Optimization of piperazine-derived ureas privileged structures for effective anti-adenovirus agents.

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ABSTRACT

In recent years, human adenovirus (HAdV) infections have shown a high clinical impact in both immunosuppressed and immunocompetent patients. The research into specific antiviral drugs for the treatment of HAdV infections in immunocompromised patients constitutes a principal objective for medicinal chemistry due to the lack of any specific

secure drug to treat these infections. In this study, we report a small-molecule library (67 compounds) designed from an optimization process of piperazine-derived urea privileged structures and their biological evaluation: antiviral activity and cytotoxicity. The active compounds selected were further evaluated to gain mechanistic understanding for their inhibition. Twelve derivatives were identified that inhibited HAdV infections at nanomolar and low micromolar concentrations (IC_{50} from 0.6 to 5.1 μM) with low cytotoxicity. In addition, our mechanistic assays suggested differences in the way the derivatives exert their anti-HAdV activity targeting transcription, DNA replication and later steps in the HAdV replication cycle. Furthermore, eight of the 12 studied derivatives blocked human cytomegalovirus (HCMV) DNA replication at low micromolar concentrations. The data provided herein indicates that the 12 thiourea/urea piperazine derivatives studied may represent potential lead compounds for clinical evaluation and development of new anti-HAdV drugs.

1. INTRODUCTION

Human adenovirus (HAdV) is a non-enveloped virus with an icosahedral capsid containing a linear and double-stranded DNA genome of 34−36 kb. This virus has been classified into more than 60 serotypes grouped into seven subgroups or species (HAdV A−G) [1]. The high clinical impact of HAdV infections has been well-documented in immunosuppressed adult patients and children for many years [2-5]. In paediatric allogenic hematopoietic stem-cell transplant (allo-HSCT) recipients, HAdV infections occur in frequencies between 3% and 47% with high rates of associated mortality [2,6,7]. These infections have frequently been associated with respiratory, gastrointestinal, and ocular diseases in healthy individuals, and are usually presented as self-limiting infections and are rarely associated with severe clinical diseases [2]. Although the incidence of HAdV community-acquired pneumonia (CAP) in immunocompetent individuals appears to be low, with the advances in molecular techniques of diagnosis, HAdV infections have been found to be increasingly involved in occasional cases and outbreaks in healthy adults [8-12]. Despite this significant clinical impact, there are as yet no specific antiviral therapies currently approved for the treatment of HAdV infections. The non-specific therapeutic options available fail to present satisfactory results in terms of efficacy and toxicity [13,14]. Therefore, the development of low-toxic chemotherapy drugs that are effective against adenoviral infections remains a priority.

Acyclic nucleoside phosphonates (ANPs) represent a key class of antiviral agents. To overcome their known drawbacks (poor bioavailability and renal toxicity), lipophilic conjugates were studied. Only brincidofovir, (**1**) (CMX001), which is a lipidic conjugate of cidofovir (**2**) that finished a phase-III clinical trial in 2016 with no reported results so far (ClinicalTrials.gov Identifier: NCT02087306) and is now being evaluated

for the treatment of serious HAdV infection or disease (ClinicalTrials.gov Identifier: NCT02596997), represents a potential candidate for use in the treatment of HAdV infections [15, 16]. Different octadecyloxyethyl (ODE) esters of these phosphonates (**3**) were also synthesized to increase bioavailability and antiviral efficacy. Following their evaluation, they demonstrated significant antiviral activities in A549 cells [17] (Figure 1).

<Figure 1>

New types of anti-HAdV agents of a non-nucleoside nature have been discovered that exhibit an inhibitory effect on HAdV. Compounds of series of 5-amino derivatives of uracil [18] (**4**, Figure 2), and compounds from a small library of 1,2,3-triazole-containing 3-hydroxy-quinazoline-2,4(1*H*,3*H*)-diones [19] (**5,** Figure 2), were identified as potent and specific *in vitro* viral inhibitors and may constitute promising candidates for the development of drugs that are effective against HAdV infections.

<Figure 2>

As a part of our research focused on obtaining novel small molecules with biological activities (mainly antimicrobial and antitumoral) [20], in a previous study, we designed, synthesized, and evaluated the antiviral activity of three generations of new 4-acyl-1-phenylaminocarbonyl-2-methylpiperazine and 4-acyl-1-phenylaminocarbonyl-2-phenylpiperazine derivatives, which present a total of 52 compounds [21]. In this study, six new 2-phenylpiperazine derivatives are identified that show antiviral activity against HAdV and HCMV at low micromolar concentrations and present low cytotoxicity (**6-11**, Figure 3). The mechanisms of action of the selected derivatives are studied, and their structure–activity relationships are established.

<Figure 3>

Currently, the main problem for the development of new drugs involves the discovery of novel lead compounds instead of employing synthesis. The attempts to increase new drug productivity through creative discovery technologies have fallen short of producing the satisfactory results. For these reasons, “privileged structure”-guided scaffold re-evolution/refining has become a primary strategy for the identification of structurally innovative chemotypes by modifying the central core structure and/or the side-chain of the existing active compounds [22].

In this context, since compounds **6-11** (based on an innovative piperazine-derived scaffold) can be considered as potentially strong candidates for the development of a new class of antiviral compounds, we decided to explore different structural modification possibilities thereon. Faced with the urgent need for a new therapeutic alternative, our purpose was to identify optimized anti-HAdV agents in an attempt to find lead candidates (closely related structures [22b]) that would help to better characterize their mechanism of antiviral activity. A description is now given of the use of these piperazine-derived urea privileged structures (with high anti-HAdV activity and chemical versatility), which were obtained by a short and high-yielding methodology, for the development and the biological characterization of a collection of 67 compounds. The new compounds were designed while preserving the three characteristic regions of our original general framework: the piperazine core, the urea function at N-1, and the amide group at N-4. However, at the same time, these parts are considered as points for the structural variation to generate chemical diversity.

2. Results & discussion

Figure 4 shows the actions carried out on the general structure of the hit compounds **6-11** in order to generate the new derivatives. In **Pathway A,** the urea function was replaced by a thiourea function. In **Pathway B,** the acyl groups at N-4 were exchanged

with three different 2-substituted acetyl groups. Finally, in **Pathway C,** the central core of 2-substituted piperazine was modified by 2,6-dimethylpiperazine and unsubstituted piperazine (Figure 4).

<Figure 4>

The anti-HAdV activity of the new compounds obtained in each route was evaluated using plaque assays. Furthermore, the evaluation of their effect on cellular viability was also carried out (determination of CC_{50}) for those compounds that showed a percentage of inhibition in the plaque assay >80%, in order to ascertain the safety profile of the most active compounds. Although cidofovir has not been approved by the health authorities to treat HAdV infections, it remains the drug of choice for the treatment of these infections. It was also evaluated in parallel to our compounds, following our own methodologies.

Route A: Replacement of the urea function with a thiourea function.

We have carried out this action on the basis of the structure of two compounds found in our previous work, **12** and **13** (Figure 5), which showed great anti-HAdV activity (94% of the HAdV infection in the plaque assay) but also resulted in being significantly cytotoxic, with CC_{50} values of 26.3 and 31.4 μM, respectively.

