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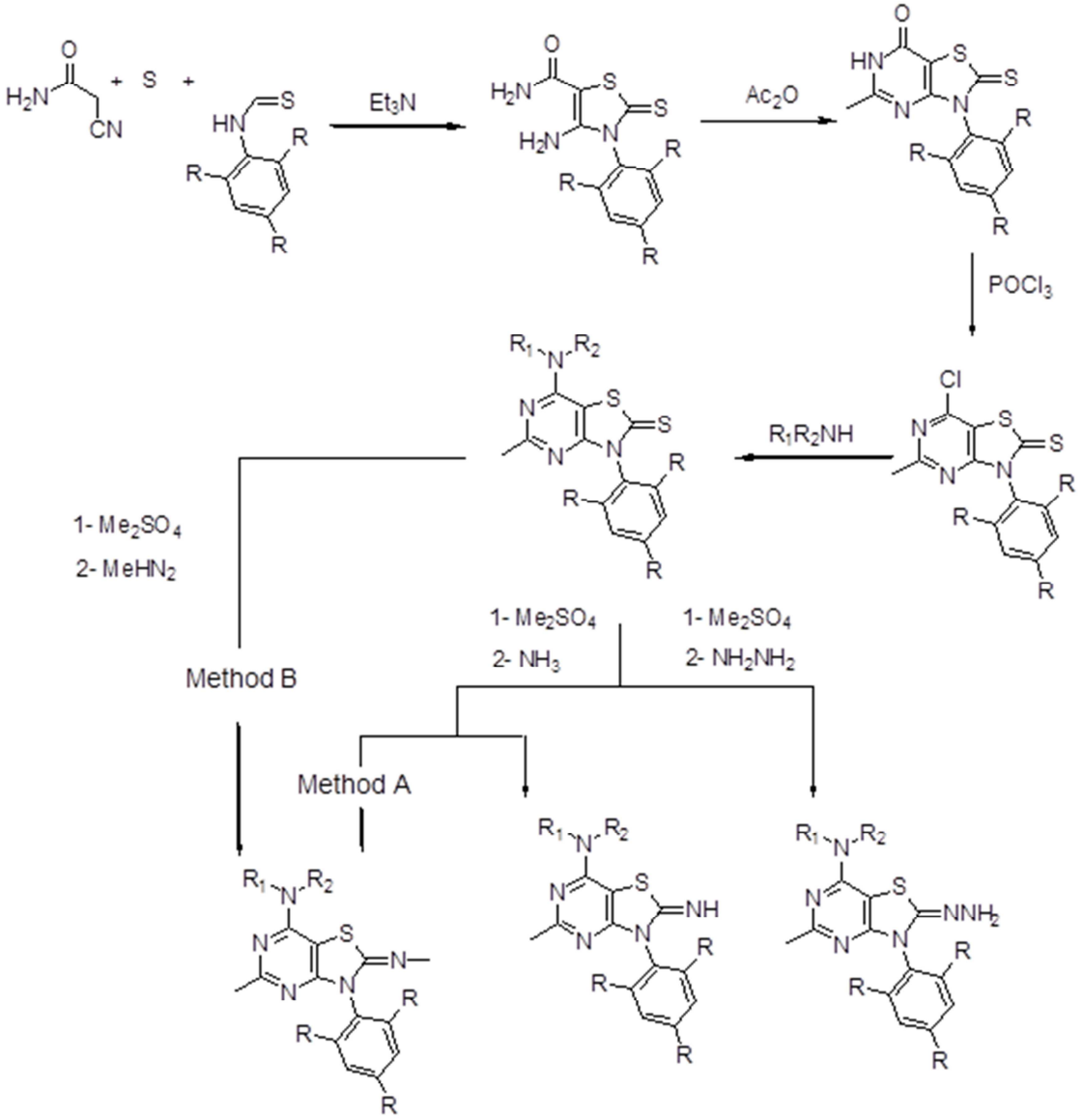

Designed CRF receptor Antagonists

Synthesis of 2-imino and 2-hydrazono thiazolo[4,5-d]pyrimidines as corticotropin releasing factor (CRF) antagonists

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Abstract:

Corticotropin-releasing factor (CRF) is an important neuropeptide hormone which controls the body's overall response to stress. It plays a crucial role in regulating the behavioral, cardiovascular, immune and gastrointestinal systems. Over-activation of the CRF system has been implicated in many disorders including anxiety, depression, drug addiction, hypertension, Irritable Bowel Syndrome (IBS), peptic ulcers, inflammation and others. Thus, binding of CRF to its receptors is an attractive target to develop new medications which aim at treating ailments associated with chronic stress. Numerous small-molecule non-peptide CRF receptor antagonists were developed and many are in various stages in clinical trials. Many showed great promise in treatment of anxiety, depression, peptic ulcers, inflammation, IBS and drug addiction. In our recent previous work, the development of two series of pyrimidine and fused pyrimidine CRF antagonists were described. In continuation of our efforts in this direction, in the current manuscript, the synthesis of a third series of CRF receptor antagonists is described. The binding affinities of select compounds for the type 1 receptor of CRF (CRF_1R) were determined and compared to a standard CRF antagonist drug antalarmin. A lead compound was identified and further evaluated by measuring its effect on the inhibition of the agonist-stimulated accumulation of second messengers.

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1. Introduction

Corticotropin-releasing factor (CRF) is an important neuropeptide hormone secreted from the hypothalamus. It is a crucial component of the hypothalamus-pituitary-adrenal (HPA) axis. CRF ultimately controls the release of cortisol by the adrenal cortex in response to stress, and thus it controls the stress-coping mechanisms. It works closely with many other systems and affects several biological processes associated with stress [1-4]. Many ailments associated with or related to anxiety or stress are assumed to be associated with excessive activation of the CRF system. Dysfunction of this system is also implicated in many diseases such as major depression, anxiety, eating disorders, drug addiction, Irritable Bowel Syndrome, inflammation, peptic ulcers and others [1-5]. The identification and characterization of a family of CRF peptides agonists and antagonists, coupled with the cloning and molecular characterization of the two main CRF receptor subtypes, CRF_1 and CRF_2, in addition to the discovery of antagonists selective to a particular CRF receptor sub-types, altogether provided new visions to further clarify the mechanism of how stress may affect different systems. It also signified the role of the CRF system and its implication in several stress-related ailments [5, 6].

