Synthesis of New Thiazolo[4,5-*d*]pyrimidines as Corticotropin Releasing Factor Modulators

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Abstract: Corticotropin-releasing factor (CRF) is a neurohormone that plays a crucial role in integrating the body's overall response to stress. It appears necessary and sufficient for the organism to mount functional, physiological and endocrine responses to stressors. CRF is released in response to various triggers such as chronic stress. The role of CRF and its involvement in these neurological disorders suggest that new drugs that can target the CRF function or bind to its receptors may represent a new development of neuropsychiatric medicines to treat various stress-related disorders including depression, anxiety and addictive disorders. Based on pharmacophore of the CRF_1 receptor antagonists, a new series of thiazolo[4,5-d] pyrimidines were synthesized as Corticotropin-releasing factor (CRF) receptor modulators and the prepared compounds carry groups shown to produce optimum binding affinity to CRF receptors. Twenty two compounds were evaluated for their CRF_1 receptor binding affinity in HEK 293 cell lines and two compounds 5o and 5s showed approximately 25% binding affinity to CRF_1 receptors. Selected compounds (5c and 5f) were also evaluated for their effect on expression of genes associated with depression and anxiety disorders such as CRF1, CREB1, MAO-A, SERT, NPY, DatSLC6a3, and DBH and significant upregulation of CRF1 mRNA has been observed with compound 5c.

Keywords: Thiazolo[4,5-*d*]pyrimidines, corticotropin releasing factor, antalarmin, anxiety, depression.

INTRODUCTION

Corticotropin-releasing factor (CRF) is a neurohormone that plays a crucial role in integrating the body's overall response to stress. It appears necessary and sufficient for the organism to mount functional, physiological and endocrine responses to stressors. CRF is released in response to various triggers such as chronic stress. This then triggers the release of corticotropin (ACTH), another hormone, from the anterior pituitary gland which then triggers the secretion of the endogenous glucocorticoids from the adrenal cortex that manages the physiological responses to stress [1, 2]. Chronic release of CRF and ACTH is believed to be directly or indirectly involved in many of the harmful physiological effects of chronic stress, such as excessive glucocorticoid release, diabetes mellitus, osteoporosis, stomach ulcers, anxiety, depression, development of high blood pressure and consequent cardiovascular problems [3].

Not surprisingly, many human psychopathologies which include hyper excitability or anxiety-like components are hypothesized to depend either casually or symptomatically on over-activation of CRF in the brain. Dysfunction in this system has been correlated with various diseases such as major depression [4], anxiety disorders [5] and eating disorders [6]. There is evidence that CRF may play a role in the stress-induced relapse of drug abuse and the anxiety-like behaviors observed during acute drug withdrawal and drug addiction [7, 8].

Administration of CRF provokes stress-like responses including simulation of the sympathetic nervous system [9, 10], inhibition of the parasympathetic nervous system with consequential increase in plasma concentration of adrenaline, noradrenaline and glucose, increase in heart rate and mean arterial blood pressure, inhibition of gastrointestinal function and acid secretion. The behavioral response to CRF administration in general is an increased arousal and emotional reactivity to the environment [2]. In addition to the neuromodulatory and neuroendocrine actions of CRF, it is also suggested that it may play a role in integrating the response of the immune system to physiological, psychological and immunological stressors [11]. Clinical data suggesting a role for CRF in major depression has been accumulating over the years. Early studies demonstrated that patients suffering from major depression disorders have elevated supra-normal concentration of CRF in their cerebrospinal fluid relative to normal volunteers [12]. Recently, there was a report of a preliminary clinical study where, in 20 depressed patients

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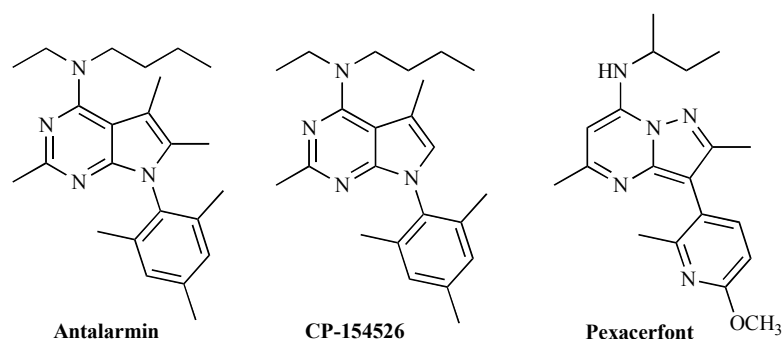
Fig. (1). Structure of prominent CRF receptor antagonists.

treated with a selective CRF_1 receptor antagonist, a significant reduction in depression and anxiety scores was observed. Moreover, CRF levels are elevated in patients suffering from anorexia nervosa [13], obsessive compulsive disorder and post-traumatic disorder. Several studies have provided evidence in support of alterations in CRF in Alzheimer's disease [14]. Alterations in brain concentration of CRF have been reported in other neurological diseases such as Parkinson's disease [15].

The role of CRF and its involvement in these neurological disorders and in behavioral, cardiovascular, gastrointestinal, immune and reproductive systems suggests that new drugs that can target the CRF function and bind to its receptors may represent a new development of neuropsychiatric medicines to treat various stress-related disorders including depression, anxiety and addictive disorders [16, 17]. The main research into CRF antagonists to date has focused on non-peptide CRF_1 receptor antagonists particularly fused pyrimidines with the aim of improving the health consequences of chronic stress and for use in the clinical management of anxiety and stress [18, 19]. Several CRF_1 receptor antagonists from the fused pyrimidine series have been developed and are widely used in research, with the best-known agents being the selective CRF_1 antagonist antalarmin, CP-154,526 and the newer drug pexacerfont (Fig. **1**).

Antalarmin showed promising results in treatment of CRF-induced hypertension [20]. Promising results for antalarmin and other CRF_1 antagonists were also observed in the area of drug addiction disorders. Antalarmin also showed anti-inflammatory effects and has been suggested as having potential uses in treatment of arthritis [21], irritable bowel syndrome [22, 23] and peptic ulcers [24]. CP-154,526 is a potent and selective CRF_1 receptor antagonist developed by Pfizer [25, 26]. CP-154,526 is under investigation for the potential treatment of alcoholism [27]. Pexacerfont (BMS-562,086) is a recently developed CRF_1 antagonist developed by Bristol-Myers-Squibb which is currently in clinical trials for the treatment of anxiety disorders [28] and has also been proposed to be useful for the treatment of depression and irritable bowel syndrome.

In this manuscript, we describe the synthesis and characterization of a new series of substituted thiazolo[4,5-d]pyrimidines carrying groups at selected positions similar to reported fused pyrimidines antagonists with superior CRF receptor antagonist activities. The synthesized compounds were evaluated for their binding affinities to CRF_1 receptors

and the selected compounds were evaluated for their effect on expression of genes associated with depression and anxiety disorders such as CRF1, CREB1, MAO-A, SERT, NPY, DatSLC6a3, and DBH, and showed a significant up-regulation of the CRF1 gene expression.
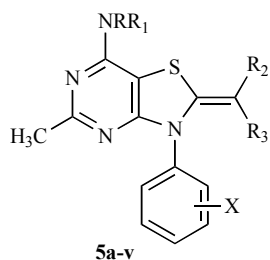
RESULTS AND DISCUSSION

Chemistry

The general synthetic scheme of the target compounds is described in Scheme **1**. The starting 4-amino-3-aryl-2-thioxo-2,3-dihydrothiazole-5-carboxamide derivatives **(1a-b)** were prepared in excellent yields by the reaction of the selected substituted phenyl isothiocyanate with cynoacetamide and sulfur in the presence of base following Gewald reaction [29-31]. Gewald first reported the synthesis of thiazole-2-thiones in 1966 and described the reaction of active-methylene containing nitriles with sulfur and phenyl isothiocyanates [29]. This synthesis has been widely utilized to synthesize various thiazoles [32-35] and was extended to include heterocyclic-containing nitriles [30]. This reaction can be carried out in dimethylformamide as a solvent at 50 ^{o}C, or in ethanol as a solvent at reflux temperature [36] or in a mixture of ethanol and dimethylformamide at reflux temperature. The selected isothiocyanates used were 2,4,6-trimethylphenyl isothiocyanate and 2-bromo-4-isopropylphenylisothiocyanate since previous studies on non-peptide CRF receptor antagonists have shown that these groups at position 3 of the thiazolo[4,5-d]pyrimidines have shown optimum CRF_1 binding antagonist activity [37, 38].