<Figure 5>

The analogues of these compounds were prepared by preserving the methyl piperazine core, the thiourea function at nitrogen 1, and the phenylamine group (Scheme 1, Table 1). In order to generate chemical diversity, two points of variation are considered in their structure: (1) different acyl functions located at the N-4: urethane (as in compound **12**) or different 2-substitued acetyl derived amides, obtained firstly by an isosteric replacement (CH_2 instead of oxygen) to give 2-*tert*-butylacetyl derivatives and secondly by the modification of the substituent at the 2 position of the acetyl moiety, to obtain 2-

cyclohexylacetyl and 2-phenylacetylderivatives; (2) the substituents on the phenyl ring of the thiourea function at N-1 (groups with different electronic properties)

Compounds **20-50** were obtained following the previously described short and high-yielding synthetic methodology that involved two reactions, by employing 2-methyl piperazine as the starting material [21] (Scheme 1, Table 1)

<Scheme 1>

From this screening, we have found 12 compounds that presented HAdV5-GFP plaque-formation inhibition >80% and showed low cytotoxicity (CC_{50}>100 μM) (Table 1). Related to the group at *para* position on the phenyl group, NO_2, Cl, CN, CF_3 and F are common substituents in the active compounds (compounds **21**, **22**, **35**, **36**, **37**, **38**, **39**, **43**, **44**, **45 and 46).** The 3,5-bistrifuoromethyl group (not previously evaluated) is present in two active compounds (**42** and **50**). Except for one case (compound **41**), all of them present electron-withdrawing groups. In terms of the type of the acyl group at N-4, none of the 2-*tert* butyl acetyl derivatives (**27-34**) gave plaque-formation inhibition >80%, while the highest number of active compounds were detected among those compounds having 2-cyclohexylacetyl moiety (**35-39**, **41** and **42**).

Those compounds with HAdV inhibition infection >90% in the plaque assay and CC_{50}>100 μM were selected for further evaluation in order to obtain mechanistic understanding for their inhibitory activity; this selection included a total of seven compounds (**22**, **37**, **38**, **41**, **43**, **45** and **50**).

<Table 1>

At this point, in order to increase the number and structural diversity of thiourea analogues, we decided to incur two different modifications on the structure of the selected seven thiourea derivatives. Firstly, during the development of our general

scaffold, we had previously observed that a benzofurane-2-carbonyl group at N-4 instead of a *tert*-butoxycarbonyl group improved the antiviral activity and preserved high CC_{50} in most of the compounds [21]. We therefore decided to incorporate this group at N-4 thereby preserving *p*-NO_2, *p*-CN, *p*-F and *p*-CF_3 substituted phenyl amine thiocarbonyl group at N-1. Secondly, since our initial prototypes (**6-11**) were based on a phenyl piperazine backbone, the preparation of the 2-phenyl piperazine derived analogues was a natural evolution for our seven selected thiourea derivatives.

In order to prepare 4-benzofurane-2-carbonyl-2-methylpiperazine analogues (**56-59**) and 2-phenyl piperazine derivatives (**60-67**), the appropriate monoacyl 2-substituted piperazine derivatives (**51-55)** were firstly synthetized (Scheme 1, Table 2).

As for the biological evaluation of these analogues, Table 2 shows the percentage of inhibition obtained for the plaque assay and the results of the cellular viability evaluation (CC_{50}) for compounds **56−67.** Compounds **56-59** showed high inhibition in the plaque assay (100%), but all were highly cytotoxic; this is the same profile as compound **13** (Figure 5), which possesses a benzoyl group at N-4. In both types of thiourea derivatives there is an aromatic amide group at N-4. However, the previously described 4-benzofurane-2-carbonyl 2-methyl piperazine urea derivatives, showed slightly lower activity (% of plaque-formation inhibition values between 70-94%) and presented low cytotoxicity [21]. Clearly this modification in our thioureas did not lead to potentially interesting analogues.

However, related to 2-phenyl piperazine derivatives (compounds **60-67**), all of them excepting **61**, **62** and **67**, did not show a high percentage of plaque-formation inhibition, with respect to their methyl analogues (**22**, **38**, **41**, **43** and **45**). Only compounds **61**, **62** and **67** presented anti-HAdV activity (80% inhibition in the plaque assay for compound **62** and 100% for compounds **61** and **67**). It is important to notice that **61** was synthesized as an analogue of the 2-methyl piperazine derivative **34**, which inhibited

HAdV plaque formation in 4.8%. In this case, the presence of the phenyl group improved the antiviral activity profile. From this group, only compound **67** was selected for further studies (CC_{50} > 100 μM).

<Table 2>

Route B: Exchange the acyl groups at N-4 with three different 2-substituted acetyl groups. From our results from Route A, the presence of 2-substituted acetyl groups at N-4 improved the profile of compound **12** and obtained analogues with high activity and without cytotoxicity (the selected seven thioureas). The subsequent modification in our prototypes (**6**-**11**) was the preparation of the 2-susbtituted acetyl-2-phenylpiperazine derivatives. Compounds **7, 10,** and **11** (4-NO_2, 2-NO_2, and 2-Cl,5-CF_3 derivatives, respectively) with lowest IC_{50} values (2.1, 2.5, and 1.1 μM, respectively) [21] were chosen as models to incur this structural modification. Scheme 2 shows the synthesis of compounds **68**-**74** (Table 3).

<Scheme 2>

Compounds **68**-**74** were obtained in a high yield and submitted to biological assays (Table 3). Another two 2-phenyl piperazine derivatives were easily prepared in order to evaluate the relevance of the benzofurane-2-carbonyl amide group and the urea function in the biological activity of our scaffold. These derivatives were designed as analogues of prototype **11**. Compounds **75** (diamide) and **76** (diurea), shown in Figure 6, were synthesized directly from 2-phenyl piperazine as precursor with an excess of the reactive agent (acyl chloride or isocyanate). These compounds were evaluated using the aforementioned biological approaches and compared to the biological data of the prototypes (Table 3).

<Figure 6>

<Table 3>

The first seven compounds from this route (**68**-**74**) were active, with a percentage of plaque-formation inhibition >80% for six of them. In terms of safety both *tert*-butylacetyl derivatives, *p*- and *o*-nitro (**68** and **69**), presented little cytotoxicity, as well as the *p*-NO_2 cyclohexylacetyl derivative (**70**). Compound **75** gave a very low % of plaque-formation inhibition; however, compound **76**, the analogue diurea derivative of prototype **11,** showed high inhibition (90.4%), similar to that of **11.** The urea function seems to be crucial for the viral-replication inhibition.

From this group of derivatives, compounds **71**, **73** and **76** (percentage of plaque-formation inhibition >90% and CC_{50}>100 μM) were selected for further biological evaluations.

Pathway C: The central core of 2-substituted piperazine was exchanged with 2,6-dimethylpiperazine and unsubstituted piperazine.

Analogues of compounds **6**-**11** were prepared based on a 2,6-dimethylpiperazine or piperazine central ring instead of the 2-phenyl piperazine ring. The rationale behind this structural modification involved the evaluation of the effect of the presence of an additional substituent in the piperazine core and of the use of an unsubstituted ring. The other parts of the general structure were preserved, with the additional introduction of two electron-donators groups (Me, OMe) as substituents at *para* position of the phenyl ring of the urea function. The benzofurane-2-carbonyl monoamide derivative **78** was prepared in the same way as other monoamides, and, by means of reaction with isocyanates, compounds **79**-**85** were obtained in high yields (Scheme 3, Table 4)

<Scheme 3>

Piperazine analogues of **7**, **10,** and **11** were first prepared from 1-Boc-piperazine (**86**) by reaction with appropriate isocyanates (obtaining compounds **87**-**89**), and secondly, through the deprotection of the Boc group by treatment with CF_3COOH, followed by reaction with benzofurano-2-carbonyl chloride, compounds **90** and **91** were produced (Scheme 4, Table 4).