The CRF role and its implication in the many disorders and behavioral, cardiovascular, gastrointestinal and immune systems suggests that new drugs that can interfere with the CRF function or CRF binding to its intended receptors may represent a different class of neuropsychiatric drugs for stress-related disorders such as anxiety and depressive and addictive disorders as well as for other peripheral disorders as Irritable Bowel Syndrome, inflammation, peptic ulcer and others [7-25]. Significant progress has been achieved in describing the structure-activity relationships of compounds acting as a selective agonists and antagonists for CRF_1 and CRF2 receptors such as the peptides astressin and anti-sauvagine1-30 [26]. However, because of the peptide nature of these compounds, their physical properties posed a boundary for their use in clinical settings. This resulted in pursuing non-peptide antagonists as a better alternative [27]. Currently, only CRF_1 selective non-peptide antagonists are available. Thus, the design of small molecule, non-peptide antagonists for the CRF receptor may afford new treatment options for many stress-related disorders. Hundreds of accounts of small molecule antagonists have appeared in the literature and the preclinical and clinical pharmacology have been reported for many of these agents. The main research into CRF antagonists to date has focused on non-peptide CRF_1 receptor antagonists to target health problems arising as a result of chronic stress and thus as a potential treatment options for anxiety-related and stress-related disorders [28,29]. Several classes of small molecule non-peptide CRF receptor antagonists have been synthesized and many are commonly used in research.

Based on all these facts, the synthesis of more non-peptide CRF_1 receptor antagonists with a diverse structure scaffolds as well as further clinical investigations may shed more light on the how CRF is implicated in stress-related disorders and may present new potential treatment options for stress-related disorders.

Several major pharmaceutical companies developed small non-peptide CRF_1 receptor antagonists including Ely-Lilly [30], Neurogen [31, 32], DuPont pharmaceutical which has been acquired by Bristol-Myers-Squibb in 2001 [33], Pfizer [34, 35], Bristol-Myers-Squibb [36-42], Glaxo Smith Kline [43-45] and Neurocrine biosciences [46-51]. The relationship between structure of non-peptide CRF_1 antagonists and their affinity to CRF_1 receptors indicates that CRF_1 antagonists are typically built of three moieties: a hydrophobic moiety up, a proton accepting moiety in the middle, and an aromatic moiety down. Thus the basic pharmacophore is proposed to be made of a monocyclic or bicyclic heterocyclic ring carrying a hydrophobic dialkylamino group on one flank and an aryl ring, usually substituted at the 2-, 4- and 6-positions, on the opposite flank (Figure 1). Having the aromatic ring orthogonal to the heterocyclic core appears to be essential for the affinity. A methyl group on the heterocyclic core also appears to enhance the CRF_1 antagonist properties [29, 52, 53]. X-ray structure of several non-peptide CRF_1 antagonists confirms that extension of the aromatic or hetero-aromatic hydrophobic unit (down area) and the alkyl groups on the amino functionality (up area) are out of the plane of the core heterocyclic unit (central area) are required for optimum CRF receptor binding affinity [54]. The most prominent CRF_1 antagonists identified from these studies were antalarmin [55-61], pexacerfont [62, 63] and verucerfont [64-66].

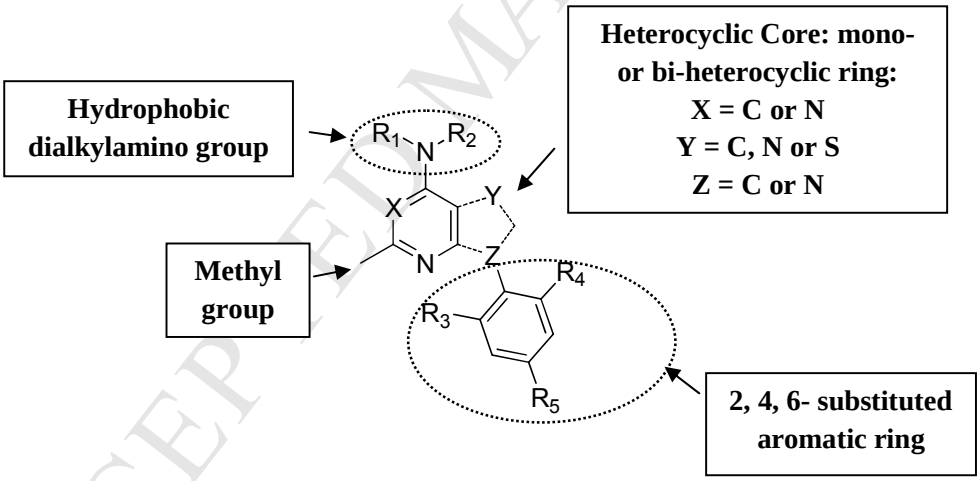

Figure 1. The proposed pharmacophoric framework of the non-peptide CRF_1 antagonists.

In our recent published work [67, 68], several series of substituted pyrimidines and fused pyrimidines were synthesized. These compounds retained the main structural features of the CRF pharmacophore, and showed structural similarities to CRF antagonists which were in clinical trials. These series were evaluated for their binding to CRF_1R in two stages. First, the ability of the test compounds at a single concentration (500-1000 nM) to inhibit the specific binding of [^{125}I]-Tyr^0 sauvagine, which is a strong agonist, was evaluated. Compounds showing promising binding to CRF_1R were subjected to a second pharmacological characterization by determination of their binding affinities (IC_{50}) for this receptor in competition experiments in comparison to the

standard drug Antalarmin. Several lead compounds were identified.To continue our work in this direction, in this manuscript the synthesis and characterization of a new serious of 2-substituted thiazolo[4,5-*d*]pyrimidines is described. The binding affinities (IC_{50} values) for the CRF_1R of representative compounds was evaluated with promising results and compared with that of antalarmin. These compounds were found to bind to CRF_1R with better affinities than our previous compounds [67, 68]. A lead compound was identified and its effect on the inhibition of the agonist-stimulated accumulation of second messenger of CRF_1 receptors was measured.