The stating thiazoles **(1a-b)** were then reacted with acetic anhydride at reflux temperature to yield 3-aryl-5-methyl-2-thioxo-2,3-dihydrothiazolo[4,5-d]pyrimidin-7(6H)-ones **(2a-b)** [30, 31, 36]. Ring closure of 4-amino-3-aryl-2-thioxo-2,3-dihydrothiazole-5-carboxamides giving 3-aryl-5-methyl-thiazolo[4,5-d]pyrimidine-7(6H)-one-2-thiones, substituted with a methyl group at position 5 by heating at reflux temperature in acetic anhydride, was reported in 1998 and was described by Fahmy *et al.* [36, 39, 40]. The 3-aryl-5-methyl-2-thioxo-2,3-dihydrothiazolo[4,5-d]pyrimidin-7(6H)-ones **(2a-b)** were subjected to chlorination using phosphorous oxychloride at reflux temperature according to the reported procedures for synthesis of thiazolo[4,5-d] pyrimidines to yield 3-aryl-7-chloro-5-methylthiazolo[4,5-d]pyrimidine-2(3H)-thiones **(3a-b)** in excellent yields [36-40]. The 3-aryl-7-(N,N-dialkylamino)-5-methylthiazolo[4,5-d]pyrimidine-
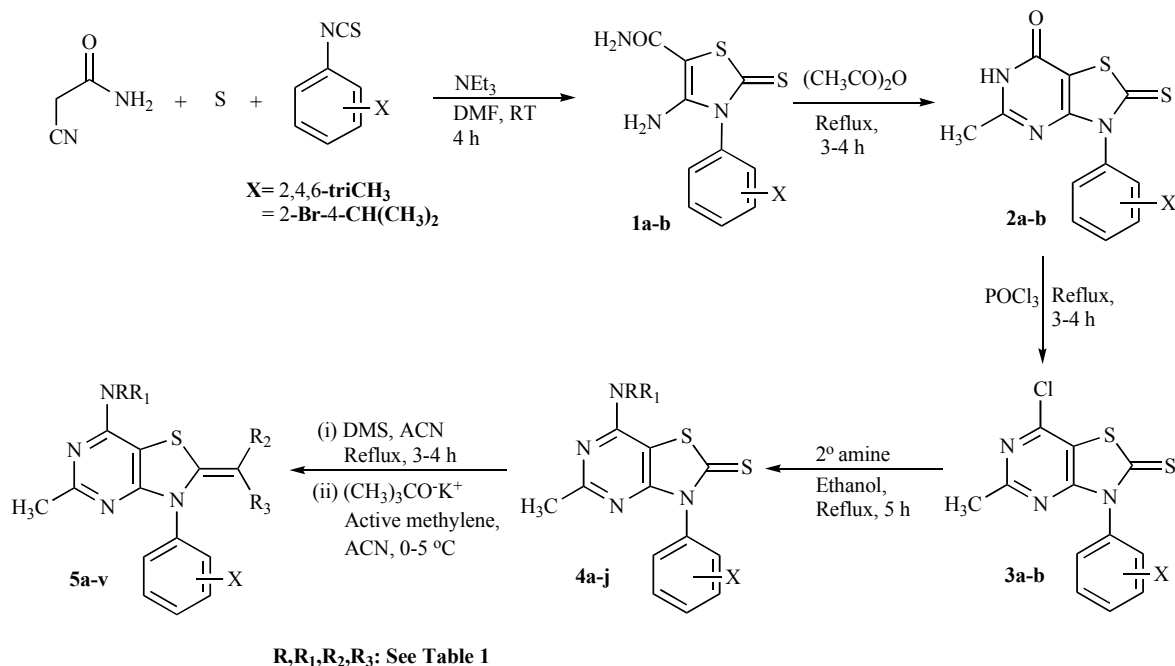
Scheme 1. Synthesis of the target 3-aryl-7-(*N,N*-dialkylamino)-5-methylthiazolo[4,5-*d*]pyrimidin-2(*3H*)-ylidenes.

Table 1. Substitutions, molecular formulae and molecular weights of target compounds (5a-v).

Compd	R	R$_1$	R$_2$	R$_3$	X	Molecular Formula (Molecular Weight)
5a	C$_3$H$_7$	C$_3$H$_7$	CN	CN	2,4,6-trimethyl	C$_{24}$H$_{28}$N$_6$S (432.6)
5b	C$_3$H$_7$	C$_3$H$_7$	CN	CO$_2$C$_2$H$_5$	2,4,6-trimethyl	C$_{26}$H$_{33}$N$_5$O$_2$S (479.6)
5c	C$_3$H$_7$	C$_3$H$_7$	CO$_2$C$_2$H$_5$	CO$_2$C$_2$H$_5$	2,4,6-trimethyl	C$_{28}$H$_{38}$N$_4$O$_4$S (526.7)
5d	C$_2$H$_4$OCH$_3$	C$_2$H$_4$OCH$_3$	CN	CN	2,4,6-trimethyl	C$_{24}$H$_{28}$N$_6$O$_2$S (464.6)
5e	C$_2$H$_4$OCH$_3$	C$_2$H$_4$OCH$_3$	CN	CO$_2$C$_2$H$_5$	2,4,6-trimethyl	C$_{26}$H$_{33}$N$_5$O$_4$S (511.64)
5f	C$_2$H$_5$	C$_4$H$_9$	CN	CN	2,4,6-trimethyl	C$_{24}$H$_{28}$N$_6$S (432.6)
5g	C$_2$H$_5$	C$_4$H$_9$	CN	CO$_2$C$_2$H$_5$	2,4,6-trimethyl	C$_{26}$H$_{33}$N$_5$O$_2$S (479.6)
5h	CH$_2$*c*-Pr	C$_3$H$_7$	CN	CN	2,4,6-trimethyl	C$_{25}$H$_{28}$N$_6$S (444.6)
5i	CH$_2$*c*-Pr	C$_3$H$_7$	CN	CO$_2$C$_2$H$_5$	2,4,6-trimethyl	C$_{27}$H$_{33}$N$_5$O$_2$S (491.6)
5j	C$_2$H$_5$	C$_2$H$_5$	CN	CN	2,4,6-trimethyl	C$_{22}$H$_{24}$N$_6$S (404.5)
5k	C$_2$H$_5$	C$_2$H$_5$	CN	CO$_2$C$_2$H$_5$	2,4,6-trimethyl	C$_{24}$H$_{29}$N$_5$O$_2$S (451.6)
5l	C$_3$H$_7$	C$_3$H$_7$	CN	CN	2-Br-4-isopropyl	C$_{24}$H$_{27}$BrN$_6$S (511.5)
5m	C$_3$H$_7$	C$_3$H$_7$	CN	CO$_2$C$_2$H$_5$	2-Br-4-isopropyl	C$_{26}$H$_{32}$BrN$_5$O$_2$S (558.5)
5n	C$_2$H$_4$OCH$_3$	C$_2$H$_4$OCH$_3$	CN	CN	2-Br-4-isopropyl	C$_{24}$H$_{27}$BrN$_6$O$_2$S (543.5)