<Scheme 4>

Finally, in the same way as for those based on a phenylpiperazine core (**75** and **76**), the benzofurane-2-carbonyl diamide derivative (**92**) and the diurea derivative (**93**) were prepared directly from piperazine (Figure 6). Table 4 shows biological data of the core-modified analogues **79**-**85**, **87**-**93**

<Table 4>

Among the 2,6-dimetilpiperazine urea derivatives, only two compounds (**80** and **82**) showed antiviral activity (percentage of plaque-formation inhibition 81.1% and 72.2 respectively), although not as high as that of the models **8** and **10** (percentage of plaque-formation inhibition > 90%,). They also showed low cytotoxicity. In general terms, this central backbone failed to provide any improvement over the prototypes. Among the piperazine derivatives, however, we have two compounds with a percentage of plaque-formation inhibition >90% and CC_{50}>100 μM (**87** and **89**). Both compounds were Boc derivatives; one with p-NO_2 phenyl urea group (**87**) and the other with 2-Cl-5-CF_3-disubstituted phenyl urea (**89**), analogues to compounds **6** and **11** respectively. In our previous work, the 2-phenyl piperazine analogue to **89**, with a 2-Cl-5-CF_3-disubstituted phenyl urea at N-1 and a Boc group at N-4, showed 100% plaque-formation inhibition

but since its CC_{50} was lower than 100 μM, it was not selected for further studies. The presence of a benzofurane-2-carbonyl group did not yield active compounds (**90** and **91**), which is in contrast to those with a phenyl piperazine as central core for which the replacement of Boc group with a benzofurane-2-carbonyl group led to the current prototypes (**7**-**11**) [21]. Both dibenzofurane-2-carbonyl derivative (**92**) and the diurea derivative (**93**) also presented low antiviral activity. In the case of diurea **93,** its percentage of plaque-formation inhibition was lower than that for compound **76** (33.9% *vs* 90.4%). All of these piperazine derivatives presented low cytotoxicity.

Since these derivatives belong to an innovative class of achiral derivatives of our privileged structure, compounds **87** and **89** were selected for further biological studies.

Compounds for further analysis were selected based on their antiviral activity in the plaque assay (>90% inhibition at 10 μM concentration and IC_{50} values) and on their cytotoxicity (CC_{50}>100 μM). A total of twelve compounds from the three pathways described below were selected in order to obtain some mechanistic understanding for their inhibitory activity. Compound **76** was not included in this selection (IC_{50} 7.0±1.0 μM, SI 28.7).

The twelve selected derivatives blocked HAdV infection in a dose-dependent manner, and presented IC_{50} values from 0.6 μM to 5.1 μM (Table 5, Supplementary Information Figure S68). Compared with the compounds selected from the previous library of 4-acyl-1-phenylaminocarbonyl-2-phenylpiperazine derivatives, all of which fall under a European patent (*Number of application: EP16382073.1.; Name of the Invention: Piperazine derivatives as antiviral agents with increased therapeutic activity; year of application: 2016*), this new library shows slight optimization in activity since the molecules reached anti-HAdV activity at the nM range in two (17%) cases (600 nM for **38** and 700 nM for **67**) and eight (67%) of the cases showed IC_{50} < 2.5 μM. In the

precursor library, the lowest values of IC_{50} registered were in the μM range, 33 % of which were < 2.5 μM. Previously reported IC_{50} values for cidofovir are significantly higher than those shown by any of these phenyl- and methylpiperazine urea/thiourea derivatives [23, 24]. Following our own methodologies, the IC_{50} value of cidofovir for HAdV5 was 24.06±5.9 μM, which is also significantly higher than the values obtained for the piperazine derivatives studied (Table 5)

<Table 5>

These selected derivatives presented a better safety profile than did the original compounds of the 4-acyl-1-phenylaminothiocarbonyl-2-methylpiperazine derivatives **12** and **13,** and presented a similar range to that of the six hit compounds selected in our previous work, with CC_{50} values between 104.3-210.4 μM *vs* 130.8-199.8 μM, respectively [21].

The anti-HAdV effect of these derivatives was subsequently evaluated using a virus burst assay to measure their effectiveness in blocking the production of new virus particles. Treatment with the compounds was associated with overall reductions in virus yield between 9.3-fold and 1,690-fold (Table 5). These values lay in the same range as those for the prototypes **6-11**, with the exception of compound **67,** which presented an extraordinary reduction of HAdV yield of 1,690-fold.

Insights into their antiviral mechanism of action

Impact on HAdV entry

As for the mechanism of action presented by these new derivatives, we also observed significant differences compared to the previous library. After the initial attachment of HAdV particles to cellular receptors by the fibre, αvβ3 or αvβ5 integrins act as co-receptors by binding to a highly conserved motif in the penton base, which is an

essential interaction for virus internalization by clathrin-mediated endocytosis [25, 26]. Once inside the endosome, the partial decapsidation of viral particles and the subsequent exposition of several viral proteins, specifically the membrane lytic protein VI, trigger the endosomolysis. The virus then escapes to the cytoplasm [27] where it is then transported via microtubules to the nuclear envelope and anchored to a nuclear pore to deliver its genome into the nucleus [28-30]. We examined whether any of these selected compounds inhibited any of the steps in the HAdV entry that would reflect in the number of HAdV genomes that reach the host nucleus after a synchronized infection [14, 31]. Thus, we demonstrated that nine out of the twelve selected compounds did not seem to block any step of the cell entry pathway up to the HAdV DNA delivery to the nucleus. Human adenovirus genomes accumulated normally at the nucleus of the host cell (Supplementary Information, Figure S69), just as with the previous 4-acyl-1-phenylaminocarbonyl-2-phenylpiperazine derivatives [21]. However, for compounds **71**, **73,** and **89**, a significant inhibition of the HAdV genome accumulation at the nucleus of the host cell were observed, suggesting that at least part of the inhibitory activity of these compounds reaches its target soon after HAdV enters the cell.

Impact on HAdV DNA replication

Once HAdV DNA, along with protein VII [29], enters the cell nucleus, then the transcription occurs of the HAdV immediate early gene E1A by the cellular RNA polymerase II, which is a prerequisite for the subsequent expression of the early genes E1B, E2, and E4 and for HAdV DNA replication [32]. Our results showed a significant inhibition of HAdV DNA replication by compounds **41**, **43**, **45, 50,** and **87** (Figure 7A). These compounds may interact directly to block the activity of viral proteins involved in HAdV DNA replication, such as HAdV DNA polymerase. Alternatively, these compounds may interfere with a previous step during the transcription of the immediate

early gene E1A. Figure 7B shows how compound **50** significantly inhibited the transcription of the immediate early HAdV gene E1A. Prior to HAdV DNA replication, the transcription of E1A by cellular RNA polymerase II takes place from the E1A promoter [32]. The E1A protein is necessary both for the subsequent expression of the early genes E1B, E2, E3, and E4 from different promoters and for HAdV DNA replication. α-Amanitin, a toxin derived from the mushroom Amanita phalloides, can interact and block the RNA polymerase II activity [33, 34]. Like α-amanitin, compound **50** displays dose-dependent inhibitory activity for the generation of new HAdV viral particles [33]. The concentrations of compound **50** and α-amanitin necessary to inhibit HAdV transcription lie within a similar range, (between 1-30 μM) [33, 34]. However, α-amanitin shows cytopathological effects (CPE) at concentrations as low as 5 μM [33]. Given the phenotypic similarities presented by these two molecules, it would be possible that compound **50** could block the host RNA polymerase II, since we also detected the inhibition of CMV infection in the presence of this compound, whose transcription is also mediated by the host RNA polymerase II [35]. Other scenarios may account for our finding that compound **50** interferes with the HAdV early transactivator, E1A. Compound **50** may also strengthen the DNA-pVII complex or interfere with any of the proteins associated with the repression/activation of the E1A transcription.

Since compounds **41**, **43**, **45,** and **87** did not block transcription of the E1A gene, they may be interfering directly with a protein involved in HAdV DNA replication**.** As a proof of concept of the possible role of HAdV and CMV DNA polymerases as targets for these compounds, we investigated whether the presence of compounds **41** and **43** affected the *in vitro* amplification efficiency of the DNA polymerase from bacteriophage Phi29, which is a closely-related DNA polymerase belonging to the same family of DNA polymerases (family B). As can be observed in Figure 7D, compound

41 showed significant inhibition of the Phi29 DNA polymerase activity, which suggests that its preferential target may be the HAdV and CMV DNA polymerases.

