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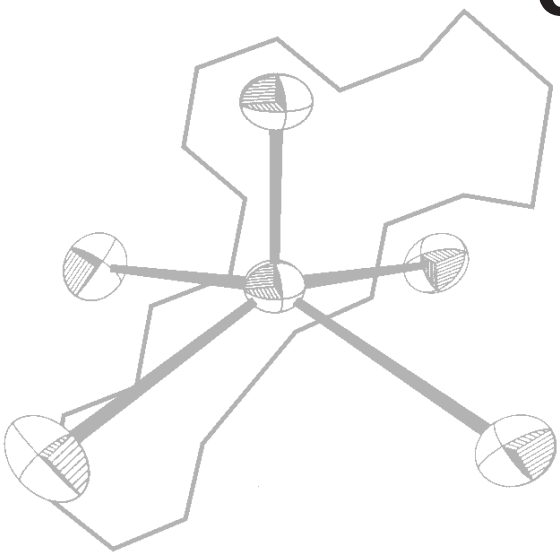

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2,7-Dimethylthiazolo[4,5-*d*]pyradazine-4-(5*H*)-thione: A Corticotrophin-Releasing Hormone Type 1 Receptor Agonist

Adam McCluskey,[A,B] *Matthew Finn*,[A] *Maria Bowman*,[C] *Roger Smith*[C] *and Paul A. Keller*[D]

[A] Medicinal Chemistry Group, Chemistry, School of Biological and Chemical Sciences, The University of Newcastle, N.S.W. 2308.
[B] Author to whom correspondence should be addressed (e-mail: amcclusk@mail.newcastle.edu.au).
[C] Department of Endocrinology, Mothers & Babies Health Research Centre, John Hunter Hospital, N.S.W. 2310.
[D] Department of Chemistry, The University of Wollongong, Wollongong, N.S.W. 2522.

Dedicated to Dr John Zdysiewicz on the occasion of his retirement

Thiazoles, including 2,7-dimethylthiazolo[4,5-*d*]pyradazine-4-(5*H*)-thione (4b) and the corresponding 5-phenylthiazolo[4,5-*d*]pyradazine-4-methylthiol (5a), were synthesized as part of an ongoing investigation into corticotrophin-releasing hormone (CRH) type 1 receptor activity. Subsequent screening indicated the successful discovery of receptor agonists. Assay results indicated a 52 and 3% increase in β-endorphin release after the administration of 100 μM (4b) and (5a), respectively. It is believed that this represents the first evidence of this class of compounds displaying CRH type 1 receptor agonist activity.

Keywords: Thiazoles; agonist; CRH; β-endorphin.

Introduction

Corticotrophin-releasing hormone (CRH), sometimes known as CRF, is a 41-residue peptide, originally isolated from ovine hypothalamus based on its ability to stimulate adrenocorticotrophin (ACTH) and β-endorphin release from cultured anterior-pituitary cells.[1] CRH is the principal neuroregulator of the basal and stress-induced secretion of ACTH, β-endorphin, and other proopiomelanocortin-related peptides from the anterior pituitary.[2]

In addition to its endocrine role in the regulation of the hypothalamic-pituitary-adrenal axis, CRH is implicated in a variety of other central and peripheral functions including food intake,[3] thermoregulation,[4] reproduction,[5] inflammation,[6] and cardiovascular function.[7] CRH exerts its effects by binding to a range of high-affinity membrane receptors, of the seven transmembrane-domain family, that are coupled, via G-coupled proteins, to second messenger-signalling mechanisms which vary with the receptor type and tissue.[8–12] CRH has been linked to many central nervous system disorders, anorexia nervosa, Alzheimer's disease and inflammatory disorders such as arthritis.[13] In the brain, CRH produces a wide spectrum of autonomic and electrophysiological effects.[14] It activates the sympathetic nervous system, with consequential increases in epinephrine, norepinephrine and glucose levels, increased heart rate and increased mean arterial blood pressure.[13] CRH, when injected peripherally, causes vasodilatation which reduces blood pressure. Additionally, we have shown that levels of plasma CRH rise exponentially in human pregnancy.[12] We have also shown that this increase can be used to predict idiopathic pre-term labour.