Table 1. contd……

Compd	R	R$_1$	R$_2$	R$_3$	X	Molecular Formula (Molecular Weight)
5o	C$_2$H$_4$OCH$_3$	C$_2$H$_4$OCH$_3$	CN	CO$_2$C$_2$H$_5$	2-Br-4-isopropyl	C$_{26}$H$_{32}$BrN$_5$O$_4$S (590.5)
5p	C$_2$H$_5$	C$_4$H$_9$	CN	CN	2-Br-4-isopropyl	C$_{24}$H$_{27}$BrN$_6$S (511.5)
5q	C$_2$H$_5$	C$_4$H$_9$	CN	CO$_2$C$_2$H$_5$	2-Br-4-isopropyl	C$_{26}$H$_{32}$BrN$_5$O$_2$S (558.5)
5r	C$_2$H$_5$	C$_4$H$_9$	CO$_2$C$_2$H$_5$	CO$_2$C$_2$H$_5$	2-Br-4-isopropyl	C$_{28}$H$_{37}$BrN$_4$O$_4$S (605.6)
5s	CH$_2$*c*-Pr	C$_3$H$_7$	CN	CN	2-Br-4-isopropyl	C$_{25}$H$_{27}$BrN$_6$S (523.5)
5t	CH$_2$*c*-Pr	C$_3$H$_7$	CO$_2$C$_2$H$_5$	CO$_2$C$_2$H$_5$	2-Br-4-isopropyl	C$_{29}$H$_{37}$BrN$_4$O$_4$S (617.6)
5u	C$_2$H$_5$	C$_2$H$_5$	CN	CN	2-Br-4-isopropyl	C$_{22}$H$_{23}$BrN$_6$S (483.4)
5v	C$_2$H$_5$	C$_2$H$_5$	CO$_2$C$_2$H$_5$	CO$_2$C$_2$H$_5$	2-Br-4-isopropyl	C$_{24}$H$_{29}$BrN$_4$O$_2$S (577.5)

2(*3H*)-thiones (**4a-j**) were prepared by the reaction of the 3-aryl-7-chloro-5-methylthiazolo[4,5-*d*]pyrimidine-2(*3H*)-thiones (**3a-b**) with the selected secondary amines under the reported reaction conditions [30, 36, 37, 39]. The products were obtained in excellent yields and the cross reaction between the aromatic bromo group and amines were not observed. This may be due the fact that the pyrimidine chloro group is more activated compared to the bromo group on the benzene ring. The selected secondary amines for this reaction were *N,N*-diethyl amine, *N,N*-di(n-propyl)amine, *N*-ethyl-*N*-(n-butyl)amine, *N,N*-bis(2-methoxyethyl) amine and *N*-[2-(c-propyl)ethyl]-*N*-(n-propyl)amine since previous reports showed an optimum CRF$_1$ receptor antagonist activity were obtained when those particular amino groups were found at position 7 of the thiazolo[4,5-*d*]pyrimidine ring [37].

The target 3-aryl-7-(*N,N*-dialkylamino)-5-methylthiazolo[4,5-*d*]pyrimidin-2(*3H*)-ylidenes (**5a-v**) derivatives were synthesized by the reaction of 3-aryl-7-(*N,N*-dialkylamino)-5-methylthiazolo[4,5-*d*] pyrimidine-2(*3H*)-thiones (**4a-j**) with dimethyl sulfate in acetonitrile at reflux temperature for 30 minutes, followed by reaction of the produced 2-methylthiazolium intermediate with the selected active methylene containing compound (malononitrile, ethyl cyanoacetate or diethyl malonate) in the presence of a base to give the target 3-aryl-7-(*N,N*-dialkylamino)-5-methylthiazolo[4,5-*d*]pyrimidin-2(*3H*)-ylidenes (**5a-v**) in moderate yields. The replacement of the 2-thioxo function of 3-aryl-7-(*N,N*-dialkylamino)-5-methylthiazolo[4,5-*d*] pyrimidine-2(*3H*)-thiones with oxygen or amino functions was first described by Gewald [30, 31] and later was extended to replace the 2-thioxo function with active methylene-containing small molecules [30, 31] and active methylene containing large heterocyclic molecules.

RECEPTOR BINDING STUDIES

The new series of 3-aryl-7-(*N,N*-dialkylamino)-5-methylthiazolo[4,5-*d*]pyrimidin-2(*3H*)-ylidenes (**5a-v**) were evaluated for their binding affinity to CRF$_1$ receptors. In this protocol [41], the ability of the tested compounds at a single concentration of 1000 nM to inhibit the specific binding of [^{125}I]-Tyr^0 sauvagine to membranes from HEK 293 cells stably expressing the receptor in binding experiments per-

formed under equilibrium conditions was evaluated (Fig. **2**). Compounds **5s** and **5o** show approximately 25% inhibition of binding of radioligand on HEK 293 cells expressing CRF$_1$ receptors.

MTT Assay

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) analysis was carried out at 24 h, 48 h and 72 h to detect the effect of **5c** and **5f** on the viability of RN46A cells. RN46A cells were treated with various concentrations of **5c** and **5f** (0.005, 0.05, 0.5, 5, 50 μM) during each stage. **5c** and **5f** showed no significant effects on the RN46A cell viability compared to the control at 24 h, 48 h, and 72 h.

