

MINI-REVIEW

β_2 -Adrenoceptor agonist activity of higenamine

Thomas J. Hudzik¹  | Metul Patel²  | Andrew Brown² 

¹Department of Research, GlaxoSmithKline, 1250 S. Collegeville Rd, Collegeville, PA, 1926, USA

²Department of Research, GlaxoSmithKline, Gunnels Wood Rd, Stevenage, SG1 2NY, UK

Correspondence

Thomas J. Hudzik, Department of Translational Neuroscience, BlueRock Therapeutics, 450 E. 29th St, New York, NY 10016 USA.
Email: Thudzik@bluerocktx.com

Abstract

Higenamine was included in the World Anti-Doping Agency (WADA) Prohibited Substances and Methods List as a β_2 -adrenoceptor agonist in 2017, thereby resulting in its prohibition both in and out of competition. The present mini review describes the physiology and pharmacology of adrenoceptors, summarizes the literature addressing the mechanism of action of higenamine and extends these findings with previously unpublished *in silico* and *in vitro* work. Studies conducted in isolated *in vitro* systems, whole-animal preparations and a small number of clinical studies suggest that higenamine acts in part as a β_2 -adrenoceptor agonist. *In silico* predictive tools indicated that higenamine and possibly a metabolite have a high probability of interacting with the β_2 -receptor as an agonist. Stable expression of human β_2 -receptors in Chinese hamster ovary (CHO) cells to measure agonist activity not only confirmed the activity of higenamine at β_2 but also closely agreed with the *in silico* prediction of potency for this compound. These data confirm and extend literature findings supporting the inclusion of higenamine in the Prohibited List.

KEYWORDS

higenamine, WADA, β_2 -adrenoceptor

1 | INTRODUCTION

Higenamine was included on the Prohibited Substances and Methods List of the World Anti-Doping Agency (WADA) as a β_2 -adrenoceptor agonist in 2017, thereby resulting in its prohibition both in and out of competition. Nevertheless, higenamine can be readily found in many different commercially available 'supplements', although the labels of such products may not state its presence or accurately state the amount contained. For example, Cohen et al.¹ identified 24 such products, finding amounts of higenamine ranging from trace up to 62 mg per 'serving'. Athletes have been and should continue to be cautioned to avoid these products, which may contain higenamine as well as other banned substances.

Higenamine [(±)-noroclaurine, 1-(4-hydroxybenzyl)-1,2,3,4-tetrahydroisoquinoline-6,7-diol] (Figure 1) is an alkaloid identified from extracts from a number of different plant species. These higenamine-

containing plant species have been utilized in traditional Chinese medicine to treat a range of medical conditions including asthma and heart failure.² First isolated in the 1970s,³ higenamine was shown to have inotropic and chronotropic effects in isolated heart preparations³⁻⁸ stimulating interest in the substance as a potential 'cardiotonic'. Additionally, higenamine was shown to have relaxant effects upon smooth muscle of the isolated trachea⁹⁻¹¹ and colon.¹⁰ Higenamine also enhances glucose uptake and metabolism.¹² Each of these effects alone and in combination has the potential to enhance athletic performance.

Adrenaline (epinephrine), the circulating hormone released in response to stress and key to the 'fight or flight' response,¹³ has a well-described pharmacology as a result of its interaction with its multiple receptor subtypes: α and β , as summarized in Table 1. Adrenoceptors are G-protein coupled receptors (GPCRs) and therefore expressed as the typical seven-transmembrane spanning structure. GPCRs activate multiple downstream signalling pathways, and selectivity for one

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signalling pathway versus another defines biased agonism, adding nuance to the physiology. Adrenoceptors and other GPCRs also can exist as homo- or hetero-oligomers, which further complicates their physiology, as reviewed by Maggio et al.¹⁵ Noradrenaline (norepinephrine) is another endogenous transmitter/hormone that interacts with adrenergic receptors, with a somewhat different profile of activity than adrenaline. β_3 -Adrenoceptors are expressed primarily in adipose tissues¹⁶ and will not be discussed further in the present review.

