teddington, Middlesex, UK). Betaxolol was from Tocris Life Sciences (Avonmouth, UK). Esmolol, timolol and all other reagents were from Sigma Aldrich (Poole, Dorset, UK).

Cell culture

CHO-β1 and CHO-β2 cells (with human β1-AR and β2-AR expression levels of 1146fmol/mg protein and 466fmol/mg protein respectively[46]) were grown in Dulbecco's modified Eagle's medium nutrient mix F12 (DMEM/F12) containing 10% foetal calf serum and 2mM L-glutamine in a 37°C humidified 5% CO_2 : 95% air atmosphere. For determination of the affinity at the rat β1-AR, CHO cells (i.e. with no transfected receptor) were transiently transfected with 10μg of rat β1-AR per T75 flask using lipofectamine and OPTIMEM as per manufacturer's instructions. After 24 hours, the transfection reagents were removed and replaced with media. The following day the cells were plated into white-sided 96-well plates and grown to confluence overnight before a whole cell binding experiment was conducted as below.

^{3}H-CGP12177 Whole Cell Binding - method

Cells were grown to confluence in sterile white-sided, tissue culture treated 96-well view plates. ^{3}H-CGP12177 whole cell binding was performed as previously described[46]. Competing ligand in 100μl, followed immediately by ^{3}H-CGP12177 in 100μl (giving a total volume of 200μl per well), was incubated with the cells for 2 hr (Figure 1). In the presence of esterases, this was extended to 4 hr and 6 hr incubations (Figure 2), with 6 hr becoming the normal for all

subsequent esterase studies (Figure 2, 3, 4 and 7, Supporting information figures S2, S3 and S4). After the incubation with ^{3}H-CGP12177, the cells were washed with 2 x 200µl 4°C PBS, 100µl Microscint 20 added to each well and the plates left for several hours before being counted on a Topcount for 2 min per well. Propranolol (10µM) was used to define non-specific binding in all experiments.

^{3}H-CGP12177 Whole Cell Binding – data analysis

The affinity of ^{3}H-CGP12177 was determined from saturation binding[45]. The affinity of the other ligands was determined from competition binding. All data points were performed in triplicate with 3-6 determinations of total and non-specific binding on every plate. A sigmoidal response curve was then fitted to the data using Graphpad Prism 5 and the IC_{50} was then determined as the concentration required to inhibit 50% of the specific binding using equation (1).

Equation (1): $$\% \text{ specific binding} = 100 - \frac{(100 \times A)}{(A + IC_{50})}$$

where A is the concentration of the competing ligand, IC_{50} is the concentration at which half of the specific binding of ^{3}H-CGP 12177 has been inhibited.

From the IC_{50} value, K_D value of ^{3}H-CGP12177 and the known concentration of ^{3}H-CGP12177 added in each experiment, a K_D value for the competing ligand (concentration at which half the receptors are bound by the competing ligand) was calculated using equation (2).

Equation (2): $$K_D = \frac{IC_{50}}{1 + ([^{3}\text{H-CGP12177}]/K_D\ ^{3}\text{H-CGP12177})}$$

For the assessment of esterase activity, ligands were incubated for 6 hrs with ^{3}H-CGP12177, and an esterase (porcine liver carboxyl esterase, human serum butyrylcholinesterase or human rbc acetylcholinesterase). Quantification of the esterase activity was then measured as a rightward shift of the competition ligand binding curve compared to the control curve measured in the same plate. Thus, ligands not sensitive to esterase activity would result in similar ^{3}H-CGP12177 competition binding curves in both the absence and presence of esterase. Ligands sensitive to the

esterase would be hydrolysed and thus have reduced β-AR binding. In this case, the competition binding curve in the presence of esterase would be right-shifted as compared to that in the absence of esterase. Thus, the degree in rightward shift of the competition binding curve gives a relative measure of esterase hydrolysis (assuming that the products of hydrolysis no longer bind to the β-AR).

^{3}H-cAMP accumulation

Cells were grown to confluence in sterile, clear plastic, tissue culture treated 48-well plates. Cells were pre-labelled with ^{3}H-adenine (2 hr incubation with 2μCi/ml ^{3}H-adenine in media, 0.5ml per well), before they were washed (1ml serum free media (sfm) per well) and 0.5ml sfm containing 1mM IBMX (3-isobutyl-1-methylxanthine) added to each well. Agonists (in 5μl sfm) were added to each well and the plates incubated for 5 hours at 37ºC[54]. The assay was terminated by adding 50μl concentrated HCl per well, the plates frozen, thawed and ^{3}H-cAMP separated from other ^{3}H-nucleotides by sequential AG 50W-4X resin and alumina column chromatography (using ^{14}C-cAMP to determine column efficiency[54]). Isoprenaline (10μM) was used to define the maximal response in each plate of each experiment.

A one-site sigmoidal concentration response curve was fitted to the data using equation (3).

Equation (3): $$\text{Response} = \frac{\text{Emax} \times [A]}{EC_{50} + [A]}$$

where Emax is the maximum response, [A] is the agonist concentration and EC_{50} is the concentration of agonist that produces 50% of the maximal response.