<Figure 7>

Compounds **22**, **37, 38 and 67** did not show any inhibition of the HAdV genome accessibility to the nucleus nor of the HAdV *de novo* DNA synthesis. The mechanism of action for these compounds may be related to later steps in the HAdV replicative cycle, such as assembly, maturation, and even release of the new viral particles. Further studies are needed to clarify the specific mechanisms for the inhibition of HAdV infection by these seven compounds. Since they have shown high variability regarding their mechanism of action, the compounds could be useful as a tool to unravel the complex events involved in the HAdV replicative cycle after HAdV entry.

The twelve derivatives presented here target different steps in the HAdV replicative cycle: inhibition of early steps during HAdV entry into the cells, inhibition of the transcription of the E1A gene, interference with the viral DNA polymerase and other not fully characterized mechanisms interfering with HAdV DNA replication and interference with later steps after HAdV DNA replication.

Synergistic activity of the selected derivatives

Since we identify different mechanisms of action for the selected compounds we hypothesized that their combination will improve their antiviral activity blocking HAdV infection at lower concentrations due to their synergistic effect. In order to assess this hypothesis, three representative derivatives of each mechanism described were selected to perform a combination study based on the Chou-Talalay method for drug combination using the CalcuSyn software [36, 37]. The three derivatives that presented different mechanisms of action in later stages after the arrival of the HAdV genomes into nucleus were: compound **38** as a compound acting in later stages after DNA

replication; compound **41** as an inhibitor of the HAdV DNA replication process (HAdV DNA polymerase); and compound **50** as an inhibitor of the E1A transcription (all of them are 4-acyl-1-phenylaminothiocarbonyl-2-methylpiperazine derivatives). The ratio for each combination was selected based on the IC_{50} values for each derivative. The data for all the combinations showed good conformity to the mass-action law (r ranged from 0.937 to 0.967) (Table 6). The combination of these three derivatives was classified as synergistic when they were combined at the different ratios and at all the inhibitory levels assayed; specifically this activity was classified as very strong synergism for the combination **38**+**41 (1:4)**, targeting HAdV DNA polymerase activity (**41**) and later steps after HAdV DNA replication (**38**) at all three levels of inhibition (IC_{50}, IC_{75} and IC_{90}), and for the combination **38**+**41**+**50** (1:4:8) at the IC_{90}, including in this combination compound **50** targeting the gen E1A transcription. For the **41**+**50** (1:2) combination the IC_{50} and IC_{75} levels of inhibition were classified as strong synergism. Finally, the combinations **41**+**50** (1:2) at IC_{90}, **38**+**50** (1:8) at all the levels of inhibition and **38**+**41**+**50** (1:4:8) at the IC_{50} and IC_{75} levels of inhibition were classified as synergism.

The existence of different mechanisms for the antiviral activity of these three compounds is this way supported by the significant combinatory index values obtained using the CalcuSyn software for all the combinations. These derivatives showed a synergistic to very strong synergistic activity when combined in pairs or including all three compounds at all the levels of inhibition assayed.

<Table 6>

Anti-HCMV activity of 4-acyl-1-phenylaminothiocarbonyl-2-methylpiperazine derivatives

The following step explored the possible inhibitory activity of the selected compounds on HCMV DNA replication since other antiviral compounds have shown broad activity against multiple dsDNA viruses including HCMV and HAdV [21, 38]. Quantification of total HCMV DNA 72 hours after the infection of MRC-5 cells revealed: significant differences between samples treated with compounds **41**, **50,** and **87;** and differences not statistically significant for compounds **43**, **45**, **71**, **73,** and **89**, and those treated with the same volume of DMSO, five of which had previously shown inhibition of the HAdV DNA replication (Figure 7C).

Study of the stability of the derivatives in hamster serum

Determination of the stability of new chemical entities in serum is important as compounds (with the exception of pro-drugs where rapid conversion in serum is desirable), which rapidly degrade in serum, generally show poor *in vivo* efficacy [39]. We assessed the stability of the compounds in Syrian hamster serum as a previous step to the evaluation of their efficacy and safety in the Syrian hamster model of HAdV infection [16]. This assay would allow us to prioritize the anti-HAdV compounds with the structural motifs most resistant to degradation by serum proteins and those compounds with the lowest binding to serum proteins, which will potentially show better *in vivo* efficacy. The four derivatives that presented the best SI (>100) were included in the evaluation (**22**, **38**, **43,** and **67**, with different modes of action to block HAdV infection, see below) together with compound **50** due to its activity as an inhibitor of the E1A transcription (again all of these five selected compounds are 4-acyl-1-phenylaminothiocarbonyl-2-methylpiperazine derivatives). Only compounds **22** and **38** (*tert*-butoxycarbonyl and 2-cyclohexylacetyl derivatives respectively) showed a significant degradation after two hours of incubation in the Syrian hamster serum, with percentages of remaining compound <70%. Compounds **43** and **67** showed no degradation after the incubation time with percentages of remaining compound of

106.7±3.8 and 100.0±9.2 respectively, whilst compound **50** showed a percentage of remaining compound of 92.8% (the three of them, 2-phenylacetyl derivatives) (Supplementary Information, Table S70).

In silico prediction of physicochemical properties: *drug-likeness* evaluation

During the drug discovery and development processes of new molecules with the final goal to identify those with the best chance to become an effective drug for patients, apart from efficacy and toxicity, many failures are related to pharmacokinetics, i.e., the distribution of the compound in the organism [40]. In this sense, the early estimation of ADME behaviors (Absorption, Distribution, Metabolism and Excretion) has decreased the fraction of global pharmacokinetics related failures in later phases of development.

Although there are different routes of drug administration, oral dosing is highly preferred for the patient's comfort and compliance. Bioavailability is highly multifactorial, but is primarily driven by gastrointestinal absorption. The concept of drug-likeness (to assess qualitatively the chance for a molecule to become an oral drug with respect to bioavailability), established from the analyses of the physiochemical properties or/and structural features of drug candidates, has been widely used to rule out compounds with properties most probably incompatible with an acceptable pharmacokinetics profile. There are different rule-based filters, with diverse ranges of properties inside of which the molecule is defined as *drug-like* [41].

Some physicochemical properties of the 12 selected compounds have been predicted using free online software (http://www.molinspiration.com) to assess their compliance with the Lipinski's rule of five [42].

As shown in Table S71 (Supporting Information) eleven out of twelve conformed well to the Lipinski's rule of five. Only compound **67** failed with two violations, LogP and Molecular Weight values exceeded the accepted ranges.

A second web tool (SwissADME, freely accessible at http://www.swissadme.ch) was also used to evaluate pharmacokinetics and *drug-likeness* of these small molecules (data no shown) [43]. Excepting compound **67** (which showed the same two Lipinski's rules violations), the rest of the compounds conformed again to both Lipinski's and Veber's rules. In addition, the displayed *Bioavailability Radar* (a rapid appraisal of drug-likeness based on six physicochemical properties: lipophilicity, size, polarity, solubility, flexibility and saturation that define the suitable physicochemical space for oral bioavailability) predicted only compound **67** as no orally bioavailable (too lipophilic, insoluble and high sized). According to the white of the BOILED-egg [41, 43], the human gastrointestinal absorption predicted was high for all compounds, except for compound **67**.

2. Conclusions

The twelve selected piperazine-derived thioureas/ureas presented here showed significant anti-HAdV activity within nanomolar and low micromolar ranges with low cytotoxicity. The strengthening of their antiviral activity in combination supports the existence of different mechanisms of action. These molecules target different steps in the HAdV replicative cycle: i) blockage of the HAdV genome accessibility to the nucleus (**71**, **73** and **89**) targeting early steps after HAdV enters the cell, ii) inhibition of the transcription of the E1A gene (**50**), iii) interference with HAdV DNA replication **(41**, **43**, **45,** and **87)** and specifically in case of compound **41** interfering with the viral DNA polymerase, iv) and other not fully characterized mechanisms interfering with later steps after HAdV DNA replication (**22**, **37, 38 and 67)**. Although further studies

are needed for the full characterization of the mechanisms of action of these molecules, these could also provide great insights towards a better understanding of the HAdV replication cycle.