Quantitative Real-Time RT-PCR

The currently used medications for treatment of mood disorders focus on neural circuitry of monoaminergic systems such as serotonin (5HT), norepinephrine (NE), and dopamine (DA) by preventing their enzymatic degradation or inhibiting reuptake from the presynaptic membranes. Genes associated with depression and anxiety disorders such as CRF1, CREB1 (cAMP responsive element binding protein 1), MAO-A (monoamine oxidase A), SERT (serotonin transporter), NPY (neuropeptide Y), DatSLC6a3 (dopamine transporter), and DBH (dopamine β-hydroxylase) were selected for this study. Being G-protein coupled receptor, CRF activates cAMP which in turn activates transcription factor CREB, thereby controlling gene expression. MAO-A is an enzyme that degrades the monoamines such as serotonin, adrenaline and dopamine, and terminates their actions, whereas NPY is neuropeptide that has been implicated in appetite, obesity and anxiety disorders. DatSLC6a3 is membrane spanning protein that pumps back dopamine from synaptic cleft to presynaptic membranes and terminates its actions. DBH is an enzyme which converts dopamine to norepinephrine. Real time PCR study was performed to evaluate the effect of the newly synthesized compounds on these targets and on their mRNA expression. Compounds **5c** (with the 2-substitution is diethylmalonate-ylidene) and **5f** (where the 2-substitution is malononitrile-ylidene) were selected as representative for the new series of compounds to be used for gene expression studies. Compound **5c** showed a significant effect on gene expression at 2.5μM and 25μM concen-
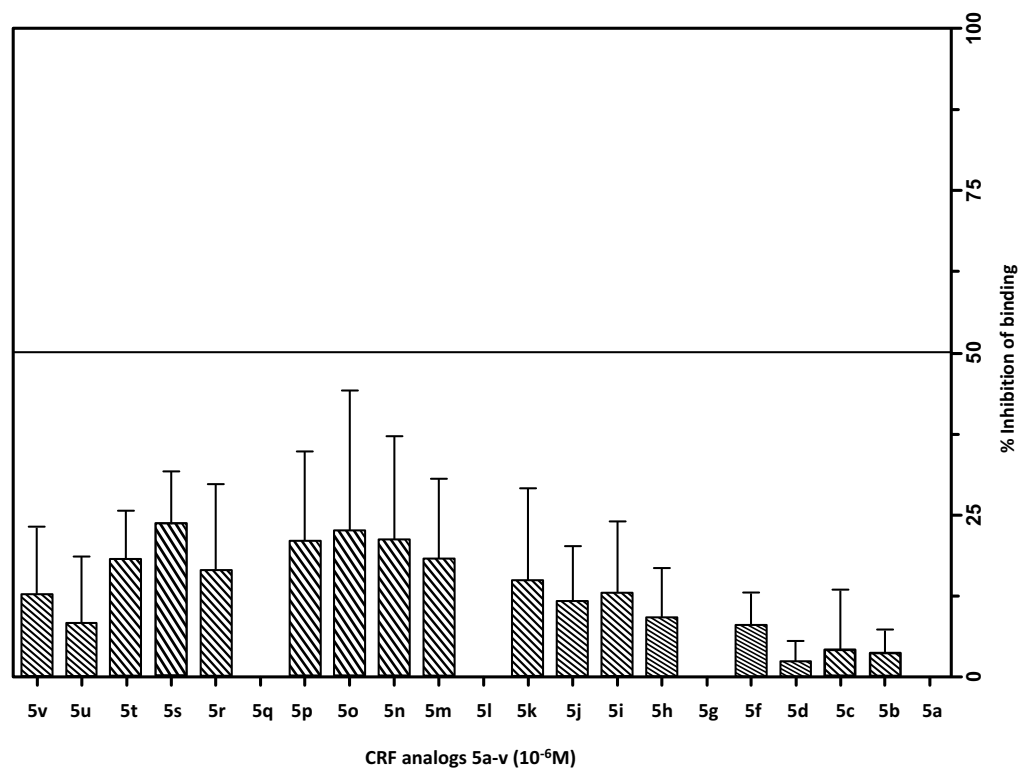
Fig. (2). Screening of compounds for binding to human CRF_1 receptor. Inhibition of $[^{125}I]$-Tyr^0-sauvagine specific binding by 1000 nM of compounds 5a-u on membranes from HEK 293 cells stably expressing the human CRF_1 receptor. The bars represent the % inhibition of radi-oligand specific binding by the compounds, determined from 2 to 5 experiments (with their means and S.E.).
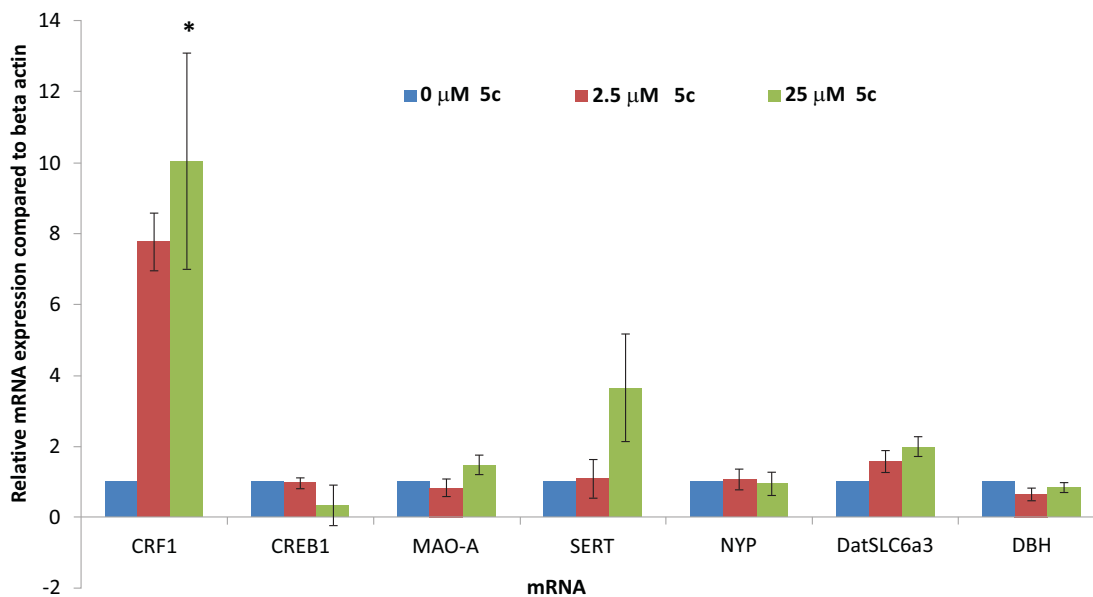

Fig. (3). Effects of 5c on mRNA expression of CRF1, CREB1, MAO-A, SERT, NYP, DatSLC6a3 and DBH in RN46A cells treated with compound 5c for 24 h. Values given are the means ± S.D (n=3). * denotes significant difference comparing with vehicle-treated cultures (p<0.05).

tration (Fig. **3**) for 24 h treatment in RN46A cell lines. However, the compound **5f** showed no significant effect on gene expression at 0.5µM and 50µM concentration (Fig. **4**).

RN46 cells were treated with varying concentrations of **5c** for 24 h, and the results show increased CRF1 mRNA expression in RN46Acells cultured in DMEM compared to cells cultured in media treated with vehicle (Fig. **3**). CRF_1

mRNA expression was increased to 7.76 fold (2.5 µM) and 10.04 fold (25 µM) compared to vehicle (control) group. Interestingly, there were no significant changes in CREB1, MAO-A, SERT, NYP, DatSLC6a3 and DBH mRNA expression following treatment with varying concentration of **5c** for 24 h which indicates a selective effect on CRF_1 mRNA expression.
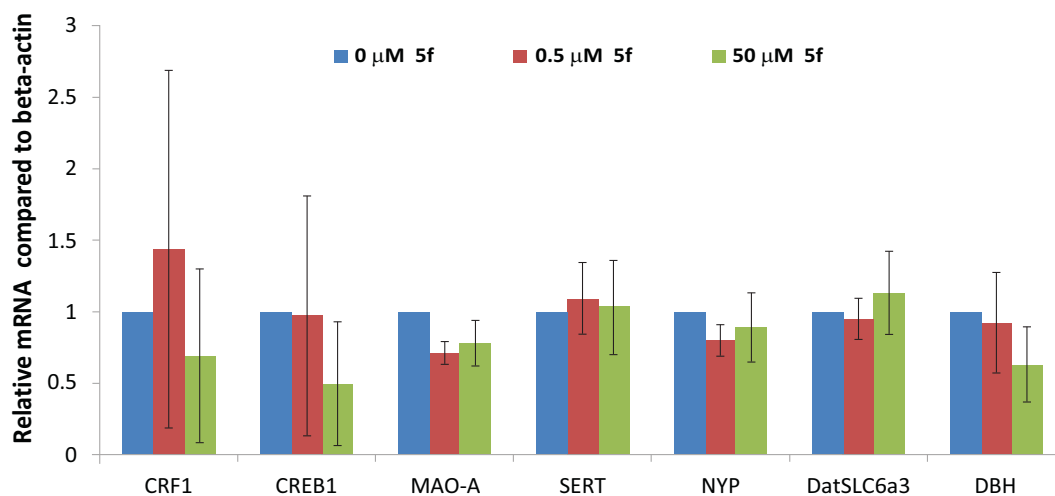
Fig. (4). Effects of 5f on mRNA expression of CRF1, CREB1, MAO-A, SERT, NYP, DatSLC6a3 and DBH in RN46A cells treated with compound 5f for 24 h. Values given are the means ± S.D.