CRH has recently been the target of a series of drug-discovery programs,[13,15] both of academic and industrial interest. Until now, these programs have resulted in the development of a series of small-molecule antagonists (pyrimidines, pyrazidines, pyrazolopyrimidines, etc)[16] of CRH type 1. Antalarmin, Fig. 1, is perhaps the best known of the CRH_1 receptor antagonists, being both potent and selective.[16]

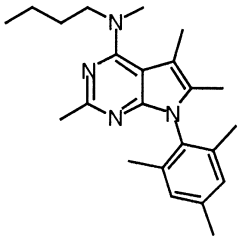

Fig. 1. Antalarmin.

Recently, sulfur-containing compounds have drawn much attention due to their significant biological and pharmacological activities.[17–22] Dury[17] reported that 5-amido-, and 5-acetylamino-4-chloropyridazinones reacted with hydrogen sulfide to give thiazolo[4,5-*d*]pyridazines, while Simiti *et. al.*[19] reported the preparation of this heterocyclic system from thiazoles and hydrazines. Thiazolo[4,5-*d*]pyridazinones bearing C–2 amino groups were first synthesized by Takaya *et. al.*[20] via cyclization of 5-amino-6-chloropyridazinones with carbon disulfide, followed by *S*-methylation with methyl iodide and then amination with amines under heating. More recently, Furukawa *et. al.*[21,22] have developed a new route, which

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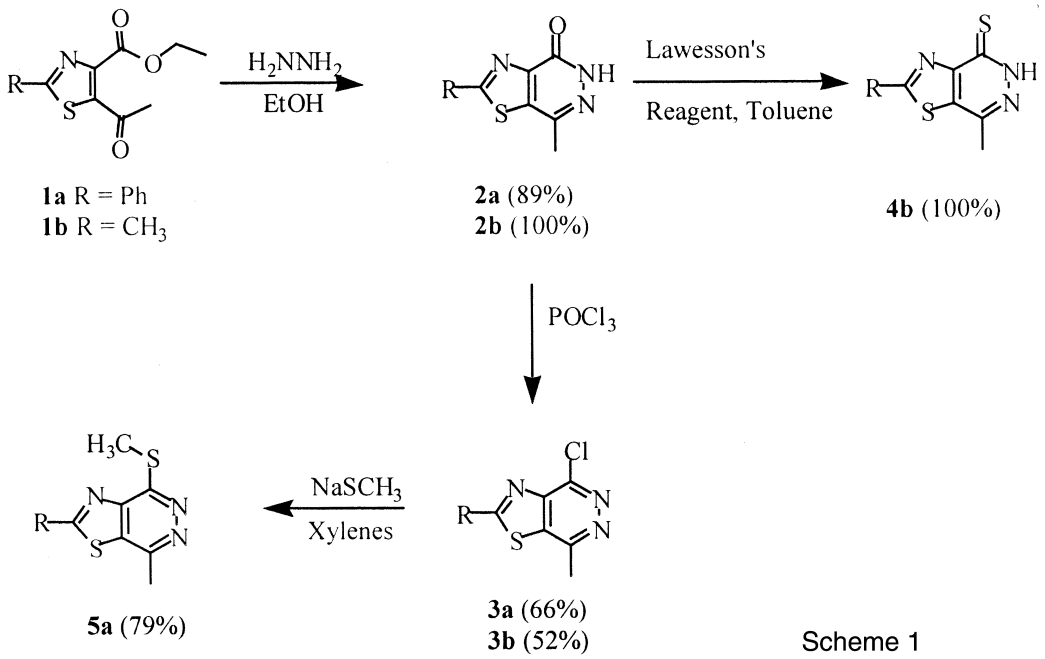

Scheme 1

entails heating 5-amino-4-chloropyridazinones with an excess of methyl dithiocarbamates, yielding the corresponding 2-aryl-aminothiazolo[4,5-*d*]pyridazin-7(6)-ones.