2. Results and discussion

2.1. Chemistry

The general synthetic scheme of the target compounds is described in Figure 2. The starting 4-amino-3-aryl-5-carboxamido-thiazole-2(*3H*)-thiones **(1a & b)** were synthesized following Gewald reaction conditions by reacting the selected substituted phenyl isothiocyanate with cyanoacetamide and sulfur base under basic catalysis [69-71]. Based on the previous SAR studies for optimum CRF_1 binding antagonist activity, the 2,4,6-trimethylphenylisothiocyanate and 2,4,6-trichlorophenylisothiocyanate were selected so that they introduce a tri-substituted phenyl at position 3 of the resulting thiazolo[4,5-*d*]pyrimidines [72, 73]. The starting thiazole-2(*3H*)-thiones **(1a & b)** were then cyclized with acetic anhydride at reflux temperature to yield 3-aryl-5-methylthiazolo[4,5-*d*]pyrimidin-7(*6H*)-one-2(*3H*)-thiones **(2a & b)** using the reaction conditions described by Badawey et al. [74, 75] and Fahmy et al. [76]. Compounds **2a & b** were then subjected to chlorination using phosphorous oxychloride at reflux temperature under the reported reactions conditions [74-76] to yield 3-aryl-7-chloro-5-methylthiazolo[4,5-*d*]pyrimidine-2*(3H*)-thiones **(3a,b)** in excellent yields.

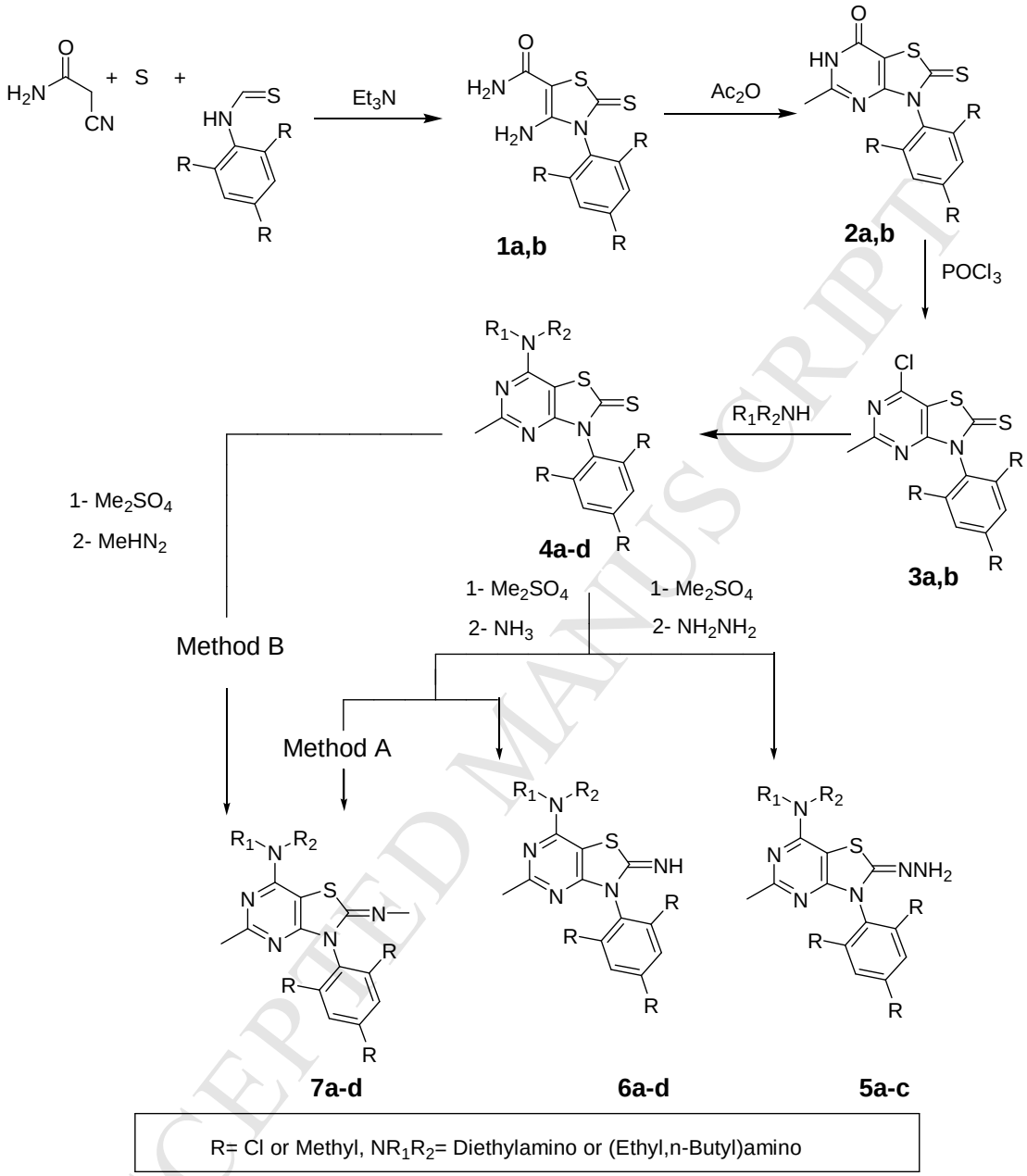

Figure 2. Synthetic scheme of compounds

Reaction of the 7-chloro derivatives (**3a, b)** with 2 equivalent of the selected secondary amines in refluxing ethanol, under the established reaction conditions [74-76], afforded the 3-aryl-7-dialkylamino-5-methylthiazolo[4,5-*d*]pyrimidine-2(*3H*)-thiones (**4a & b**). Diethyl amine and [ethyl,n-butyl]amine were selected, since previous reports showed optimum CRF_1 receptor antagonist activities of derivatives with those particular amino groups at position 7 of the thiazolo[4,5-*d*]pyrimidine ring [72, 73].