Although, **5c** showed modest inhibition to $[^{125}I]$-Tyr^0-sauvagine binding to CRF1 receptor in HEK 293 cells at 1000 nM, it upregulated the CRF$_1$ mRNA expression in RN46A cells compared to other genes. At this time, the molecular mechanism responsible for upregulation of CRF$_1$ mRNA compared to other genes is not clear. However, it clearly indicates that these compounds have an effect on the CRF and that future studies are needed to further explore the cellular mechanism of action.

CONCLUSIONS

A new series of thiazolo[4,5-d]pyrimdines were synthesized as CRF receptor antagonists. Twenty two compounds were screened for the binding affinities to CRF receptors, where two compounds (**5o** and **5s**) showed approximately 25% inhibition of radiolabeled $[^{125}I]$-Tyr^0-sauvagine to CRF$_1$ receptor in HEK 293 cells at 1000 nM. Two representative compounds of the new series (**5c** and **5f**) were also screened for their effects on mRNA gene expression of neurotransmitters implicated in anxiety and depression in RN46A cells (CRF1, CREB1, MAO-A, SERT, NYP, DatSLC6a3 and DBH). Compound **5c** showed significant up-regulation of the CRF1 compared to control and compared to other genes which indicates selective effect on CRF1 gene expression.

MATERIALS AND METHODS

Chemistry

All Chemicals, secondary amines and dry solvents were purchased from Sigma Aldrich USA, Acros organics, Fisher scientific USA. The substituted phenyl isothiocyantaes were purchased from Oakwood Products, Inc. SC, USA. Flash column chromatography separation was performed using Acros organics silica gel 40-60 μm, 60A using combination of ethyl acetate and hexane. Whatman TLC plates were used 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 were uncorrected. %CHN Analyzer by combustion/TCD and %S by O flask combustion/IC were used for elemental analysis

and performed by Micro Analysis Inc., Wilmington DE, and all samples were within ±0.4%. ^{1}H NMR spectra were obtained on a Bruker Avance 400 MHz instrument using CDCl$_3$ as solvent unless otherwise stated. Chemical shifts are relative to TMS as an internal standard. Mass spectra were recorded on Finnigan LCQTM DECA by Thermo Quest San Jose, CA. All the reactions were carried out in flame dried glassware under an atmosphere of nitrogen unless otherwise stated.

4-amino-3-aryl-2-thioxo-2,3-dihydrothiazole-5-carboxamide (1a-b)

The thiazole-2-thiones were prepared from cyanoacetamide, sulfur, and the selected aromatic isothiocyanate under basic conditions in DMSO according to the reported procedures [37, 38].

3-aryl-5-substituted-2-thioxo-2,3-dihydrothiazolo[4,5-d] pyrimidin-7(*6H*)-ones (2a-b)

The thiazolo[4,5-d]pyrimidin-7(*6H*)-ones were prepared from thiazoles **1a-b** and acetic anhydride according to the reported procedures [37, 38].

3-aryl-7-chloro-5-methylthiazolo[4,5-d]pyrimidine-2(*3H*)-thiones (3a-b)

The chloro derivatives were prepared from **2a-b** and phosphorous oxychloride according to the reported procedures [37, 38].

3-aryl-7-(N,N-dialkylamino)-5-methylthiazolo[4,5-d] pyrimidine-2(*3H*)-thiones (4a-j) [37, 38]

The amino derivatives were prepared from chloro derivatives **3a-b** and the selected secondary amines in ethanol according to the reported procedures [37, 38].

3-aryl-7-(*N,N*-dialkylamino)-5-methylthiazolo[4,5-d] pyrimidin-2(*3H*)-ylidenes (5a-v)

The **4a-j** (1.0 mmol) and dimethyl sulfate (3.0 mmol) in acetonitrile (20 mL) were stirred at reflux temperature for

about 3 to 4h and disappearance of **4a-j** was monitored by TLC using ethyl acetate and hexanes. In another flask, appropriate active methylene compound (1.5 mmol) and potassium *tert*-butoxide (3.0 mmol) in acetonitrile (10 mL) were stirred at 0-5 °C for 30 minutes. Then, the above reaction mass was slowly added at 0-5 °C and stirred. Completion of the reaction was monitored by the TLC using ethyl acetate and hexane. The reaction was worked up by diluting with water followed by extraction with ethyl acetate. The combined organic layer was washed with water, brine solution, dried over sodium sulfate and concentrated under vacuum to get crude residue followed by flash chromatographic separation using gradient ethyl acetate and hexane to afford pure **5a-v** derivatives.

2-(7-(Dipropylamino)-3-mesityl-5-methylthiazolo[4,5-d]pyrimidin-2(3H)-ylidene)malononitrile [5a]: Yield: white solid (51%); MP: 184 °C; ^{1}H NMR (400 MHz, CDCl$_3$) δ 6.96 (s, 2 H), 3.52 - 3.43 (m, 4 H), 2.32 (s, 3 H), 2.28 (s, 3 H), 1.95 (s, 6 H), 1.65 (qd, *J* = 7.5, 15.3 Hz, 4 H), 0.94 (t, *J* = 7.3 Hz, 6 H). MS m/z: 433.33 (MH)$^+$ (C$_{24}$H$_{28}$N$_6$S).

Ethyl 2-cyano-2-(7-(dipropylamino)-3-mesityl-5-methylthiazolo[4,5-d]pyrimidin-2(3H)-ylidene)acetate [5b]: Yield: off-white solid (23%); MP: 226 °C; ^{1}H NMR (400 MHz, CDCl$_3$) δ 7.01 (s, 2 H), 4.26 (q, *J* = 7.1 Hz, 2 H), 3.66 - 3.57 (m, 4 H), 2.39 (s, 3 H), 2.34 (s, 3 H), 1.99 (s, 6 H), 1.75 (dq, *J* = 7.5, 15.3 Hz, 4 H), 1.30 (t, *J* = 7.2 Hz, 3 H), 1.02 (t, *J* = 7.4 Hz, 6 H). MS m/z: 480.23 (MH)$^+$ (C$_{26}$H$_{33}$N$_5$O$_2$S).

Diethyl 2-(7-(dipropylamino)-3-mesityl-5-methylthiazolo[4,5-d]pyrimidin-2(3H)-ylidene)malonate [5c]: Yield: white solid (31%); MP: 125 °C; ^{1}H NMR (400 MHz, CDCl$_3$) δ 7.01 (s, 2 H), 4.26 (q, *J* = 7.1 Hz, 4 H), 3.66 - 3.57 (m, 4 H), 2.39 (s, 3 H), 2.34 (s, 3 H), 1.99 (s, 6 H), 1.75 (dq, *J* = 7.5, 15.3 Hz, 4 H), 1.30 (t, *J* = 7.2 Hz, 6 H), 1.02 (t, *J* = 7.4 Hz, 6 H). MS m/z: 528.29 (MH)$^+$ (C$_{28}$H$_{38}$N$_4$O$_4$S).

2-(7-[bis(2-methoxyethyl)amino]-3-mesityl-5-methylthiazolo[4,5-d]pyrimidin-2(3H)-ylidene)malononitrile [5d]: Yield: off-white solid (37%); MP: 162 ° C; ^{1}H NMR (400 MHz, CDCl$_3$) δ 7.04 (s, 2 H), 3.92 (t, *J* = 5.3 Hz, 4 H), 3.66 (t, *J* = 5.4 Hz, 4 H), 3.39 (s, 6 H), 2.40 (s, 3 H), 2.36 (s, 3 H), 2.03 (s, 6 H). MS m/z: 464.6 (MH)$^+$ (C$_{24}$H$_{28}$N$_6$O$_2$S).