Herein we wish to report a new lead, in the discovery of a thiazolo[4,5-*d*]pyradazine-derived agonist for CRH_1 receptors.

Results and Discussion

As part of ongoing studies into the prevention of pre-term delivery in humans we have synthesized a range of small heterocyclic molecules, including those based on the thiazolo[4,5-*d*]pyridazine skeleton, as shown in Scheme 1.[23]

The syntheses were conducted essentially according to the method of Makki.[24] Starting from the commercially available ethyl 5-acetyl-2-phenylthiazole-4-carboxylate (1a) or ethyl 5-acetyl-2-methylthiazole-4-carboxylate (1b), and treatment with hydrazine hydrate (1.1 equiv., in refluxing ethanol, 2 h), afforded the corresponding substituted methyl 4-oxothiazolo[4,5-*d*]pyridazines (2a and 2b). Chlorination of the enol with $POCl_3$ proceeded smoothly, giving (3a and 3b) and allowing subsequent displacement of the chlorine atom. This displacement could be readily accomplished in a two-stage procedure with thiourea and base. Alternatively, treatment of (2b) with Lawesson's reagent in refluxing toluene gave thiol analogue (4b), quantitatively.[25]

Treatment of a refluxing xylene mixture of (3a) with sodium thiomethoxide, resulted in the desired *S*-methyl analogue (5a) without the potential complication of *N*-methylation.

In order to assess the agonist/antagonistic effects of the compounds synthesized, we made use of a β-endorphin stimulation assay in mouse AtT20 cells which express type 1 receptors (see experimental).

As can be seen from Fig. 2, a 100 μM concentration of antalarmin results in a 54% decrease in the CRH-stimulated release of β-endorphin; characteristic of its antagonistic effects. However, both (4b) and (5a), when tested at the same concentration (100 μM), resulted in a 52 and 3% increase in β-endorphin levels, respectively, relative to the CRH stimulated levels. The latter result is unlikely to be significant and is probably a function of experimental error. Experiments with (5a) at higher concentrations failed to show any concentration dependent response in this assay (data not shown).

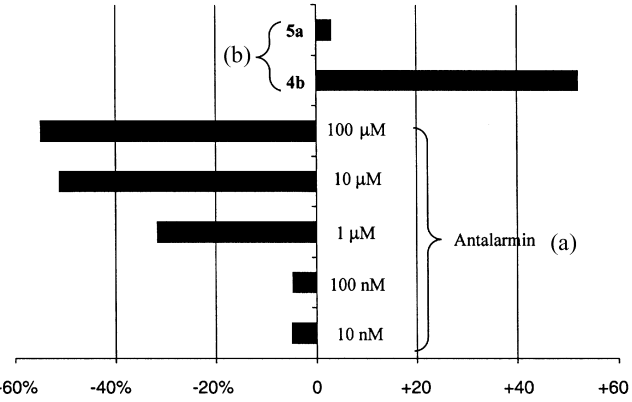

Fig. 2. % Change in β-endorphin release after addition of test compounds: (a) effects of Antalarmin at different doses on the CRH (10 nM) stimulation of β-endorphin in cultured AtT20 cells; (b) effect of added thiazoles (4b) and (5a) (at 100 μM concentration) on β-endorphin concentrations in the presence of 10 nM CRH. A negative change in β-endorphin levels indicates an antagonistic effect. Conversely a positive change in β-endorphin levels indicates agonist effect. Data is the average of two experiments conducted in duplicate.

Conclusion

We believe that these thiazoles represent the first evidence of the potential of small molecules to act as agonists of CRH_1 receptors. We also believe that the results presented herein

will allow a greater understanding of CRH receptors and should act as a stepping-stone to more potent analogues that will be of use as pharmacological tools.