The target 7-dialkyl-5-methyl-3-(substituted phenyl)-thiazolo[4,5-*d*]pyrimidin-2(*3H*)-hydrazones **(5a-c)** were synthesized by the reaction of the corresponding 3-aryl-7-dialkylamino-5-methylthiazolo[4,5-*d*]pyrimidine-2(*3H*)-thiones **(4a-c)** with dimethyl sulfate in acetonitrile at reflux temperature, followed by reaction of the produced 2-methylthiazolium intermediate with excess hydrazine following the reported reaction conditions [76]. Nucleophilic substitution of the 2-thioxo function of 3-aryl-7-dialkylamino-5-methylthiazolo[4,5-*d*]pyrimidine-2(*3H*)-thiones with oxygen or amino functions was first described by Gewald [69, 70] and later with active methylene-containing molecules [69]. Moreover, in the current study, this reaction was extended to utilize ammonia as a nucleophile. Thus, the 2-imino target compounds **(6a-d)** were prepared using the same reaction conditions but using ammonia (7 Normal solution in methanol). The corresponding 2-methylimino derivatives **(7a-d**) were also produced, possibly due to *in situ* alkylation of the 2-imino products **(6a-d)** by the excess dimethyl sulfate in the reaction mixture. To further confirm this finding, the same 2-methylimino derivatives **(7a-d**) were also prepared from compounds **4 a-c** using methylamine instead of ammonia.

2.2. Biological evaluation

Three representative compounds (Figure 3) were evaluated for theirabilities to displace radiolabelled sauvagine binding at binding affinities concentration of 300 nM. The binding affinities of these compounds is illustrated in Figure 4.

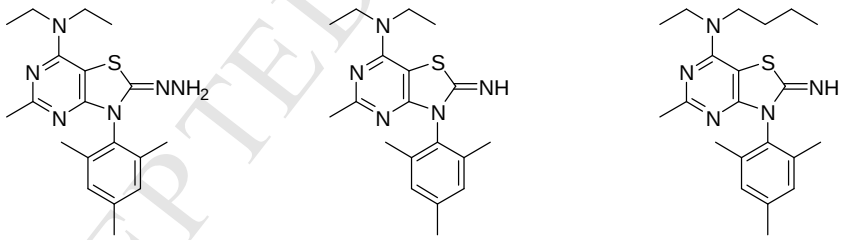

Figure 3. Structures of the representative test compounds

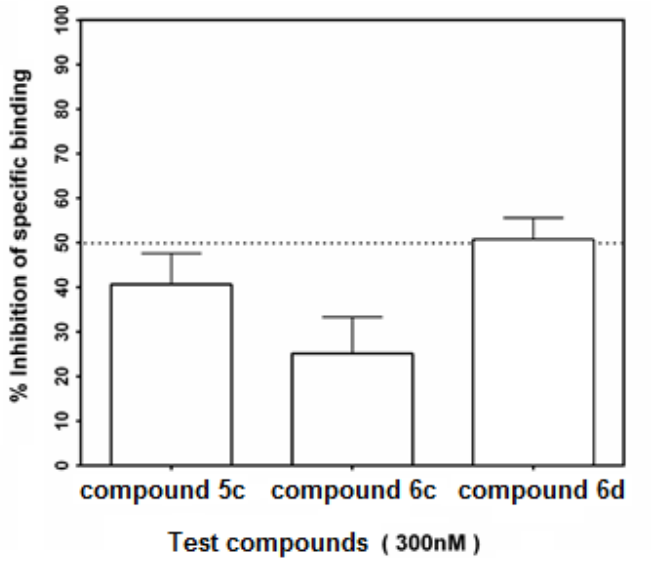

Figure 4. % Inhibition of [^{125}I]-Tyr^{0}-sauvagine specific binding by 300 nM of analogs 1-3 on membranes from HEK 293 cells stably expressing the human CRF_1 receptor. In the absence of compounds the inhibition is 0%.

In a second assay, a specific, aquantitative assay to measure their competitive binding compared with a standard drug antalarmin at different concentrations was carried out. In this experiment we determined the Log IC_{50} values of these compounds, which under the experimental conditions of this study are similar to their binding affinities (Log $_{Ki}$ values). It showed that the selected compounds displayed Log IC_{50} ± S.E values of -6.35 ± 0.158 (compound **5c**), -6.05 ± 0.139 (compound **6c**) and -6.77 ± 0.224 (compound **6d**) compared to -7.73 ± 0.074 value for Antalarmin (Figure 5).

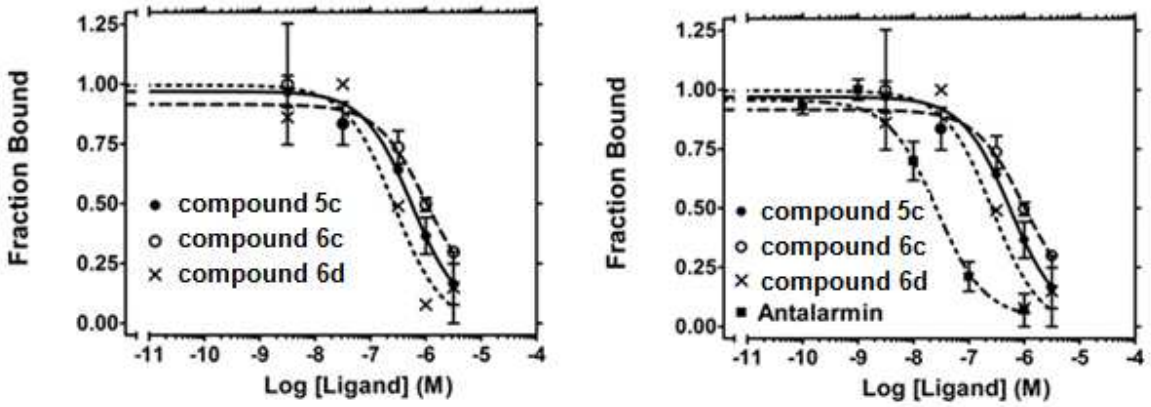

Figure 5. Competition binding isotherms of analogs 1-3 to human CRF_1 receptor: Competition of [^{125}I]-Tyr^0-sauvagine specific binding by increasing concentrations of test compounds performed on membranes from HEK 293 cells stably expressing the human CRF_1 receptor.