Herein we have identified new mechanisms of action respect to the prototypes used for the generation of this new library of compounds, which were targeting HAdV replication and later steps. The selected compounds also improve the antiviral activity of the ones they come from, showing anti-HAdV activity at nanomolar concentrations. Based on their mechanisms of action and their antiviral activity and serum stability, compounds **22**, **41**, **43**, **50, 67** and **73** have been selected for further *in vivo* studies in the immunosuppressed Syriam Hamster model of HAdV infections towards improving our knowledge regarding their efficacy and safety as the first step in their development as therapeutic alternatives for the treatment of HAdV infections.

4. Experimental Section

4.1 Chemistry. General Chemistry Methods. All reagents, solvents, and starting materials were obtained from commercial suppliers and used without further purification. The crude reaction mixtures were concentrated under reduced pressure by removing the organic solvents in a rotary evaporator. Reactions were monitored by thin layer chromatography (TLC) using Kieselgel 60 F_{254} (E. Merck) plates and UV detector for visualization. Flash column chromatography was performed on Silica Gel 60 (E. Merck). All reported yields are of purified products. Melting points were obtained on a Stuart Melting Point Apparatus SMP 10 and are uncorrected. Mass spectra were recorded on a Micromass AUTOSPECQ mass spectrometer: EI at 70 eV and CI at 150 eV, HR mass measurements with resolutions of 10,000. FAB mass spectra were recorded using a thioglycerol matrix. NMR spectra were recorded at 25 ºC on a Bruker AV500 spectrometer at 500 MHz for ^{1}H and 125 MHz for ^{13}C. COSY, DEPT, HSQC,

and NOESY experiments were performed to assign the signals in the NMR spectra. The chemical shifts (δ) reported are given in parts per million (ppm) on the δ scale relative to TMS, and the coupling constants (J) are in hertz (Hz). ^{1}H chemical shift values (δ) are referenced to the residual nondeuterated components of the NMR solvents (δ = 2.54 ppm for DMSO, δ = 7.26 ppm for $CDCl_3$). The ^{13}C chemical shifts (δ) are referenced to $CDCl_3$ (central peak, δ = 39.5 ppm for DMSO and 77.16 ppm for $CDCl_3$) as the internal standard. The spin multiplicities are reported as s (singlet), d (doublet), t (triplet), q (quadruplet), m (multiplet), or br s (broad singlet).. The purity of final compounds was evaluated by C, H and N analysis. The purity of all the final compounds was confirmed to be ≥95% by combustion.

General Procedure 1. Chemoselective *N*-acylation reaction of 2-Substituted piperazines or 2,6-Disubstituted piperazine (16-19, 51-55, 78). 2-Substituted piperazine or 2,6-Disubstituted piperazine (5.0 mmol) was dissolved in dry dichloromethane (100 mL) and cooled to 0 ºC. A solution of the appropriate acylating agent (5.0 mmol) in dichloromethane (20 mL) was added dropwise in 30 minutes, and then pyridine (7.5 mmol). The reaction mixture was kept into an ice-water bath with stirring 12 hours and left at room temperature until TLC showed that all the starting material had reacted. The reaction mixture was evaporated to dryness to obtain the corresponding monoacylderivative. Column chromatography gave the pure compound in high yield.

1-*tert*-Butoxycarbonyl-3-methylpiperazine (16) [21]. The product was obtained as a syrup and purified by column chromatography using dichloromethane-methanol (15:1) as eluent (750 mg, 75% yield). MS (CI): *m/z* 201 (20%) $[M+H]^+$. ^{1}H NMR (500 MHz, DMSO-d_6) δ 3.75–3.71 (m, 2H), 2.85–2.82 (m, 1H), 2.75–2.69 (m, 1H), 2.60–2.54 (m, 3H), 2.39–2.34 (m, 1H), 1.41 (s, 9H), 0.96 (d, J = 6.3 Hz, 3H). ^{13}C NMR (125 MHz,

DMSO-d_6) δ 154.5, 79.3, 51.2, 50.5, 45.5, 44.4, 28.6, 19,3. HRMS (*m/z*): calcd for $C_{10}H_{20}N_2O_2$ 200.1528 $[M]^{+\cdot}$; found 200.1525.

1-(3,3-Dimethylbutanoyl)-3-methylpiperazine (17). The product was obtained as a syrup and purified by column chromatography using ethyl acetate-methanol (3:1) as eluent (792 mg, 80% yield). MS (CI): *m/z* 199 (100%) $[M+H]^+$. ^{1}H NMR (500 MHz, $CDCl_3$) δ 4.57 (d, J = 12.4 Hz, 1H), 3.85 (m, 1H), 3.52–3.08 (m, 2H), 3.06–2.62 (m, 3H), 2.23 (s, 2H), 1.33 (br s, 3H), 1.02 (s, 9H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 170.3, 51.4, 45.8, 44.6, 44.5, 39.3, 31.5, 30.0, 17.3. HRMS (*m/z*): calcd for $C_{11}H_{22}N_2ONa$ 222.1624 $[M+Na]^+$; found 222.1619.

1-(2-Cyclohexylacetyl)-3-methylpiperazine (18). The product was obtained as a syrup and purified by column chromatography using ethyl acetate-methanol (4:1) as eluent (1.1 g, 97% yield). ^{1}H NMR (500 MHz, DMSO-d_6) δ 4.26 (d, J = 11.7 Hz, 1H), 3.76 (d, J = 11.4 Hz, 1H), 3.05–2.88 (m, 2H), 2.70–2.58 (m, 3H), 2.26–2.14 (m, 3H), 1.75–1.60 (m, 6H), 1.30–1.10 (m, 3H), 1.05–0.91 (m, 5H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 169.7, 52.0, 50.8, 50.3, 47.7, 45.4, 44.8, 41.0, 34.4, 32.7, 25.8, 25.6, 18.7, 18.4. HRMS (*m/z*): calcd for $C_{13}H_{25}N_2O$ 225.1961 $[M+H]^{+\cdot}$; found 225.1964.

3-Methyl-1-(2-phenylacetyl)piperazine (19). The product was obtained as a syrup and purified by column chromatography using ethyl acetate-methanol (4:1) as eluent (1.1 g, 98% yield). ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.33–7.22 (m, 5H), 4.25 (d, J = 12.7 Hz, 1H), 3.09–2.62 (m, 3H), 2.58–2.53 (m, 1H), 2.49–2.34 (m, 1H), 1.75-1.60 (m, 6H), 1.03, 0.99 (2s, J = 6.4 Hz, 3H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 168.7, 135.9, 135.8, 128.9, 128.8, 128.3, 128.2, 126.3, 51.5, 50.5, 50.1, 47.1, 45.0, 44.6, 44.2 40.6, 18.1, 17.7. HRMS (*m/z*): calcd for $C_{13}H_{19}N_2O$ 219.1492 $[M+H]^+$; found 219.1486.

1-(Benzofuran-2-carbonyl)-3-methylpiperazine (51). The product was obtained as a solid and purified by column chromatography using dichloromethane-methanol (40:1) as eluent (903 mg, 74% yield), mp 101–103 ºC. ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.7–7.5 (m, 5H), 4.47 (br s, 2H), 3.10 (d, J = 11.4 Hz, 1H), 2.94–2.86 (m, 2H), 1.97 (br s, 2H), 1.13 (d, J = 5.0 Hz, 3H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 159.8, 154.6, 149.1, 127.0, 126.4, 123.6, 122.2, 111.9, 111.8, 51.1, 46.1, 19.4. Anal. Calcd $C_{14}H_{16}N_2O_2$: C, 68.55; H, 6.99; N, 11.42. Found: C, 68.32; H, 6.62; N, 11.22.