Acknowledgments

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Experimental

General Methods

^{1}H and ^{13}C nuclear magnetic resonance (n.m.r.) spectra were recorded at 300.13 and 75.47 MHz respectively, on a Bruker Advance 300 MHz spectrometer. Residual protonated solvent peaks were used as internal standards. Melting points are uncorrected and were measured using a Buchi melting point apparatus. Flash chromatography was performed according to the method of Still *et. al.*[26] using silica gel (Aldrich Chemical Co. 200–400 mesh, 60Å). All solvents were distilled from glass prior to use. Ethyl 5-acetyl-2-phenylthiazole-4-carboxylate (1a) and ethyl 5-acetyl-2-methylthiazole-4-carboxylate (1b) were purchased from the Maybridge Chemical Co. and used without further purification.

Synthesis

*7-Methyl-2-phenyl-4-oxothiazolo[4,5-*d*]pyridazine* (*2a*)

A solution of ethyl 5-acetyl-2-phenylthiazole-4-carboxylate (1a) (2.75 g, 10 mmol) in ethanol (25 ml) was heated under reflux with hydrazine monohydrate (1.00 g) for 2 h. The ethanol was removed under vacuum, and the resulting solid residue recrystallized from ethanol to yield white needles (2.163 g, 89%), m.p. 316–318°C. ^{1}H n.m.r. ($CDCl_3$) δ 12.90, s, 1H, NH; 7.70–8.25, m, 5H, Ph; 2.64, s, 3H, CH_3. ^{13}C n.m.r. ($CDCl_3$) δ 174.5, 160.2, 143.4, 142.2, 136.3, 135.8, 133.7, 131.4, 115.3, 24.5.

*2,7-Dimethyl-4-oxothiazolo[4,5-*d*]pyridazine* (*2b*)

As for (2a), starting from ethyl 5-acetyl-2-methylthiazole-4-carboxylate (1b) (2.13 g, 10 mmol). Recrystallization from ethanol afforded white needles (1.81 g, 100%), m.p. 254–255°C. ^{1}H n.m.r. ($CDCl_3$) δ 12.94, s, 1H, NH; 2.95, s, 3H, CH_3; 2.60, s, 3H, CH_3. ^{13}C n.m.r. ($CDCl_3$) δ 171.7, 157.4, 148.6, 140.9, 112.3, 21.6, 20.7.

*4-Chloro-7-methyl-2-phenylthiazolo[4,5-*d*]pyridazine* (*3a*)

A suspension of (2a) (0.243 g, 1 mmol) in freshly distilled $POCl_3$ (5 ml) was heated at reflux for 1 h. The excess $POCl_3$ was then removed under vacuum. The residual syrup was poured slowly onto finely crushed ice (50 g) and extracted with ether (10 × 15 ml), the solvent removed and the residual solid recrystallized from methanol to yield yellow needles (0.173 g, 66%) m.p. 218–220°C. ^{1}H n.m.r. ($CDCl_3$) δ 7.65–8.25, m, 5H, Ph; 2.75, s, 3H, CH_3. ^{13}C n.m.r. ($CDCl_3$) δ 175.3, 155.1, 148.5, 133.8, 132.5, 130.2, 129.1, 120.1, 112.3, 22.7.

*4-Chloro-2,7-dimethylthiazolo[4,5-*d*]pyridazine* (*3b*)

As for (3a), starting from 2,7-dimethyl-4-oxothiazolo[4,5-*d*]pyridazine (2b) (0.181 g, 1 mmol). Recrystallization from methanol yielded yellow needles (0.104 g, 52%), m.p. 146–148°C. ^{1}H n.m.r. ($CDCl_3$) δ 2.68, s, 3H, CH_3; 2.46, s, 3H, CH_3. ^{13}C n.m.r. ($CDCl_3$) δ 164.5, 155.8, 148.6, 145.8, 136.6, 21.5, 20.1.