A third biological evaluation to screen the compound **6d** with the highest binding affinity (lowest IC_{50}) was carried out by measuring its ability to antagonize agonist-stimulated accumulation of cAMP, which is the second messenger that mediates the effects resulting from stimulating CRF_1 receptors,. Results of this evaluation were very promising (Figure 6). The Lead compound (**6d)**, at concentration of 1μM, was able to significantly and substantially reduce the potency of sauvagine to cause accumulation of cAMP. In the presence of compound **6d**, $EC_{50\%}$ of sauvagine was 1.114 nM (Log $EC_{50\%}$ = -8.953), whereas, in its absence, $EC_{50\%}$ was only 0.203 nM (Log $EC_{50\%}$ = -9.692). This clearly indicates the potential of this class of compounds for development as possible new class of medications for stress-related illnesses and that further structural modifications may lead to compounds with superior CRF_1 antagonist properties.

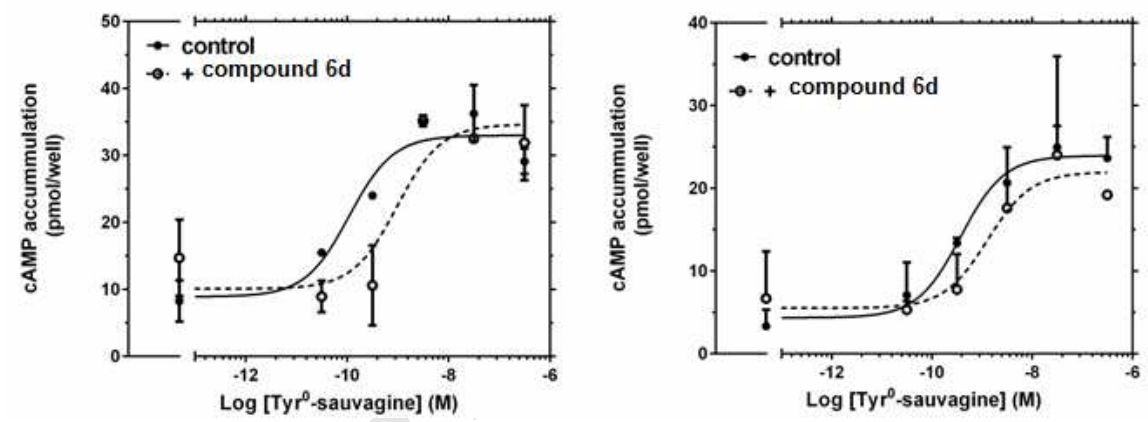

Figure 6. Inhibition of sauvagine-induced cAMP accumulation by compound **6d**

3. Conclusion

A novel series of thiazolo[4,5-*d*]pyrimidines-2-imine and 2-hydrazones were synthesized as CRF_1 receptor antagonists. These compounds were designed to have the structural features known for optimum binding affinity for CRF_1 receptors, particularly a tri-substituted phenyl at position-3, a methyl group at position-5 and a dialkylamino group at position-7. Select compounds were evaluated for their binding affinities for CRF_1 receptors in comparison to antalarmin, a standard CRF_1 antagonist drug. A lead compound was identified and was evaluated for its effects to antagonize sauvagine in stimulating the accumulation of cAMP. It showed an almost 5-fold inhibition which confirmed the potential of the new series for further development as CRF_1 receptor antagonists.

4. Experimental

4.1. Chemistry

4.1.1 General information

All chemicals were purchased from commercial sources. Flash column chromatography separation was performed using Acros organics silica gel 40-60 µm, 60 Å using combination of ethyl acetate and hexanes. Preparative thin layer chromatography was performed using UNIPLATE ^{TM}1500µm silica gel plates with UV 254 preparative layer. Whatman and sigma TLC plates were utilized for thin layer chromatography and visualization was done using UV fluorescence at 254 nm. Melting points were recorded on a Mel-Temp, Laboratory devices, Inc and are uncorrected. %CHN Analyzer by combustion/ TCD and %S by O flask combustion/IC were used for elemental analysis of final compounds and performed by Micro Analysis Inc., Wilmington DE, USA and are within 0.4%. ^{1} H and ^{13}C NMR spectra were obtained on a BrukerAvance 400 MHz & 600MHz instrument using DMSO-d6 as solvent unless otherwise stated. ^{1}H NMR Spectra are reported in order; multiplicity, number of protons and signals were characterized as s (singlet), d (doublet), dd (doublet of doublet), t (triplet), m (multiplet), br s (broad signal), q (quartet), quin (quintet), tquin (triplet of quintet), sxt (sextet), spt (septet). Chemical shifts are relative to TMS as an internal standard. Mass spectra were recorded on ThermoFinnigan MAT95XL high resolution magnetic sector mass spectrometer, using electrospray ionization method. The IR spectra were recorded on ZnSe crystal at 8 cm^{-1} resolution in Nicolet 380 ATR-FTIR spectrophotometer (Thermo electron Corporation, Madison, WI).

4.1.2 Synthesis of compounds

Compounds 1-4 were synthesized as previously reported [68, 73].

*7-Dialkylamino-5-methyl-3-(substitute-phenyl)thiazolo[4,5-d]pyrimidine-2(3H)-hydrazones(**5a-c**)*

Dimethyl sulfate (3.0 mmol) was added to a solution of **4** (1.0 mmol) in acetonitrile (20 mL). The reaction mixture was stirred at reflux temperature for 10-12h. Additional dimethyl sulfate (3.0 mmol) was added then the mixture was stirred at reflux temperature and monitored by TLC till completion of the reaction (10-12h). The reaction was allowed to cool down to room temperature. Then the reaction mixture was slowly added drop-wise toa solution of hydrazine hydrate 100% (10 mmol) in methanol (10 mL) while stirring at 0-5 ^{o} C to.The reaction mixture was allowed to reach room temperature during stirring and monitored with TLC till completion (6-10h). After completion, the reaction mixture was poured onto cold water (200 mL) and extracted with ethyl acetate (100 mL x 3). The combined organic layers were washed with water

and brine then dried over anhydrous sodium sulfate and evaporated under reduced pressure. The obtained crude residue was then purified by preparative TLC using ethyl acetate / hexane (1:12).