Table 1 summarizes the major subtypes of adrenoceptor and some of the effects of agonists.

There are far more examples of non-selective ligands than of selective ligands for adrenoceptors. That is, many ligands can activate or block both α_1 - and α_2 -receptors (e.g., phentolamine and oxymetazoline), both β_1 - and β_2 -adrenoceptors (e.g., isoproterenol and propranolol) or both α - and β -adrenoceptors (e.g., adrenaline).

2 | PHYSIOLOGY OF β_2 -ADRENOCEPTORS

The structure of β_2 -adrenoceptors has been well characterized,^{17–19} and how the structure lends insight into the signalling properties of its associated ligands²⁰ has been a subject of ongoing, fruitful discovery in biochemical physiology and pharmacology. As with other GPCRs, the

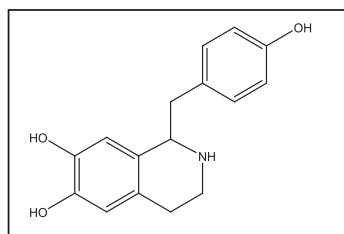


FIGURE 1 Higenamine chemical structure

adrenoceptor is coupled within the membrane to a variety of subtypes of G proteins as well as kinases that constitute the intracellular signalling mechanisms of the receptor. Extracellular loops form the ligand binding domains of the receptor, and receptor mutagenesis studies have contributed greatly to our understanding of which regions confer selectivity, activity and signalling preferences, for example, Breyer et al.²¹ These domains are not static but rather can reconfigure depending upon ligand properties.²² β_2 -Adrenoceptors internalize in response to agonist stimulation²³—an adaptational property inherent in many receptor systems. There is relatively high homology between the human form of the adrenoceptor and that of guinea pig and rat.^{24,25}

β_2 -Adrenoceptors are expressed in numerous tissues in the body including the heart and brain, although their densities are highest in the smooth muscle of the lungs, uterus, major blood vessels and blood cell types and, along with other adrenoceptors, play a key role in haemodynamic homeostasis. β_2 -Adrenoceptor in the heart likely contributes to cardiac contractility,²⁶ although β_1 -adrenoceptor is the predominant subtype in the heart in terms of density. In the smooth muscle of the bladder, β_2 -adrenoceptors regulate bladder tone,²⁷ similar to its action in the colon.²⁸ Synaptic brain β_2 -adrenoceptors likely underlie regulation of attention²⁹ and mood.³⁰ β_2 -Adrenoceptors are also present in brain vasculature and play a role in maintaining vascular tone³¹ and also participate in glucose uptake and utilization in a range of tissues and cell types, for example.^{32,33} β_2 -Adrenoceptor function in the trachea and lung involves relaxation of the tissue, and therefore, the constriction of the airways characteristic of asthma can be effectively treated with β_2 -adrenoceptor agonists.^{10,34} Similarly, in the uterus, contraction can be lessened in specific instances, such as premature labour. These are the primary therapeutic uses of β_2 -adrenoceptor agonists.^{35,36}

As stated earlier, there is evidence that β_2 -adrenoceptors can form homo- and hetero-oligomers with other GPCRs, which can alter

TABLE 1 Effects of stimulation of adrenoceptor subtypes—adapted from Westfall and Westfall¹⁴