1-*tert*-Butoxycarbonyl-3-phenylpiperazine (**52**) [21].The product was obtained as a syrup and purified by column chromatography using hexane−ethyl acetate (2:1) as eluent (864 mg, 66% yield), mp 103−105 °C. MS (CI): *m/z* 263 (100%) $[M+H]^+$. 1H NMR (500 MHz, $CDCl_3$) δ 7.4−7.3 (m, 5H), 4.05 (br s, 2H), 3.70 (dd, J = 2.4 Hz, J = 10.5 Hz, 1H,), 3.07 (m, 1H), 2.9−2.8 (m, 2H), 2.72 (br s, 1H), 1.90 (br s, 1H), 1.47 (s, 9H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 154.8, 141.5, 128.5, 127.8, 127.0, 79.7, 60.3, 51.5, 46.1, 43.4, 28.5. HRMS (*m/z*): calcd for $C_{15}H_{23}N_2O_2$ 263.1754 $[M+H]^+$; found 263.1748.

1-(3,3-Dimethylbutanoyl)-3-phenylpiperazine (53). The product was obtained as a syrup and purified by column chromatography using ethyl acetate-methanol (20:1) as eluent (1.0 g, 77% yield). MS (CI): *m/z* 261 (100%) $[M+H]^+$. ^{1}H NMR (500 MHz, DMSO-d_6) δ 7,45–7,27 (m, 5H), 4,44–4.39 (m, 1H), 3,94–3,85 (m, 1H), 3,58 (dd, J = 2.3 Hz, J = 10.3 Hz, 1H), 3,31 (br s, 1H), 3,11–2,99 (m, 2H), 2,91–2,68 (m, 1H), 2,61–2,57 (m, 1H), 2,31–2,20 (m, 2H), 1,02, 1,00 (2s, 9H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 169.3, 169.2, 142.2, 141.9, 128.2, 128.1, 127.4, 127.3, 127.0, 126.9, 60.1, 59.4, 53.5, 48.1, 46.4, 45.9, 45.5, 43.8, 43.6, 41.1, 40.1, 30.9, 29.8. HRMS (*m/z*): calcd for $C_{16}H_{25}N_2O$ 261.1961 $[M+H]^+$; found 261.1956.

1-(2-Cyclohexylacetyl)-3-phenylpiperazine (54). The product was obtained as a syrup and purified by column chromatography using ethyl acetate-methanol (30:1) as eluent (815 mg, 57% yield). MS (CI): *m/z* 287 (100%) $[M+H]^+$. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.40–7.27 (m, 5H), 4.67–4.63 (m, 1H), 3.84–3.79 (m, 1H), 3.69–3.67 (m, 1H), 3.26–3.02 (m, 2H), 2.91–2.83 (m, 1H), 2.59–2.54 (m, 1H), 2.29–2.16 (m, 2H), 2.10–1.91 (m, 1H), 1.85–1.65 (m, 5H), 1.33–1.21 (m, 2H), 1.18–1.10 (m, 1H), 1.02–0.92 (m, 2H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 170.9, 141.3, 141.1, 128.7, 128.5, 128.2, 127.8, 127.0, 126.9, 61.4, 60.2, 53.6, 48.8, 46.7, 46.3, 46.1, 41.8, 40.8, 40.7, 35.3, 35.2, 33.5, 33.4, 33.3, 26.3, 26.2. HRMS (*m/z*): calcd for $C_{18}H_{27}N_2O$ 287.2118 $[M+H]^+$; found 287.2111.

3-Phenyl-1-(2-phenylacetyl)piperazine (55). The product was obtained as a syrup and purified by column chromatography using ethyl acetate-methanol (100:1) as eluent (784 mg, 56% yield).). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.39–7.12 (m, 10H), 4.68–4.59 (m, 1H), 3.83–3.62 (m, 3H), 3.29–3.13 (m, 1H), 3.11–2.92 (m, 2H), 2.84–2.56 (m, 2H), 2.18 (br s, 1H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 169.5, 169.4, 141.0, 140.8, 135.3, 135.1, 128.9, 128.7, 128.6, 128.5, 128.0, 127.9, 127.0, 126.9, 126.8, 60.8, 60.0, 53.8, 48.9, 46.5, 46.2, 45.9, 42.0, 41.4, 41.2. HRMS (*m/z*): calcd for $C_{18}H_{21}N_2O$ 281.1648 $[M+H]^+$; found 281.1644.

1-(Benzofuran-2-carbonyl)-3,5-dimethylpiperazine (77). The product was obtained as a syrup and purified by column chromatography using dichloromethane-methanol (20:1) as eluent (942 mg, 73% yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.76 (d, J = 7.8 Hz, 1H), 7.68 (dd, J = 8.1 Hz, J = 0.6 Hz, 1H), 7.48–7.44 (m, 1H), 7.41 (s, 1H), 7.37–7.33 (m, 1H), 4.46–4.10 (m, 2H), 2.98–2.75 (m, 3H), 1.16–0.94 (m, 6H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 158.6, 153.9, 148.2, 126.7, 126.4 123.6, 122.4, 111.8, 110.7, 54.9, 18.2. HRMS (*m/z*): calcd for $C_{15}H_{19}N_2O_2$ 259.1441 $[M+H]^+$; found 259.1444.

General Procedure 2. Synthesis of the thiourea / urea derivatives (12, 20–50, 56-67, **68-74**, **79-85**, **87-91).** To a solution of the monoacyl derivative (**16–19, 51–55, 78** and **86**) (0.75 mmol) in dry dichloromethane (10 mL) was added the corresponding isothiocyanate/isocyanate (0.9 mmol). The reaction mixture was stirred at room temperature until TLC showed that all the starting material had reacted. The reaction mixture was evaporated to dryness**.** The compounds were purified by flash chromatography on silica gel using the appropriate eluent. For compounds **75** and **92** 1.8 mmol of the appropriate isocyanate were employed.

4-*tert*-Butoxycarbonyl-2-methyl-1-[(4-nitrophenyl)aminothiocarbonyl]piperazine (12) [21]. The product was obtained as a solid and purified by column chromatography using hexane-ethyl acetate (2:1) as eluent (234 mg, 82% yield), mp 174–176 ºC. MS (FAB): *m/z* 403 (95%) $[M+Na]^+$. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9,72 (br s, 1H), 8.16–8.11 (m, 2H), 7.61–7.59 (m, 2H), 5.14–5.05 (m, 1H), 4.43–4.35 (m, 1H), 4.00–3.86 (m, 1H), 3.83–3.75 (m, 1H), 3.22–3.12 (m, 1H), 3.06–2.93 (m, 1H), 1.44 (s, 9H), 1.21 (d, J = 6.7 Hz, 3H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 181.3, 154.5, 147.7, 142.4, 123.9, 122.9, 79.2, 51.8, 43.1, 27.9, 26.8, 14.7. HRMS (*m/z*): calcd for $C_{17}H_{24}N_4O_4SNa$ 403.1410 $[M+Na]^+$; found 403.1405. Anal. Calcd $C_{17}H_{24}N_4O_4S$: C, 53.67; H, 6.36; N, 14.73; S, 8.43. Found: C, 53.60; H, 6.58; N, 14.56; S, 8.27.

4-*tert*-Butoxycarbonyl-1-[(4-chlorophenyl)aminothiocarbonyl]-2-methylpiperazine (20). The product was obtained as a solid and purified by column chromatography using hexane-ethyl acetate (2:1) as eluent (202 mg, 73% yield), mp 143–144 ºC. MS (FAB): *m/z* 392 (100%) $[M+Na]^+$. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.30 (d, J = 8.4 Hz, 2H), 7.16–7.33 (m, 3H), 5.13–4.76 (m, 1H), 4.45–3.79 (m, 3H), 3.39–3.32 (m, 1H), 3.21 (dd, J = 3.5 Hz, J = 13.5 Hz, 1H), 3.12–2.91 (m, 1H), 1.48 (s, 9H), 1.28 (d, J = 6.7 Hz, 3H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 183.3, 155.0, 138.5, 131.0, 129.8, 129.2, 126.9, 125.4,

80.5, 52.4, 43.8, 28.4, 15.1. HRMS (*m/z*): calcd for $C_{17}H_{24}ClN_3O_2SNa$ 392.1170 $[M+Na]^+$; found 392.1163. Anal. Calcd $C_{17}H_{24}ClN_3O_2S$: C, 55.20; H, 6.54; N, 11.36; S, 8.67. Found: C, 55.48; H, 6.67; N, 11.44, S, 9,01.