*7-Diethylamino-5-methyl-3-(2,4,6-trichlorophenyl)-2,3-thiazolo[4,5-d]pyrimidine-2(3H)-hydrazone (**5a**):*

Yield: semisolid (70%);^{1}H NMR (400 MHz, $CDCl_3$) δ 7.49 (s, 2H), 4.41 (br. s., 2H), 3.63 (q, J = 8 Hz, 4H), 2.31 (s, 3H), 1.28 (t,J = 6 Hz, 6H); ^{13}C NMR (400 MHz, $CDCl_3$) δ 164.67, 157.91, 155.27, 153.68, 136.25, 135.81, 130.91, 129.02, 89.26, 42.91, 25.76, 14.44; HRMS (ES^+) calculated for $C_{16}H_{18}N_6Cl_3S$ $[M+H]^+$: 431.0374; found: 431.03925.

*7-(Butyl,ethyl)amino-5-methyl-3-(2,4,6-trichlorophenyl)thiazolo[4,5-d]pyrimidine-2(3H)-hydrazone (**5b**).*

Yield: semisolid (80%);^{1}H NMR (400 MHz, $CDCl_3$) δ 7.48 (s, 2H), 4.42 (br. s., 2H), 3.63 (q, J = 8 Hz, 2H), 3.55 (t,J = 8 Hz, 2H), 2.30 (s, 3H), 1.69-1.64 (m, 2H), 1.43-1.38 (m, 2H), 1.26 (t,J = 8 Hz, 3H), 0.99 (t,J = 6 Hz, 3H); ^{13}C NMR (400 MHz, $CDCl_3$) δ 164.57, 157.91, 155.38, 153.39, 136.27, 135.78, 130.94, 129.00, 89.26, 48.15, 43.46, 31.38, 25.73, 20.11, 14.34, 14.03; HRMS (ES^+) calculated for $C_{18}H_{22}N_6Cl_3S$ $[M+H]^+$: 459.0687; found: 459.06924.

*7-Diethylamio-3-mesityl-5-methylthiazolo[4,5-d]pyrimidin-2(3H)-hydrazone (**5c**).*

Yield: semisolid (66%);^{1}H NMR (400 MHz, $CDCl_3$) δ 6.98 (d,J = 8 Hz, 2H), 4.38 (br. s., 2H), 3.65 (q, J = 8 Hz, 4H), 2.33, 2.32 (s, 3H), 2.31, 2.30 (2s, 3H), 2.06 (s, 6H), 1.28 (t,J = 8 Hz, 6H); ^{13}C NMR (400 MHz, $CDCl_3$) δ 164.71, 159.01, 155.22, 138.79, 136.28, 136.18, 131.46, 129.40, 129.07, 89.19, 42.81, 25.90, 21.30, 17.99, 17.91, 14.49, 14.44; HRMS (ES^+) calculated for $C_{19}H_{27}N_6S$ $[M+H]^+$: 371.2012; found: 371.20151.

*7-Dialkylamino-5-methyl-3-(substitutedphenyl)thiazolo[4,5-d]pyrimidin-2(3H)-imine (**6a-d**) and 7-dialkylamino-5-methyl)-3-(substituted-phenyl)thiazolo[4,5-d]pyrimidin-2(3H)-methylimine (**7a-d**) [Method A]*

Dimethyl sulfate (3.0 mmol) was added to a solution of **4** (1.0 mmol) in acetonitrile (20 mL). The reaction mixture was stirred at reflux temperature for 10-12 h. Additional dimethyl sulfate (3.0 mmol) was then added and the mixture was stirred at reflux temperature and monitored by TLC till completion of the reaction (10-12 h). The reaction mixture was allowed to cool down to room temperature. A solution of 7N ammonia in methanol (10 mmol) was then slowly added to the reaction mixture while stirring at 0-5 ^{o} C. The reaction mixture was allowed to reach room temperature during stirring and monitored with TLC till completion (6-8 h). After completion, the reaction mixture was evaporated under reduced pressure, diluted with water and extracted with dichloromethane. The combined organic layer was washed with water, brine solution, dried over anhydrous sodium sulfate and concentrated under vacuum. The crude residue

was then purified by preparative TLC using ethyl acetate / hexane (1:12) to afford the imino and methylimino derivatives.

7-Dialkylamino-5-methyl)-3-(substituted-phenyl)thiazolo[4,5-d]pyrimidin-2(3H)-methylimine (7a-d) [Method B]

Dimethyl sulfate (3.0 mmol) was added to a solution of **4** (1.0 mmol) in acetonitrile (20 mL). The reaction mixture was stirred at reflux temperature for 10-12 h. Additional dimethyl sulfate (3.0 mmol) was added then the mixture was stirred at reflux temperature and monitored by TLC till completion of the reaction (10-12 h). The reaction was allowed to cool down to room temperature. A solution of 2M methylamine in THF (12 eq.) was then slowly added to the reaction mixture while stirring at 0-5 ^{o} C. The reaction mixture was allowed to reach room temperature during stirring and monitored with TLC till completion (4-8 h). After completion, the reaction mixture was evaporated under reduced pressure, diluted with water and extracted with dichloromethane. The combined organic layer was washed with water, brine solution, dried over anhydrous sodium sulfate and concentrated under vacuum. The crude residue was then purified by preparative TLC using ethyl acetate / hexane (1:12). Yield: semisolid (40 %).

*7-Diethylamino-5-methyl-3-(2,4,6-trichlorophenyl)thiazolo[4,5-d]pyrimidin-2(3H)-imine (**6a**).*

Yield: semisolid (40%);^{1}H NMR (400 MHz, $CDCl_3$) δ 7.50 (s, 2H), 3.57 (q, J = 8 Hz, 4H), 2.31 (s, 3H), 1.25 (t,J = 8 Hz, 6H); ^{13}C NMR (400 MHz, $CDCl_3$) δ 164.72, 157.15, 154.89, 136.38, 136.10, 130.24, 129.05, 89.53, 42.86, 25.70, 14.37; HRMS (ES^+) calculated for $C_{16}H_{17}N_5Cl_3S$ $[M+H]^+$:416.0265; found: 416.02695.