Ethyl 2-(7-[bis(2-methoxyethyl)amino]-3-mesityl-5-methylthiazolo[4,5-d]pyrimidin-2(3H)-ylidene)-2-cyanoacetate [5e]: Yield: off-white solid (32%); MP: 135 °C; ^{1}H NMR (400 MHz, CDCl$_3$) δ 7.03 (s, 2 H), 4.27 (q, *J* = 7.1 Hz, 2 H), 4.00 (t, *J* = 5.5 Hz, 4 H), 3.70 (t, *J* = 5.4 Hz, 4 H), 3.39 (s, 6 H), 2.40 (s, 3 H), 2.36 (s, 3 H), 1.99 (s, 6 H), 1.31 (t, *J* = 7.2 Hz, 3 H). MS m/z: 512.20 (MH)$^+$ (C$_{26}$H$_{33}$N$_5$O$_4$S).

2-(7-[Butyl(ethyl)amino]-3-mesityl-5-methylthiazolo[4,5-d]pyrimidin-2(3H)-ylidene)malononitrile [5f]: Yield: white solid (55%); MP: 171 °C; ^{1}H NMR (400 MHz, CDCl$_3$) δ 7.04 (s, 2 H), 3.67 (q, *J* = 7.1 Hz, 2 H), 3.64 - 3.54 (m, 2 H), 2.40 (s, 3 H), 2.36 (s, 3 H), 2.03 (s, 6 H), 1.74 - 1.65 (m, 2 H), 1.44 (dq, *J* = 7.4, 14.9 Hz, 2 H), 1.31 (t, *J* = 7.2 Hz, 3 H), 1.02 (t, *J* = 7.4 Hz, 3 H). MS m/z: 433.26 (MH)$^+$ (C$_{24}$H$_{28}$N$_6$S).

Ethyl-2-[7-(butyl(ethyl)amino]-3-mesityl-5-methylthiazolo[4,5-d]pyrimidin-2(3H)-ylidene)-2-cyanoacetate [5g]: Yield: off-white solid (33%); MP: 180 °C; ^{1}H NMR (400 MHz, CDCl$_3$) δ 7.03 (s, 2 H), 4.27 (q, *J* = 7.1 Hz, 2 H), 3.74 (q, *J* = 7.1 Hz, 2 H), 3.70 - 3.60 (m, 2 H), 2.40 (s, 3 H), 2.36 (s, 3 H), 2.00 (s, 6 H), 1.76 - 1.67 (m, 2 H), 1.46 (dq, *J* = 7.3, 15.0 Hz, 2 H), 1.32 (q, *J* = 7.1 Hz, 6 H), 1.01 (t, *J* = 7.3 Hz, 3 H). MS m/z: 480.23 (MH)$^+$ (C$_{26}$H$_{33}$N$_5$O$_2$S).

2-(7-[(cyclopropylmethyl)(propyl)amino]-3-mesityl-5-methylthiazolo[4,5-d]pyrimidin-2(3H)-ylidene) malononitrile [5h]: Yield: white solid (33%); MP: 115 °C; ^{1}H NMR (400MHz, CDCl$_3$) δ 7.04 (s, 2 H), 3.65 - 3.59 (m, 2 H), 3.57 (d, *J* = 6.5 Hz, 2 H), 2.40 (s, 3 H), 2.36 (s, 3 H), 2.03 (s, 6 H), 1.76 (dq, *J* = 7.4, 15.4 Hz, 2 H), 1.17 - 1.09 (m, 1 H), 1.02 (t, *J* = 7.4 Hz, 3 H), 0.67 - 0.60 (m, 2 H), 0.37 (q, *J* = 5.0 Hz, 2 H). MS m/z: 445.20 (MH)$^+$ (C$_{25}$H$_{28}$N$_6$S).

Ethyl 2-cyano-2-(7-[(cyclopropylmethyl)(propyl)amino]-3-mesityl-5-methylthiazolo[4,5-d]pyrimidin-2(3H)-ylidene)acetate [5i]: Yield: off-white solid (23%); MP: 247°C; ^{1}H NMR (400 MHz, CDCl$_3$) δ 7.03 (s, 2 H), 4.27 (q, *J* = 7.1 Hz, 2 H), 3.73 - 3.67 (m, 2 H), 3.63 (d, *J* = 6.5 Hz, 2 H), 2.40 (s, 3 H), 2.36 (s, 3 H), 2.00 (s, 6 H), 1.79 (dq, *J* = 7.6, 15.3 Hz, 2 H), 1.32 (t, *J* = 7.2 Hz, 3 H), 1.21 - 1.13 (m, 1 H), 1.05 (t, *J* = 7.3 Hz, 3 H), 0.65-0.58 (m, 2 H), 0.41-0.34 (m, 2 H). MS m/z: 492.23 (MH)$^+$ (C$_{27}$H$_{33}$N$_5$O$_2$S).

2-(7-(Diethylamino)-3-mesityl-5-methylthiazolo[4,5-d]pyrimidin-2(3H)-ylidene)malononitrile [5j]: Yield: white solid (39%); MP: 236 °C; ^{1}H NMR (400 MHz, CDCl$_3$) δ 7.04 (s, 2 H), 3.68 (q, *J* = 7.1 Hz, 4 H), 2.40 (s, 3 H), 2.37 (s, 3 H), 2.03 (s, 6 H), 1.32 (t, *J* = 7.2 Hz, 6 H). MS m/z: 405.27 (MH)$^+$ (C$_{22}$H$_{24}$N$_6$S).

Ethyl 2-cyano-2-(7-diethylamino-3-mesityl-5-methylthiazolo[4,5-d]pyrimidin-2(3H)-ylidene)acetate [5k]: Yield: white solid (32%); MP: 221 °C; ^{1}H NMR (400 MHz, CDCl$_3$) δ 7.03 (s, 2 H), 4.27 (q, *J* = 7.1 Hz, 2 H), 3.74 (q, *J* = 7.1 Hz, 4 H), 2.40 (s, 3 H), 2.36 (s, 3 H), 2.00 (s, 6 H), 1.33 (q, *J* = 7.1 Hz, 9 H) . MS m/z: 452.24 (MH)$^+$ (C$_{24}$H$_{29}$N$_5$O$_2$S).

2-(3-[2-bromo-4-isopropylphenyl]-7-dipropylamino-5-methylthiazolo[4,5-d]pyrimidin-2(3H)-ylidene)malononitrile [5l]: Yield: off-white solid (27%); MP: 193 °C; ^{1}H NMR (400 MHz, CDCl$_3$) δ 7.62 (d, *J* = 1.8 Hz, 1 H), 7.38 (dd, *J* = 1.8, 8.1 Hz, 1 H), 7.31 (d, *J* = 8.3 Hz, 1 H), 3.55 (dd, *J* = 5.7, 8.7 Hz, 4 H), 3.02 (spt, *J* = 6.7 Hz, 1 H), 2.37 (s, 3 H), 1.72 (dq, *J* = 7.4, 15.4 Hz, 4 H), 1.33 (d, *J* = 7.1 Hz, 6 H), 1.01 (t, *J* = 7.4 Hz, 6 H). MS m/z: 511.27 (MH)$^+$, 513.3 (MH+2)$^+$ (C$_{24}$H$_{27}$BrN$_6$S).