4-*tert*-Butoxycarbonyl-1-[(4-cyanophenyl)aminothiocarbonyl]-2-methylpiperazine (21). The product was obtained as a solid and purified by column chromatography using hexane-ethyl acetate (3:1) as eluent (205 mg, 76% yield), mp 132–134 ºC. MS (FAB): *m/z* 383 (100%) $[M+Na]^+$. ^1H NMR (500 MHz, $CDCl_3$) δ 7.61 (d, J = 8.4 Hz, 2H), 7.32 (d, J = 8.4 Hz, 2H), 7.22 (br s, 1H), 5.18–4.86 (m, 1H), 4.40–3.73 (m, 4H), 3.44–3.37 (m, 1H), 3.26–3.19 (m, 1H), 3.14–2.90 (m, 1H), 1.48 (s, 9H), 1,32 (d, J = 6.8 Hz, 3H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 182.5, 154.9, 144.0, 133.2, 126.5, 122.7, 118,6. 80.5, 52.8, 44.1, 28.4, 15.3. HRMS (*m/z*): calcd for $C_{18}H_{24}N_4O_2SNa$ 383.1512 $[M+Na]^+$; found 383.1500. Anal. Calcd $C_{18}H_{24}N_4O_2S$: C, 59.97; H, 6.71; N, 15.54; S, 8.90. Found: C, 60.03; H, 6.81; N, 15.20; S, 9.15.

4-*tert*-Butoxycarbonyl-1-[(4-fluorophenyl)aminothiocarbonyl]-2-methylpiperazine (22). The product was obtained as a solid and purified by column chromatography using hexane-ethyl acetate (3:1) as eluent (209 mg, 79% yield), mp 74–76 ºC. MS (FAB): *m/z* 376 (100%) $[M+Na]^+$. ^1H NMR (500 MHz, $CDCl_3$) δ 7.22–7.14 (m, 2H), 7.10 (s, 1H), 7.06–7.02 (m, 2H), 5.14–4.75 (m, 1H), 4.51–3.69 (m, 3H), 3.41–3.32 (m, 1H), 3.24–3.16 (m, 1H), 3.14–2.75 (m, 1H), 1.47 (s, 9H), 1.28 (d, J = 6.8 Hz, 3H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 183.6, 155.0, 135.9, 126.6, 115,9. 80.4, 52.3, 43.6, 28.4, 15.1. HRMS (*m/z*): calcd. for $C_{17}H_{24}FN_3O_2SNa$ 376.1465 $[M+Na]^+$; found 376.1456. Anal. Calcd $C_{17}H_{24}FN_3O_2S$: C, 57.77; H, 6.84; N, 11.89; S, 9.07. Found: C, 58.00; H, 6.82; N, 11.68; S, 9.48.

4-*tert*-Butoxycarbonyl-2-methyl-1-[(4-trifluoromethylphenyl)aminothiocarbonyl]piperazine (23). The product was obtained as a solid and purified by column chromatography using hexane-ethyl acetate (4:1) as eluent (220 mg, 73% yield), mp 141–143 ºC. MS (FAB): *m/z* 426 (100%) $[M+Na]^+$. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.52 (s, 1H), 7.65 (d, J = 8.3 Hz, 2H), 7.55 (d, J = 8.3 Hz, 2H), 5.11 (br s, 1H), 4.45–4.38 (m, 1H), 3.98–3.84 (m, 1H), 3.82–3.75 (m, 1H), 3.25–3.12 (m, 1H), 3.09–2.93 (m, 1H), 1.45 (s, 9H), 1,20 (d, J = 6.7 Hz, 3H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 181.7, 154.2, 144.9, 125.1, 125.0, 124.5, 79.2, 51.4, 42.7, 28.0, 14.8. HRMS (*m/z*): calcd for $C_{18}H_{24}F_3N_3O_2SNa$ 426.1434 $[M+Na]^+$; found 426.1428. Anal. Calcd $C_{18}H_{24}F_3N_3O_2S$: C, 53.58; H, 6.00; N, 10.41. Found: C, 53.12; H, 6.41; N, 10.31.

4-*tert*-Butoxycarbonyl-1-[(4-methoxyphenyl)aminothiocarbonyl]-2-methylpiperazine (24). The product was obtained as a solid and purified by column chromatography using hexane-ethyl acetate (3:1) as eluent (205 mg, 75% yield), mp 127–129 ºC. MS (FAB): *m/z* 388 (100%) $[M+Na]^+$. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.15 (s, 1H), 7.16 (d, J = 9.0 Hz, 2H), 6.89 (d, J = 9.0 Hz, 2H), 5.05 (br s, 1H), 4.48–4.38 (m, 1H), 3.95–3.80 (m, 1H), 3.76 (s, 3H), 3.28–2.92 (m, 3H), 1.44 (s, 9H), 1,16 (d, J = 6.8 Hz, 3H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 182.0, 156.6, 154.3, 133.8, 127.6, 113.2, 79.1, 55.2, 50.8, 42.1, 28.0, 14.8. HRMS (*m/z*): calcd for $C_{18}H_{27}FN_3O_3SNa$ 388.1665 $[M+Na]^+$; found 388.1669.

4-*tert*-Butoxycarbonyl-2-methyl-1-[(4-methylphenyl)aminothiocarbonyl]piperazine (25). The product was obtained as a solid and purified by column chromatography using hexane-ethyl acetate (6:1) as eluent (149 mg, 75% yield), mp 155–157 ºC. MS (FAB): *m/z* 372 (100%) $[M+Na]^+$. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.18 (s, 1H), 7.15 (d, J =

8.4 Hz, 2H), 7.11 (d, *J* = 8.4 Hz, 2H), 5.06 (br s, 1H), 4.46–4.37 (m, 1H), 3.95–3.81 (m, 1H), 3.79–3.72 (m, 1H), 3.21–2.88 (m, 2H), 2.29 (s, 3H), 1.44 (s, 9H), 1,16 (d, *J* = 6.6 Hz, 3H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 181.9, 154.2, 138.4, 133.7, 128.5, 125.8, 79.1, 50.9, 42.2, 28.0, 20.5, 15.1. HRMS (*m/z*): calcd for $C_{18}H_{27}N_3O_2SNa$ 372.1716 $[M+Na]^+$; found 372.1710.

1-[{3,5-Bis(trifluoromethyl)phenyl}aminothiocarbonyl]-4-*tert*-butoxycarbonyl-2-methylpiperazine (26). The product was obtained as a solid and purified by column chromatography using hexane-ethyl acetate (7:1) as eluent (251 mg, 71% yield), mp 134–136 ºC. MS (FAB): *m/z* 494 (100%) $[M+Na]^+$. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.72 (s, 1H), 8.09 (s, 2H), 7.78 (s, 1H), 5.10 (br s, 1H), 4.55–4.42 (m, 1H), 4.04–3.74 (m, 2H), 3.26–2.91 (s, 2H), 1.45 (s, 9H), 1,21 (d, *J* = 6.7 Hz, 3H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 181.0, 154.2, 143.1, 130.1, 129.8, 129.5, 129.3, 126.5, 124.8, 124.4, 122.2, 116.7, 79.2, 51.5, 42.7, 28.0, 14.9. HRMS (*m/z*): calcd for $C_{19}H_{23}F_6N_3O_2SNa$ 494.1307 $[M+Na]^+$; found 494.1300. Anal. Calcd $C_{19}H_{23}F_6N_3O_2S$: C, 48.40; H, 4.92; N, 8.91. Found: C, 48.56; H, 4.64; N, 8.88.