*7-(Butyl,ethyl)amino-5-methyl-3-(2,4,6-trichlorophenyl)thiazolo[4,5-d]pyrimidin-2(3H)-imine (**6b**)*

.Yield: semisolid (36%);^{1}H NMR (400 MHz, $CDCl_3$) δ 7.51 (s, 2H), 3.58 (q, J = 8 Hz, 2H), 3.42 (t,J = 8 Hz, 2H), 2.30 (s, 3H), 1.67-1.63 (m, 2H), 1.37-1.42 (m, 2H), 1.24 (t,J = 8 Hz, 3H), 0.99 (t,J = 8 Hz, 3H); HRMS (ES^+) calculated for $C_{18}H_{21}N_5Cl_3S$ $[M+H]^+$: 444.0578; found: 444.05704.

*7-Diethylamino-3-mesityl-5-methylthiazolo[4,5-d]pyrimidin-2(3H)-imine (**6c**).*

Yield: semisolid (41%);^{1}H NMR (400 MHz, $CDCl_3$) δ 6.93 (s, 2H), 3.51 (q, J = 8 Hz, 4H), 2.25 (s, 3H), 2.22 (s, 3H), 1.99 (s, 6H), 1.18 (t,J = 8 Hz, 6H); ^{13}C NMR (400 MHz, $CDCl_3$) δ 164.66, 158.07, 155.08, 139.24, 136.90, 129.89, 129.62, 126.86, 89.34, 42.78, 25.86, 21.27, 17.80, 14.41; HRMS (ES^+) calculated for $C_{19}H_{26}N_5S$ $[M+H]^+$: 356.1903; found: 356.19012.

*7-(Butyl, ethyl)amino-3-mesityl-5-methylthiazolo[4,5-d]pyrimidin-2(3H)-imine (**6d**).*

Yield: semisolid (42%);^{1}H NMR (400 MHz, $CDCl_3$) δ 7.01 (s, 2H), 3.60 (q, J = 8 Hz, 2H), 3.49 (t,J = 8 Hz, 2H), 2.33 (s, 3H), 2.29 (s, 3H), 2.07 (s, 6H), 1.67-1.64 (m, 2H), 1.43-1.37

(m, 2H), 1.24 (t,J = 8 Hz, 3H), 0.99 (t,J = 8 Hz, 3H); ^{13}C NMR (400 MHz, $CDCl_3$) δ 164.56, 158.09, 155.19, 139.22, 136.91, 129.61, 89.29, 48.09, 43.32, 31.56, 25.85, 21.27, 20.06, 17.81, 14.17, 14.00; HRMS (ES^+) calculated for $C_{21}H_{30}N_5S$ $[M+H]^+$: 384.2216; found: 384.22189.

*7-Diethylamino-5-methyl-3-(2,4,6-trichlorophenyl)thiazolo[4,5-d]pyrimidin-2(3H)-methylimine (**7a**).*

Yield: (30 %); MP: 172; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.47 (s, 2H), 3.62 (q, J = 8 Hz, 4H), 3.06 (s, 3H), 2.31 (s, 3H), 1.26 (t,J = 6 Hz, 6H);^{13}C NMR (400 MHz, $CDCl_3$) δ 164.54, 157.53, 155.02, 151.93, 136.35, 135.59, 131.42, 128.95, 89.03, 42.91, 40.38, 25.80, 14.46; HRMS (ES^+) calculated for $C_{17}H_{19}N_5Cl_3S$ $[M+H]^+$: 430.0421; found: 430.04169

*7-(Butyl,ethyl)amino-5-methyl-3-(2,4,6-trichlorophenyl)thiazolo[4,5-d]pyrimidin-2(3H)-methylimine (**7b**)*

Yield: (31%); MP: 136;^{1}H NMR (400 MHz, $CDCl_3$) δ 7.47 (s, 2H), 3.63 (q, J = 8 Hz, 2H), 3.55 (t,J = 8 Hz, 2H), 3.06 (s, 3H), 2.30 (s, 3H), 1.70-1.65 (m, 2H), 1.43-1.38 (m, 2H), 1.26 (t,J = 8 Hz, 3H), 0.99 (t,J = 8 Hz, 3H); ^{13}C NMR (400 MHz, $CDCl_3$) δ 164.45, 157.53, 155.12, 151.85, 136.32, 135.55, 131.44, 128.95, 88.99, 48.13, 43.43, 40.41, 31.39, 25.78, 20.09, 14.31, 14.01; HRMS (ES^+) calculated for $C_{19}H_{23}N_5Cl_3S$ $[M+H]^+$: 458.0734; found: 458.07184.

*7-Diethylamino-3-mesityl-5-methylthiazolo[4,5-d]pyrimidin-2(3H)-methylimine (**7c**).*

Yield: (31%); MP: 132;^{1}H NMR (400 MHz, $CDCl_3$) δ 6.89 (s, 2H), 3.57 (q, J = 8 Hz, 4H), 2.96 (s, 3H), 2.22 (s, 6H), 1.96 (s, 6H), 1.20 (t,J = 8 Hz, 6H); ^{13}C NMR (400 MHz, $CDCl_3$) δ 164.58, 158.68, 154.97, 138.50, 136.32, 131.91, 129.32, 128.47, 126.92, 88.93, 42.80, 40.56, 25.95, 21.32, 17.96, 14.49; HRMS (ES^+) calculated for $C_{20}H_{28}N_5S$ $[M+H]^+$: 370.2060; found: 370.20651

*7-(Butyl,ethyl)amino-3-mesityl-5-methylthiazolo[4,5-d]pyrimidin-2(3H)-methylimine (**7d**).*

Yield: semisolid (30%);^{1}H NMR (400 MHz, $CDCl_3$) δ 6.97 (s, 2H), 3.65 (q, J = 8 Hz, 2H), 3.56 (t,J = 8 Hz, 2H), 3.04 (s, 3H), 2.30 (s, 3H), 2.29 (s, 3H), 2.04 (s, 6H), 1.71-1.65 (m, 2H), 1.44-1.38 (m, 2H), 1.26 (t,J = 8 Hz, 3H), 1.00 (t,J = 8 Hz, 3H); ^{13}C NMR (400 MHz, $CDCl_3$) δ 164.46, 158.69, 155.06, 153.22, 138.45, 136.34, 129.30, 88.90, 48.05, 40.63, 31.44, 25.92, 21.31, 20.12, 17.96, 14.34, 14.02; HRMS (ES^+) calculated for $C_{22}H_{32}N_5S$ $[M+H]^+$: 398.2373; found: 398.23746.