All data are presented as mean ± s.e.mean of triplicate determinations and n in the text refers to the number of separate experiments.

Chemistry methods

A range of ester ligands and some of their hydrolysis products were synthesised in house using well established methods – see Supporting Information for structures and analytical data for all compounds.

In vivo methods - Animals and surgery

In vivo experiments were carried out in conscious, surgically-prepared rats. Adult, male, Sprague-Dawley rats (Charles River, Margate, Kent, UK), weighing 300-350g, were housed in groups in a temperature-controlled (21-23°C) environment with a 12 hr light-dark cycle (lights on at 06.00h) and free access to food (Teklad Global 18% Protein Rodent Diet, Bicester, Oxon U.K.) and water for at least 7 days after arrival from the supplier before any surgical intervention. Surgery was performed under general anaesthesia (fentanyl and medetomidine, 300µg/kg of each i.p., supplemented as required), with reversal of anaesthesia and post-operative analgesia provided by atipamezole (1 mg/kg s.c.) and buprenorphine (0.02 mg/kg s.c.). In the surgical procedure, catheters were implanted in the distal abdominal aorta via the caudal artery to monitor heart rate (HR), and in the right jugular vein for drug administration. The catheters emerged from the nape of the neck and were secured to a custom-designed harness. The arterial catheters were connected to a fluid-filled swivel for overnight infusion of heparinised saline (15 units/mL; 0.4ml/hr) to maintain patency.

During the experiments, the animals were fully conscious and unrestrained in home cages, with free access to food and water. All procedures were carried out with approval of the University of Nottingham Local Ethical Review Committee, under Home Office Project and Personal Licence Authority.

In vivo methods - Experimental protocol and heart rate recordings

After a period of baseline recording, fenoterol (50µg/kg/hr) was given as a continuous infusion to provide a background increase in heart rate. Thirty minutes after the onset of fenoterol, ligands or vehicle (saline) were given as i.v. injections (0.1ml) at t=0 and heart rate was recorded continuously for 2 hrs. Heart rate was recorded using a customized, computer-based system (Instrument Development Engineering Evaluation (IDEEQ), Maastrich Instruments Bv, The Netherlands). Data are shown as the change (mean ± s.e.mean) in heart rate from immediately prior to ligand administration.

Supporting information

Figure S1. chemical structure of esmolol, betaxolol and timolol

Figure S2. effect of porcine liver carboxyl esterase (Sigma E3019) on the binding of esmolol and betaxolol to CHO-β1-cells showing concentration dependence of esterase activity

Figure S3. effect of porcine liver carboxyl esterase (Sigma E2884) on the binding of esmolol and betaxolol to CHO-β1-cells showing concentration dependence of esterase activity

Figure S4. effect of porcine liver carboxyl esterase (Sigma E2884) on the binding of esmolol and bexatolol to CHO-β1 cells showing time dependence of esterase activity

Table S1 – affinity of ester ligand hydrolysis products

Analytical Data for all novel compounds and General Experimental Chemistry Details

Abbreviations

β-AR: β-adrenoceptor
CHO: Chinese hamster ovary
sfm: serum free media
rbc: red blood cell

Authorship contributions

JGB, SMG, and PMF designed the research study. JGB and SMG performed the research. PMF, CF, and BK designed and synthesized the ester compounds. CF and MB synthesized the hydrolysis products. JGB and SMG analysed the data. JGB, SJH, BK and KSJT wrote the paper.

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Conflicts of interest

There were no conflicts of interest during the time this work was carried out. Since completion of this project, JGB, BK, SJH, PMF received an MRC MICA grant in conjunction with Heptares on orexin receptors; JGB is an advisor for CuraSen Therapeutics; CF works for Charnwood Molecular; KSJT has worked for Chronos Therapeutics and now works for Gifford Bioscience; BK has been involved in projects with Promega, Addex and Excellerate; SJH has been involved in collaborative projects with Promega, Heptares, GSK and AstraZeneca.

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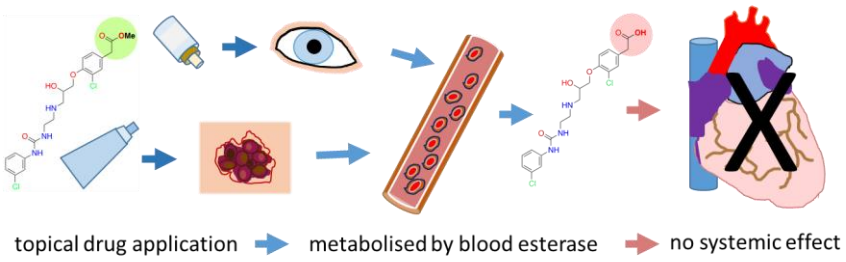
Using esterase selectivity to determine the *in vivo* duration of systemic availability and abolish systemic side-effects of β-blockers.

Jillian G Baker, Christophe Fromont, Marjorie Bruder, Kevin SJ Thompson, Barrie Kellam, Stephen J Hill, Sheila M Gardiner, Peter M Fischer



Topical drugs applied to the eyes or skin, become degraded immediately upon entry into the blood stream so are unable to cause systemic side-effects.