	α_1	α_2	β_1	β_2
Localization	Brain Eye Gut vascular beds Lung Bladder	Brain (presynaptic) Spinal cord Synapses upon peripheral organ systems	Heart Brain	Heart Smooth muscle Lung Vasculature Uterus Brain Striate muscle
Effects	<ul style="list-style-type: none"> • Stimulant effects • Pupillary dilation • Vasoconstriction • Bronchoconstriction • Increased blood pressure 	<ul style="list-style-type: none"> • Sedation • Inhibition of norepinephrine, insulin and acetylcholine release • Decreased blood pressure 	<ul style="list-style-type: none"> • Positive chronotropic effect • Positive inotropic effect • Neuromodulation 	<ul style="list-style-type: none"> • Cardiac stimulation • Decreased smooth muscle tone: aorta, bronchiole (increase oxygen utilization), uterus and bladder colon • Decreased pulmonary inflammation • Increased attention • Increased glucose uptake (muscle)
Pharmacology				
Selective agonists	Phenylephrine	Clonidine	Xamoterol	Albuterol
Selective antagonists	Prazosin	Yohimbine	Bisoprolol	ICI-118,551

Note: Potentially performance-enhancing effects are in bold.

ligand selectivity as well as signalling properties, depending on the resultant structure, reviewed by Wnorowski and Jozwiak.³⁷ There have been several receptors suggested to oligomerize with β_2 , including opioid receptors, other adrenoceptors (β_1 , β_3 and α subtypes) and the cannabinoid CB₁ receptor. The functional consequences of hetero-oligomerization are another rich area for future study but also highlight the nuanced complexity of the living, integrated receptor relative to the study of isolated receptors.

3 | POTENTIAL β_2 -ADRENOCEPTOR AGONIST ENHANCEMENT OF ATHLETIC PERFORMANCE

Inasmuch as agonism at β_2 -adrenoceptors can alter central nervous system, cardiovascular and respiratory function as well as glucose uptake, under appropriate conditions such as supratherapeutic doses and by routes other than inhaled where bio-distribution may occur more widely, a rationale for performance enhancement can be hypothesized, although the exact mechanisms of performance enhancement are still not fully understood. Numerous studies have attempted to address the performance-enhancing effects of β_2 -agonists, with somewhat mixed results. Nevertheless, in a meta-analysis of 34 studies composed of 323 study participants, Riiser et al.³⁸ concluded that the preponderance of the data for β_2 -agonists supports the assertion that they do significantly enhance anaerobic performance. Although this effect on anaerobic performance appears to be paradoxical given the primary pharmacology of the drug class in the lung, the findings support the rationale for inclusion in the Prohibited Substances List.

4 | HIGENAMINE AS A β_2 -ADRENOCEPTOR AGONIST

There is substantial literature evidence that higenamine interacts with β_2 -adrenoceptors. This has been demonstrated by recombinant expression of β_2 -adrenoceptors in immortalized mammalian cells, in cells endogenously expressing β_2 -adrenoceptors in isolated smooth muscle preparations, in whole-animal preparations and in human clinical studies.

4.1 | Cellular systems expressing β_2 -adrenoceptors

Bai et al.¹⁰ first showed that in Chinese hamster ovary (CHO) cells stably expressing rat β_2 -adrenoceptors, a number of reference β -adrenoceptor agonists including salbutamol, epinephrine and isoprenaline all produced full agonist stimulation of the receptor. Bai et al. also directly compared the activities of higenamine with the β_2 -selective drug, salbutamol, showing that higenamine was a full β_2 -adrenoceptor agonist with a potency of about 10-fold less than salbutamol. Agonism by both higenamine and salbutamol was right

shifted by the β_2 -selective antagonist alprenolol, consistent with the measured effects being mediated by β_2 -adrenoceptors. In agreement with these findings, our laboratory demonstrated that higenamine stimulates CHO cells expressing the *human* β_2 -adrenoceptor (described below). Complimentary studies on β_2 -adrenoceptor expressed in a different cell line (SK-N-MC) result in similar conclusions: higenamine stimulated β_2 -adrenoceptors, and its effects were abolished in the presence of the β_2 -selective antagonist, ICI-118,551.⁸ Finally, Kato et al.^{12,39} showed that higenamine and a number of synthetic analogues enhanced glucose uptake in a rat muscle cell line and did so with a potency and efficacy similar to that of epinephrine and norepinephrine.