*2,7-Dimethylthiazolo[4,5-*d*]pyradazine-4-thiol* (*4b*)

To a refluxing solution of (2b) (0.181 g, 1 mmol) in anhydrous toluene (50 ml), was added Lawesson's reagent (0.808 g, 2 mmol). The mixture was allowed to reflux for 24 h, after which it was cooled and the solvent removed under vacuum. The residual solid was purified by silica-gel flash chromatography using $CHCl_3$/MeOH (19:1) as eluent and then recrystallized from dimethyl sulfoxide (DMSO) to yield (4b), as pale yellow needles (0.197 g, 100%), m.p. 320–322°C (Found: C, 42.3; H, 3.4; N, 21.6%. Calc. for $C_7H_7N_3S_2$: C, 42.6; H, 3.6; N, 21.3%). ^{1}H n.m.r. (d_6-DMSO) δ 2.90, s, 3H, CH_3; 2.60, s, 3H, CH_3. ^{13}C n.m.r. (d_6-DMSO) δ 158.6, 155.8, 148.6, 145.8, 136.6, 21.5, 20.1.

*7-Methyl-2-phenylthiazolo[4,5-*d*]pyridazine-4-thiomethane* (*5a*)

A xylene solution (25 ml) of (3a) (0.261 g, 1 mmol) and sodium thiomethoxide (0.067 g, 0.95 mmol) was heated under reflux under a nitrogen atmosphere for 2 h, cooled and the solvent removed under vacuum. Recrystallization from DMSO yielded tan coloured crystals (0.205 g, 79%), m.p. 150–152°C (Found: C, 56.8; H, 3.7; N, 15.3%. Calc. for $C_{13}H_{11}N_3S_2$: C, 57.1; H, 4.1; N, 15.4%). ^{1}H n.m.r. ($CDCl_3$) δ 7.50–7.70, m, 5H, Ph; 2.95, s, 3H, CH_3; 2.85, s, 3H, CH_3. ^{13}C n.m.r. ($CDCl_3$) δ 174.1, 151.7, 149.1, 133.2, 132.7, 130.0, 128.9, 125.0, 22.5, 21.5.

Biochemistry

β-Endorphin Stimulation Experiments

The cells were washed with Dulbecco's modified Eagle's medium (DMEM) containing bovine serum albumin (0.2%, w/v) (Sigma Chemical Company Ltd, St. Louis, U.S.A.)] and then incubated for 90 min at 37°C in a humidified atmosphere of 5% CO_2 in air. The supernatant was removed and the cells were treated with 0.5 ml of incubation medium containing the specified drug together with 10 nM CRH (Peninsula Laboratories, Belmont, CA, U.S.A.). Following a 60 min incubation, the supernatant was collected and frozen at –80°C until assayed.

AtT20 cells were cultured in multiwell plates and stimulated with CRH (10 nM), test compounds plus 10 nM CRH, or 10 nM CRH plus antalarmin. The cells were incubated and the level of β-endorphin stimulated in AtT20 cells, which express type 1 receptors,[27] were briefly recorded. A decrease in β-endorphin levels (i.e. negative) is indicative of antagonistic activity (cf. dose response with antalarmin, a known potent CRH type 1 antagonist) (Fig. 2); and an increase in β-endorphin levels (i.e. positive) is indicative of agonist activity. Note in Fig. 2, that the CRH stimulated β-endorphin release (i.e. the concentration of β-endorphin measure above the basal level) has been normalized to 0%, the percent increase or decrease is then reported relative to the stimulated level.

AtT20 Cell Cultures

AtT20 mouse anterior-pituitary tumour cells were grown and subcultured in DMEM (4500 mg glucose/l, with glutamine) (Gibco, Grand Island, NY, U.S.A.), buffered with Hepes and $NaHCO_3$ (BDH Chemicals) at pH 7.4, supplemented with 10% foetal bovine serum (Cytosystems, N.S.W., Australia), 5% horse serum (Gibco) and penicillin-streptomycin (100 IU ml^{-1}, 100 mg ml^{-1}). Cells were plated in 24-well plates (nunclon–22, falcon–23) at an initial density of 50,000 cells/well and were used 3 days after subculturing (~60% confluency).

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