Ethyl 2-(3-[2-bromo-4-isopropylphenyl]-7-dipropylamino-5-methylthiazolo[4,5-d]pyrimidin-2(3H)-ylidene)-2-cyanoacetate [5m]: Yield: white solid (28%); MP: 236°C; ^{1}H NMR (400 MHz, CDCl$_3$) δ 7.61 (d, *J* = 1.8 Hz, 1 H), 7.36 (dd, *J* = 1.6, 8.2 Hz, 1 H), 7.32 (d, *J* = 8.1 Hz, 1 H), 4.26 (q, *J* = 6.9 Hz, 2 H), 3.62 (dt, *J* = 3.8, 7.7 Hz, 4 H), 3.03 (spt, *J* = 6.9 Hz, 1 H), 2.37 (s, 3 H), 1.74 (sxt, *J* = 7.6 Hz, 4 H), 1.33 (d, *J* = 6.8 Hz, 6 H), 1.30 (t, *J* = 7.2 Hz, 3 H), 1.02 (t, *J* = 7.4 Hz, 6 H). MS m/z: 558.2 (MH)$^+$, 560.2 (MH+2)$^+$ (C$_{26}$H$_{32}$BrN$_5$O$_2$S).

2-(7-[Bis(2-methoxyethyl)amino]-3-(2-bromo-4-isopropylphenyl)-5-methylthiazolo[4,5-d]pyrimidin-2(3H)-ylidene)malononitrile [5n]: Yield: off-white solid (34%); MP: 149 °C; ^{1}H NMR (400 MHz, CDCl$_3$) δ 7.63 (d, *J* = 1.8 Hz, 1 H), 7.40 - 7.36 (m, 1 H), 7.30 (d, *J* = 8.1 Hz, 1 H), 3.91 (t, *J* = 5.4 Hz, 4 H), 3.68 - 3.63 (m, 4 H), 3.38 (s, 6 H), 3.02

(spt, *J* = 6.9 Hz, 1 H), 2.37 (s, 3 H), 1.33 (d, *J* = 7.1 Hz, 6 H). MS m/z: 543.13 (MH)$^+$, 545.13 (MH+2)$^+$ ($C_{24}H_{27}BrN_6O_2S$).

Ethyl-2-(7-[bis(2-methoxyethyl)amino]-3-(2-bromo-4-isopropylphenyl)-5-methylthiazolo[4,5-*d*] pyrimidin-2(3*H*)-ylidene)-2-cyanoacetate [5o]: Yield: off-white solid (33%); MP: 152 ^{o}C; ^{1}H NMR (400 MHz, CDCl$_3$) δ 7.62 (d, *J* = 1.8 Hz, 1 H), 7.37 (dd, *J* = 1.8, 8.3 Hz, 1 H), 7.31 (d, *J* = 8.1 Hz, 1 H), 4.26 (dq, *J* = 1.0, 7.1 Hz, 2 H), 3.98 (t, *J* = 5.4 Hz, 4 H), 3.72 - 3.66 (m, 4 H), 3.39 (s, 6 H), 3.03 (spt, *J* = 6.8 Hz, 1 H), 2.37 (s, 3 H), 1.34 (d, *J* = 7.1 Hz, 6 H), 1.30 (t, *J* = 7.1 Hz, 3 H). MS m/z: 590.2 (MH)$^+$, 592.2 (MH+2)$^+$ ($C_{26}H_{32}BrN_5O_4S$).

2-(3-{2-Bromo-4-isopropylphenyl}-7-[butyl(ethyl)amino]-5-methylthiazolo[4,5-*d*]pyrimidin-2(3*H*)-ylidene) malononitrile [5p]: Yield: white solid (37%); MP: 165 ^{o}C; ^{1}H NMR (400 MHz, CDCl$_3$) δ 7.63 (d, *J* = 1.8 Hz, 1 H), 7.38 (dd, *J* = 1.9, 8.2 Hz, 1 H), 7.31 (d, *J* = 8.1 Hz, 1 H), 3.66 (q, *J* = 7.1 Hz, 2 H), 3.63 - 3.52 (m, 2 H), 3.09 - 2.96 (m, 1 H), 2.37 (s, 3 H), 1.73 - 1.63 (m, 2 H), 1.48 - 1.38 (m, 2 H), 1.33 (d, *J* = 7.1 Hz, 6 H), 1.31 - 1.24 (m, 3 H), 1.01 (t, *J* = 7.3 Hz, 3 H). MS m/z: 511.27 (MH)$^+$, 513.3 (MH+2)$^+$ ($C_{24}H_{27}BrN_6S$).

Ethyl 2-(3-{2-bromo-4-isopropylphenyl}-7-[butyl(ethyl)amino]-5-methylthiazolo[4,5-*d*]pyrimidin-2(3*H*)-ylidene)-2-cyanoacetate [5q]: Yield: white solid (44%); MP: 184°C; ^{1}H NMR (400 MHz, CDCl$_3$) δ 7.61 (d, *J* = 1.8 Hz, 1 H), 7.37 (dd, *J* = 1.8, 8.3 Hz, 1 H), 7.32 (d, *J* = 8.3 Hz, 1 H), 4.26 (dq, *J* = 0.8, 7.1 Hz, 2 H), 3.73 (q, *J* = 7.1 Hz, 2 H), 3.69 - 3.62 (m, 2 H), 3.03 (spt, *J* = 6.9 Hz, 1 H), 2.37 (s, 3 H), 1.70 (td, *J* = 7.6, 15.3 Hz, 2 H), 1.45 (dd, *J* = 7.4, 15.0 Hz, 2 H), 1.34 (d, *J* = 6.8 Hz, 6 H), 1.32 - 1.27 (m, 6 H), 1.01 (t, *J* = 7.3 Hz, 3 H). MS m/z: 558.3 (MH)$^+$, 560.3 (MH+2)$^+$ ($C_{26}H_{32}BrN_5O_2S$).

Diethyl 2-(3-{2-bromo-4-isopropylphenyl}-7-[butyl(ethyl)amino]-5-methylthiazolo[4,5-*d*]pyrimidin-2(3*H*)-ylidene)malonate [5r]: Yield: off-white solid (17%); MP: 137 ^{o}C; ^{1}H NMR (400 MHz, CDCl$_3$) δ 7.61 (d, *J* = 1.8 Hz, 1 H), 7.37 (dd, *J* = 1.8, 8.3 Hz, 1 H), 7.32 (d, *J* = 8.3 Hz, 1 H), 4.26 (q, *J* = 7.1 Hz, 4 H), 3.73 (q, *J* = 7.1 Hz, 2 H), 3.69 - 3.62 (m, 2 H), 3.03 (spt, *J* = 6.9 Hz, 1 H), 2.37 (s, 3 H), 1.70 (td, *J* = 7.6, 15.3 Hz, 2 H), 1.45 (dd, *J* = 7.4, 15.0 Hz, 2 H), 1.34 (d, *J* = 6.8 Hz, 6 H), 1.32 - 1.27 (m, 9 H), 1.01 (t, *J* = 7.3 Hz, 3 H). MS m/z: 605.2 (MH)$^+$, 607.2 (MH+2)$^+$ ($C_{28}H_{37}BrN_4O_4S$).

2-(3-[2-bromo-4-isopropylphenyl]-7-{(cyclopropylmethyl)(propyl)amino}-5-methylthiazolo[4,5-*d*] pyrimidin-2(3*H*)-ylidene)malononitrile [5s]: Yield: white solid (35%); MP: 168 ^{o}C; ^{1}H NMR (400 MHz, CDCl$_3$) δ 7.63 (d, *J* = 1.8 Hz, 1 H), 7.40 - 7.36 (m, 1 H), 7.31 (d, *J* = 8.1 Hz, 1 H), 3.65 - 3.59 (m, 2 H), 3.57 (d, *J* = 6.5 Hz, 2 H), 3.08 - 2.97 (m, 1 H), 2.37 (s, 3 H), 1.80 - 1.70 (m, 2 H), 1.33 (d, *J* = 6.8 Hz, 6 H), 1.01 (t, *J* = 7.4 Hz, 3 H), 0.66 - 0.59 (m, 2 H), 0.39 - 0.33 (m, 2 H). MS m/z: 523.27 (MH)$^+$, 525.3 (MH+2)$^+$ ($C_{25}H_{27}BrN_6S$).