4.2. Biological Evaluation

4.2.1. CRF_1 Receptor binding study

Binding studies was performed according to our reported procedure [67,68] in membrane homogenates from human embryonic kidney cells (HEK 293) stably expressing CRF_1 receptors

and using [^{125}I]-Tyr^{0}-sauvagine as radioligand. Membrane homogenates were prepared according to the method of Gkountelias [77]. CRF_1-expressing HEK 293 cells, grown in DMEM/F12 (1:1) containing 3.15 g/L glucose, 10% bovine calf serum and 300 μg/ml of the antibiotic, Geneticin at 37^{o} C and 5% CO_2, were washed with phosphate-buffered saline (PBS) (4.3 mM Na_2HPO_4.7 H_20, 1.4 mM KH_2PO_4, 137 mMNaCl, 2.7 mMKCl, pH 7.2-7.3 at R.T). Then the cells were briefly treated with PBS containing 2 mM EDTA (PBS/EDTA), and then dissociated in PBS/EDTA. Cells suspensions were centrifuged at 1000 x g for 5 min at room temperature, and the pellets were homogenized in 1.5 ml of buffer H (20 mM HEPES, containing 10 mM $MgCl_2$, 2 mM EGTA, 0.2 mg/ml bacitracin and 0.93 μg/ml aprotinin pH 7.2 at 4^{o} C) using a Janke& Kunkel IKA Ultra Turrax T25 homogenizer, at setting ~20, for 10-15 sec, at 4^{o} C. The homogenates were centrifuged at 16000 x g, for 10 min, at 4^{o} C. The membrane pellets were re-suspended by homogenization, as described above, in 1 ml buffer B (buffer H containing 0.1% BSA, pH 7.2 at 20^{o} C). The membrane suspensions were then diluted in buffer B and aliquots of suspensions (50 μl) were added into tubes containing buffer B and 20-25 pM [^{125}I]-Tyr^{0}sauvagine without or with the new test compounds at the single concentration of 300 nM or with increasing concentrations of the test compounds (competition binding experiments) in a final volume of 0.2 ml. The mixtures were incubated at 20-21° C for 120 min and then were filtered through Whatman 934AH filters, presoaked for 1 h in 0.3% polyethylene imine at 4° C. The filters were washed 3 times with 0.5 ml of ice-cold PBS, pH 7.1 containing 0.01% Triton X-100 and assessed for radioactivity in a gamma counter. The amount of membranes used were adjusted to insure that the specific binding is always equal to or less than 10% of the total concentration of the added radio-ligand. Specific [^{125}I]-Tyr^{0}-sauvagine binding was defined as total binding less nonspecific binding in the presence of 1000 nMantalarmin. Data for competition binding was analyzed by nonlinear regression analysis, using Prism 4.0 (GraphPad Software, San Diego, CA). IC_{50} values were obtained by fitting the data from competition studies to a one-site competition model.

4.2.2. cAMP Accumulation Assays:

The cAMP Accumulation Assays was carried out according to the reported procedure [78]. HEK293 cells stably expressing the CRF_1R were plated in 96-well cell culture plates (pretreated with 0.1 mg/ml poly-L-lysine). After overnight incubation (37°C, 5% CO2) of cells (95–100% confluence) the medium was removed, and 100 μl of assay buffer (25 mM HEPES, pH 7.4, 2 mM choline, 288 mM sucrose, 0.9 mM CaCl2, 0.5 mM MgCl2, and 1 mM 3-isobutyl-1-methylxanthine) were added. After 1 h of incubation at 37°C, more assay buffer without (basal levels) or with increasing concentrations of CRF in the presence or absence of 1μM non-peptide CRF antagonist was added to a total volume of 200 μl, and the incubation was continued for 30 min at 37 °C. At the end of the incubation, the assay buffer was removed. The cells were placed on ice and lysed with 3% trichloroacetic acid. Lysates were incubated on ice for 30–60 min and stored at -20 °C. After 1–5 days, frozen lysates were thawed and centrifuged at 1800xg for 10 min at 4 °C, and the supernatants were neutralized with 2 N NaOH. Quantification of cAMP in

the neutralized supernatants was performed using a competitive binding assay. In brief, supernatants were transferred to polypropylene mini-tubes (20 µl/tube) containing buffer A (100 mMTris-HCl, pH 7.4, 100 mMNaCl, and 5 mM EDTA) with 1–1.5 nM [2,8-3H]cAMP. Subsequently, cAMP-binding protein (~100 mg of crude bovine adrenal cortex extract in 500 ml of buffer A) was added to each tube. After incubation on ice for 3 h, the mixtures were filtered through Whatman 934AH glass fiber filters using buffer C (120 mMNaCl and 10mM TrisHCl, pH 7.4, at 4°C) as washing buffer. The amount of cAMP in each sample (one-tenth of a well) was determined by comparison with a standard curve of known concentrations of unlabeled cAMP (1–100 pmol/tube). The logEC50 values were obtained by fitting the data to a one-site sigmoidal model using nonlinear regression analysis

4.2.3. Statistical Analysis

Prism 4.0 was used for all statistical analysis (GraphPad Software, San Diego, CA). Competition binding were analyzed by nonlinear regression analysis, and IC_{50} values were obtained by fitting the data from competition studies to a one-site competition model by nonlinear regression.

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Highlights:

- 2-iminothiazolo[4,5-*d*]pyrimdines as CRF receptor antagonists.
- 2-hydrazonothiazolo[4,5-*d*]pyrimdines as CRF receptor antagonists.
- Inhibition of binding of radiolabelled sauvagive to CRF 1 receptors by thiazolo[4,5-*d*]pyrimdines.
- Inhibition of suavagine-induced accumulation of cAMP by thiazolo[4,5-*d*]pyrimdines.