4.2 | Isolated smooth muscle preparations and whole-animal studies

Bai et al.¹⁰ also showed that in isolated guinea pig trachea, a tissue rich in β_2 -adrenoceptors, higenamine dose-dependently reduced acetylcholine-stimulated contraction of the tissue. This tracheal relaxation has been replicated in a number of different laboratories^{9,11} with the former additionally showing blockade of higenamine by ICI-118,551 and by the uncoupling of β_2 -signalling mechanisms by addition of pertussis toxin, again, strongly indicating β_2 -agonism. Liu et al.²⁸ utilizing guinea pig colonic mucosa showed that higenamine could decrease the short circuit current in the tissue, an effect also blocked by ICI-118,551.

In rabbits cannulated in the right common carotid artery, Chang et al.²⁸ showed that higenamine could reduce diastolic blood pressure. In a guinea pig model of histamine-induced asthma, both higenamine and salbutamol inhibited asphyxia convulsion.¹⁰ Both these effects are consistent with activation of β_2 -adrenoceptors.

4.3 | Clinical studies

The 'cardiotonic' effects of higenamine have been shown in both a healthy population⁴⁰ and patients with various forms of heart disease.⁴¹ In both populations, there were dose-related inotropic and chronotropic effects measured following intravenous (IV) infusion, which allowed for careful control of pharmacokinetic (PK)/pharmacodynamic (PD) measurements. One study failed to see changes in cardiac parameters following daily oral higenamine administration, but, unlike those cited above, this study did not conduct the measurements

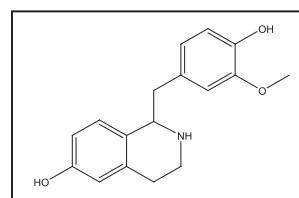


FIGURE 2 Structure of potential M3 higenamine metabolite

necessarily at a time proximal to the administration of drug,⁴² which has a short half-life.⁴⁰ Although these cardiac effects are possibly mediated by β_1 -adrenoceptors, contribution β_2 -adrenoceptors cannot be ruled out. Specific endpoints for β_2 -adrenoceptor activation (e.g., effects on lung function) were not measured in this study, and hence, no conclusions can be made about relative β_2/β_1 -activation. This study nevertheless shows that higenamine can engage β -adrenoceptors in humans. As mentioned above, potential cardiac effects of higenamine have been demonstrated in both in vitro and in vivo preparations, showing various potential roles for both β_2 - as well as β_1 -adrenoceptors.

4.4 | Studies conducted at GSK

In 2019, we conducted in silico predictive modelling of likely human adrenoceptor targets based upon the chemical structure of higenamine, followed by direct in vitro test of agonist activity using cells expressing recombinant human β_2 -adrenoceptors.

4.5 | In silico prediction

The predicted pharmacology of higenamine was generated using CLARITY[®] software (Version 4.0 for Unix, Copyright © 2019 Chemotargets SL, Barcelona, Spain). This approach compares the chemical structure of a test article with structures and associated pharmacological activities in the ChemEMBL database as described previously.⁴³ Where ChemEMBL contains separate compounds with differing modalities acting at the same target (in this case agonists and antagonists at β -adrenoceptors or 5-hydroxytryptamine receptor 2A), CLARITY

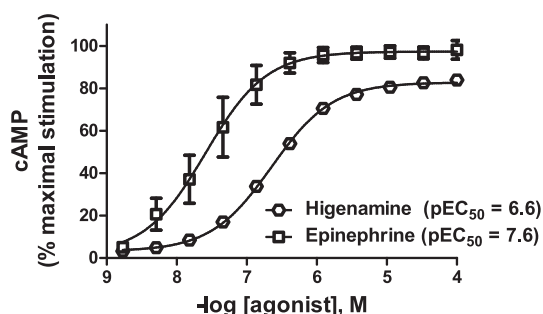


FIGURE 3 Comparison on the effects of higenamine to epinephrine against human β_2 -adrenoceptor