Diethyl 2-(3-[2-bromo-4-isopropylphenyl]-7-{(cyclopropylmethyl)(propyl)amino}-5-methylthiazolo[4,5-*d*] pyrimidin-2(3*H*)-ylidene)malonate [5t]: Yield: white solid (38%); MP: 108 ^{o}C; ^{1}H NMR (400 MHz, CDCl$_3$) δ 7.63 (d, *J* = 1.8 Hz, 1 H), 7.40 - 7.36 (m, 1 H), 7.31 (d, *J* = 8.1 Hz, 1 H), 4.26 (q, *J* = 7.1 Hz, 4 H), 3.65 - 3.59 (m, 2 H), 3.57 (d, *J* = 6.5 Hz, 2 H), 3.08 - 2.97 (m, 1 H), 2.37 (s, 3 H), 1.80 - 1.70 (m, 2 H), 1.33 (d, *J* = 6.8 Hz, 6 H), 1.25 (t, *J* = 7.2 Hz, 6 H), 1.01 (t, *J* = 7.4 Hz, 3 H), 0.66 - 0.59 (m, 2 H), 0.39 - 0.33 (m, 2 H). MS m/z: 617.27 (MH)$^+$, 619.3 (MH+2)$^+$ ($C_{29}H_{37}BrN_4O_4S$).

2-(3-[2-Bromo-4-isopropylphenyl]-7-diethylamino-5-methylthiazolo[4,5-*d*]pyrimidin-2(3*H*)-ylidene) malononitrile [5u]: Yield: off-white solid (110 mg, 21%); MP: 193°C; ^{1}H NMR (400 MHz, CDCl$_3$) δ 7.63 (d, *J* = 1.8 Hz, 1 H), 7.38 (dd, *J* = 1.6, 8.2 Hz, 1 H), 7.31 (d, *J* = 8.3 Hz, 1 H), 3.67 (q, *J* = 7.1 Hz, 4 H), 3.08 - 2.97 (m, 1 H), 2.38 (s, 3 H), 1.35 - 1.28 (m, 12 H). MS m/z: 483.3 (MH$^+$), 485.4 (MH+2)$^+$ ($C_{22}H_{23}BrN_6S$).

Diethyl 2-(3-[2-bromo-4-isopropylphenyl]-7-diethyl-amino-5-methylthiazolo[4,5-*d*]pyrimidin-2(3*H*)-ylidene) malonate [5v]: Yield: white solid (23%). %); MP: 153 ^{o}C; ^{1}H NMR (400 MHz, CDCl$_3$) δ 7.63 (d, *J* = 1.8 Hz, 1 H), 7.38 (dd, *J* = 1.6, 8.2 Hz, 1 H), 7.31 (d, *J* = 8.3 Hz, 1 H), 4.26 (q, *J* = 7.1 Hz, 4 H), 3.67 (q, *J* = 7.1 Hz, 4 H), 3.08 - 2.97 (m, 1 H), 2.38 (s, 3 H), 1.35 - 1.28 (m, 12 H), 1.25 (t, *J* = 7.2 Hz, 6 H. MS m/z: 577.4 (MH)$^+$, 579.5 (MH+2)$^+$ ($C_{24}H_{29}BrN_4O_2S$).

Cell Culture

RN46A raphe-derived cell line was graciously provided by Scott R. Whittemore (University of Louisville School of Medicine). RN46A cells were cultured at 33°C in a humidified 5% CO$_2$ atmosphere and grown in Dulbecco's modified Eagles medium (DMEM) containing 100 units/ml penicillin, 100µg/ml streptomycin and 250 µg/ml G418. Nutrient Mixture F-12 Ham was purchased from Sigma-Aldrich. Fetal Bovine Serum (FBS) was purchased from Atlanta Biologicals Corp. Penicillin/streptomycin and G418 were ordered from Sigma-Aldrich.

MTT Assay

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was carried out using RN46A cells in 96-well culture plates at a density of 6×10^3 cells/well and cultured in Dulbecco's modified Eagles medium (DMEM) containing 100 units/ml penicillin, 100µg/ml streptomycin and 250 µg/ml G418 and then supplemented with varying concentrations of **5c** and **5f** (0.005, 0.05, 0.5, 5, 50 µM). Medium was removed at different time points (24 h, 48 h, and 72 h) and MTT (0.5 mg/ml in DMEM, 50 µl/well) was added. The plates were incubated at 37 °C for 4 h, followed by the addition of DMSO (150 µl/well), and incubated at 37 °C for 1 h. Optical density (OD) was measured at 570 nm with 650 nm as background.

Quantitative Real-Time RT-PCR

Real-Time reverse transcriptase polymerase chain reaction (RT-PCR) analysis was used to measure mRNA expression of human genes CRF$_1$, CREB$_1$, MAO-A, SERT, NYP, DatSLC6a3 and DBH under the control of β-actin. Briefly, RN46A cells following treatment with **5c** and **5f** for 24 h, total RNA was isolated with Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. RNA was quantified using absorption of light at 260

and 280 nm, and sample integrity was checked by 1.5% agarose gel electrophoresis. 0.8 μg of total RNA from each sample was used for reverse transcription reaction using the TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions. PCR was done using the SYBR green Master Mix (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions. The temperature cycling conditions of amplification were as follows: an initial step of denaturation at 95 °C for 10 min, 40 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and elongation at 72 °C for 30 s. Real-Time RT-PCR was performed using Mx3000P Real-Time Thermocyclers (Statagene, La Jolla, CA, USA). The relative mRNA levels of these genes were calculated by Pfaffl's mathematical method and normalized with control-treated groups [42].

CRF$_1$ Receptor Binding Study

Binding studies were performed 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 [41] 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 °C and 5% CO$_2$, were washed with phosphate-buffered saline (PBS) (4.3 mM Na$_2$HPO$_4$.7H$_2$0, 1.4 mM KH$_2$PO$_4$, 137 mM NaCl, 2.7 mM KCl, pH 7.2-7.3 at R.T). Then the cells were briefly treated with PBS containing 2 mM EDTA (PBS/EDTA), and then dissociated in PBS/EDTA. Cells suspensions were centrifuged at 1000 x g for 5 min at room temperature, and the pellets were homogenized in 1.5 ml of buffer H (20 mM HEPES, containing 10 mM MgCl$_2$, 2 mM EGTA, 0.2 mg/ml bacitracin and 0.93 μg/ml aprotinin pH 7.2 at 4 °C) using a Janke & Kunkel IKA Ultra Turrax T25 homogenizer, at setting ~20, for 10-15 sec, at 4 °C. The homogenates were centrifuged at 16000 x g, for 10 min, at 4 °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 °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 non-peptide **5a-v** at the single concentration of 1000 nM in a final volume of 0.2 ml. The mixtures were incubated at 20-21 °C for 120 min and then 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 was adjusted to insure that the specific binding was always equal to or less than 10% of the total concentration of the added radioligand. Specific [^{125}I]-Tyr^0-sauvagine binding was defined as total binding less nonspecific binding in the presence of 1000 nM antalarmin.

Statistical Analysis

Student t test was used for data analysis and significance was considered at $P < 0.05$ (*).

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