Protein name	Gene name	Predicted pXC ₅₀	Predicted modality
Oestrogen receptor β	ESR2	7.2	Not predicted
Adenylate cyclase type 5	ADCY5	6.9	Not predicted
5-Hydroxytryptamine receptor 2A	HTR2A	6.7	Agonist
β_2 -Adrenoceptor	ADRB2	6.6	Agonist
Oestrogen receptor	ESR1	6.3	Not predicted
β_1 -Adrenoceptor	ADRB1	6	Agonist
β_3 -Adrenoceptor	ADRB3	6	Agonist

predicts both XC₅₀ (half-maximal effective concentrations in assay for target X interaction) and modality. Where ChemEMBL contains XC₅₀ information without associated functional information (e.g., binding affinity as in this case for oestrogen receptor β), CLARITY predicts XC₅₀ only. Metabolites and metabolite activities are also predicted. Results for higenamine are summarized below (XC₅₀ half-maximal effective concentrations are shown as pXC₅₀, the negative log of XC₅₀, so that pXC₅₀ = 6 equates to XC₅₀ of 1 μ M). Given the complexity of the algorithms utilized to make such predictions, it is not surprising that other possible pharmacology was identified. Typically, these data are used to make the prediction, but obviously, to test the prediction, one must go on to set up the appropriate assays. In this case, our question was rather focused upon β_2 -adrenoceptors, and hence, we did not follow-up on testing the other potential targets for higenamine's activity. The in silico search also predicted a primary metabolite for higenamine (Figure 2), which would be predicted to possess similar adrenoceptor activity as the parent molecule, higenamine, and, as shown in Table 3, possible higher agonist potency.

4.6 | In vitro characterization of higenamine

The predicted β_2 -adrenoceptor agonist activity of higenamine was confirmed using CHO cells stably expressing the human β_2 -adrenoceptor, which provides a robust high-throughput assay to support routine secondary pharmacology tests⁴⁴ and is considered a gold-standard expression system. Known β_2 -adrenoceptor agonists give reproducible pEC₅₀ agonist potency values in this assay. Cells were incubated with higenamine or epinephrine in the presence of phosphodiesterase inhibitor IBMX (500 μ M) to construct concentration–response curves (Figure 3). After 45 min, cells were lysed (0.35% Triton X-100), and β_2 -adrenoceptor agonist activity was determined by measurement of cAMP, using the LANCE[®] time-resolved fluorescence resonance energy transfer (TR-FRET) immunoassay (PerkinElmer, Beaconsfield, UK; see Doucette et al.⁴⁵ for detailed description of the TF-FRET protocol). Interestingly, the in silico model prediction closely approximated the measured potency of higenamine at human β_2 -adrenoceptor (predicted potency 250 nM [pEC₅₀ = 6.6; Table 2]; measured potency 230 nM [pEC₅₀ = 6.6, n = 4; Figure 3]). In this assay, higenamine behaved as a near-full agonist, with a maximum effect >80% relative to adrenaline/epinephrine. Experimental details are further described in Section 5.1.

TABLE 2 In silico predicted activity for higenamine (predicted pXC₅₀ \geq 6)

TABLE 3 Predicted activity for the methylated metabolite of higenamine (M3)

Protein name	Gene name	Predicted pXC ₅₀	Predicted modality
5-Hydroxytryptamine receptor 2B	HTR2B	7.3	No prediction
β ₂ -Adrenoceptor	ADRB2	7.1	Agonist
Oestrogen receptor β	ESR2	7.1	No prediction

5 | CONCLUSIONS

β₂-Adrenoceptor agonists may, under the appropriate conditions, enhance sports performance. Given the cardiovascular effects of agonists, they could also present medical complications for the athlete if misused. Here, we reviewed the evidence that higenamine is an agonist at the β₂-adrenoceptor of both human and rodent. It produced agonist activity comparable with standard β₂-agonists in a number of different functional assays, effects that were reversible by selective β₂-antagonists. These data strongly support activity of higenamine at β₂-adrenoceptors. There is further evidence that higenamine may also stimulate the β₁-adrenoceptors, which might be expected given the sequence homology of the β₁ and β₂. Effects at additional receptor systems, including α-receptors, 5-HT receptors or oestrogen receptors, also cannot be ruled out, but further characterization of these potential effects was beyond the scope of the present work.

Additionally, it is not known whether higenamine accumulates in tissues other than blood after repeated administration. This factor could significantly extend its pharmacological activity, for example, in the lung, although dedicated studies would be required to demonstrate this. The appearance of the M3 metabolite of higenamine and its contribution to the pharmacology of higenamine in humans is also unknown at this time. This metabolite has been measured in vivo following administration of higenamine,⁴⁶ and because the CLARITY predictive model suggested possible β₂-adrenoceptor activity (Table 3), the metabolite in turn could potentially increase and extend the effects of the parent compound and hence should be evaluated for activity in its own right.

5.1 | Detailed methods

CHO cells, stably expressing recombinant human adrenergic β₂-receptor, were maintained in culture in Dulbecco's modified Eagle medium (DMEM)/F12 supplemented with 5% foetal calf serum (FCS), 2-mM L-glutamine, 200-μg/ml geneticin and 100-μg/ml hygromycin B in 95%:5% air:CO₂ at 37°C. Cells were harvested at 80% confluence, resuspended at 3 × 10⁷ cells/ml in 10% DMSO and 90% DMEM/F12 and frozen as aliquots in 1-ml tubes using a controlled-rate freezer. Cells were maintained at -140°C for long-term storage. Adrenergic β₂-receptor-evoked increase in cellular cAMP was determined using a LANCE™ cAMP assay kit according to manufacturer's instructions (PerkinElmer). Briefly, cells were thawed at 37°C, diluted in phosphate-buffered saline (PBS) and centrifuged (1000 rpm, 5 min). Cells were resuspended in stimulation buffer (Hank's Buffered Saline Solution [HBSS] containing 0.01% bovine serum albumin [BSA], 500-μM IBMX and 5-mmol/L HEPES adjusted to pH 7.4 with KOH),

counted and 80% or greater viability confirmed (Vi-CELL; Beckman Coulter). Cells were adjusted to 2 × 10⁶ cells/ml and dispensed to white low-volume Greiner polypropylene 384-well microtitre plates (10,000 cells per well in 5 μl), containing 0.1 μl per well test compound in 100% DMSO. Antibody solution (stimulation buffer containing Alexa Fluor 647-labelled anti-cAMP antibody; 5 μl per well) was then added, and plates were incubated (30 min; room temperature). Detection reagent was freshly prepared by diluting europium-streptavidin conjugate 1:2250 into detection buffer (50-mM HEPES, 10-mM CaCl₂ and 0.35% Triton X-100), mixing and then adding biotin-cAMP conjugate to 1:750 dilution (solutions supplied in LANCE kit). Detection reagent was dispensed to assay plates (10 μl per well), and plates were covered and incubated at room temperature for 4 h. cAMP was quantified by TR-FRET between Alexa Fluor 647 and europium, using a PerkinElmer Wallac EnVision 2104 Multilabel Reader with dual-emission filters (acceptor = 665 nm; donor = 615 nm). Intraplate normalization was performed by reference to DMSO-treated and isoproterenol hemisulphate-treated cells (n = 16 wells of each per plate) and normalized data subjected to four-parameter sigmoidal concentration-response curve fitting using ActivityBase (IDBS), to obtain values for pEC₅₀ (negative log₁₀ of molar EC₅₀).

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ORCID

Thomas J. Hudzik  <https://orcid.org/0000-0002-3982-4475>

Metul Patel  <https://orcid.org/0000-0002-4401-6616>

Andrew Brown  <https://orcid.org/0000-0002-5616-3664>

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