4-(3,3-Dimethylbutanoyl)-2-methyl-1-[(4-nitrophenyl)aminothiocarbonyl]piperazine (27). The product was obtained as a solid and purified by column chromatography using hexane-ethyl acetate (1:1) as eluent (193 mg, 68% yield), mp 195–198 ºC. MS (FAB): *m/z* 401 (100%) $[M+Na]^+$. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.76 (s, 1H), 8.18 (d, *J* = 8.8 Hz, 2H), 7.61 (d, *J* = 8.8 Hz, 2H), 5.12 (br s, 1H), 4.48–4.43 (m, 1H), 4.27 (d, *J* = 13.5 Hz, 1H), 4.08–3.88 (m, 1H), 3.02–2.85 (m, 1H), 2.42–2.08 (m, 2H), 1,22, 1,15 (2d, *J* = 6.7 Hz, 3H), 1.03, 1.02 (2s, 9H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 181.5, 170.4, 170.3, 147.8, 142.2, 123.9, 123.0, 52.1, 51.8, 49.1, 45.1, 44.4, 43.6, 43.4, 43.1, 40.7, 31.0, 30.9, 29.7, 15.1, 14.7. HRMS (*m/z*):

calcd for $C_{18}H_{26}N_4O_3SNa$ 401.1618 $[M+Na]^+$; found 401.1615. Anal. Calcd $C_{18}H_{26}N_4O_3S$: C, 57.12; H, 6.92; N, 14.80. Found: C, 57.47; H, 7.07; N, 14.56.

1-[(4-Chlorophenyl)aminothiocarbonyl]-4-(3,3-dimethylbutanoyl)-2-methylpiperazine (28). The product was obtained as a solid and purified by column chromatography using hexane-ethyl acetate (2:1) as eluent (193 mg, 70% yield), mp 77–80 ºC. MS (FAB): *m/z* 390 (100%) $[M+Na]^+$. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.76 (s, 1H), 8.18 (d, J = 8.8 Hz, 2H), 7.61 (d, J = 8.8 Hz, 2H), 5.12 (br s, 1H), 4.48–4.43 (m, 1H), 4.27 (d, J = 13.5 Hz, 1H), 4.08–3.88 (m, 1H), 3.02–2.85 (m, 1H), 2.42–2.08 (m, 2H), 1,22, 1,15 (2d, J = 6.7 Hz, 3H), 1.03, 1.02 (2s, 9H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 181.6, 181.4, 170.4, 170.2, 128.4, 127.8, 127.2, 51.5, 51.2, 49.1, 45.6, 44.4, 43.6, 43.2, 43.0, 42.6, 40.7, 31.0, 30.9, 29.7, 15.1, 14.7. HRMS (*m/z*): calcd for $C_{18}H_{26}ClN_3OSNa$ 390.1377 $[M+Na]^+$; found 390.1373. Anal. Calcd $C_{18}H_{26}ClN_3OS$: C, 58.76; H, 7.12; N, 11.42. Found: C, 58.45; H, 7.03; N, 11.12.

1-[(4-Cyanophenyl)aminothiocarbonyl]-4-(3,3-dimethylbutanoyl)-2-methylpiperazine (29). The product was obtained as a solid and purified by column chromatography using hexane-ethyl acetate (1:1) as eluent (177 mg, 66% yield), mp 158–160 ºC. MS (FAB): *m/z* 381 (100%) $[M+Na]^+$. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.59 (s, 1H), 7.76–7.72 (m, 2H), 7.58–7.53 (m, 2H), 5.10 (br s, 1H), 4.46–4.33 (m, 1H), 4.26 (d, J = 13.3 Hz, 1H), 4.04–3.87 (m, 1H), 3.02–2.87 (m, 1H), 2.42–2.08 (m, 2H), 1,22, 1,15 (2d, J = 6.2 Hz, 3H), 1.03, 1.02 (2s, 9H). ^{13}C NMR (125 MHz, DMSO-d_6) δ, 170.0, 145.6, 132.2, 123.9, 119.1, 105.3, 51.9, 51.6, 49.1, 45.1, 44.6, 43.6, 43.4, 42.9, 40.7, 31.0, 30.9, 29.7, 15.2, 14.8. HRMS (*m/z*): calcd for $C_{19}H_{26}N_4OSNa$ 381.1720 $[M+Na]^+$; found 381.1714. Anal. Calcd $C_{19}H_{26}N_4OS$: C, 63.65; H, 7.31; N, 15.63. Found: C, 63.28; H, 7.35; N, 15.24.

1-[(4-Fluorophenyl)aminothiocarbonyl]-4-(3,3-dimethylbutanoyl)-2-methylpiperazine (30). The product was obtained as a solid and purified by column chromatography using hexane-ethyl acetate (1:1) as eluent (158 mg, 60% yield), mp 76–80 ºC. MS (FAB): *m/z* 374 (100%) $[M+Na]^+$. ^1H NMR (500 MHz, DMSO-d_6) δ 9.26 (br s, 1H), 7.33–7.26 (m, 2H), 7.18–7.11 (m, 2H), 5.06 (br s, 1H), 4.51–4.38 (m, 1H), 4.22 (d, J = 13.5 Hz, 1H), 4.05–3.84 (m, 1H), 3.06–2.88 (m, 1H), 2.43–2.09 (m, 2H), 1,20, 1,14 (2d, J = 6.6 Hz, 3H), 1.04, 1.02 (2s, 9H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 181.9, 170.4, 170.2, 160.3, 158.3, 137.3, 128.0, 127.9, 114.7, 114.5, 51.4, 51.1, 49.2, 45.1, 44.4, 43.6, 43.4, 43.0, 42.3, 40.7, 31.0, 30.9, 29.7, 15.2, 14.8. HRMS (*m/z*): calcd. for $C_{18}H_{26}FN_3OSNa$ 374.1673 $[M+Na]^+$; found 374.1670. Anal. Calcd $C_{18}H_{26}FN_3OS$: C, 61.51; H, 7.46; N, 11.96. Found: C, 61.30; H, 7.29; N, 11.69.

4-(3,3-Dimethylbutanoyl)-2-methyl-1-[(4-trifluoromethylphenyl)aminothiocarbonyl]piperazine (31). The product was obtained as a solid and purified by column chromatography using hexane-ethyl acetate (2:1) as eluent (220 mg, 73% yield), mp 79–82 ºC. MS (FAB): *m/z* 424 (100%) $[M+Na]^+$. ^1H NMR (500 MHz, DMSO-d_6) δ 9.53 (s, 1H), 7.66 (d, J = 8.6 Hz, 2H), 7.56 (d, J = 8.4 Hz, 2H), 5.12 (br s, 1H), 4.49–4.36 (m, 1H), 4.25 (d, J = 13.3 Hz, 1H), 4.05–3.87 (m, 1H), 3.05–2.86 (m, 1H), 2.45–2.08 (m, 2H), 1,22, 1,16 (2d, J = 6.7 Hz, 3H), 1.04, 1.03 (2s, 9H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 181.6, 181.4, 170.4, 170.2, 144.9, 125.5, 125.0, 124.6, 124.5, 124.0, 123.8, 123.3, 51.8, 51.5, 49.1, 45.1, 44.4, 43.6, 43.4, 43.3, 42.8, 40.7, 31.0, 30.9, 29.7, 15.2, 14.8. HRMS (*m/z*): calcd for $C_{19}H_{26}F_3N_3OSNa$ 424.1641 $[M+Na]^+$; found 424.1631. Anal. Calcd $C_{19}H_{26}F_3N_3OS$: C, 56.84; H, 6.53; N, 10.47. Found: C, 56.86; H, 6.33; N, 10.12.

4-(3,3-Dimethylbutanoyl)-1-[(4-methoxyphenyl)aminothiocarbonyl]-2-methylpiperazine (32). The product was obtained as a solid and purified by column

chromatography using hexane-ethyl acetate (1:1) as eluent (169 mg, 62% yield), mp 138–140 ºC. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.15 (s, 1H), 7.16 (d, J = 8.5 Hz, 2H), 6.88 (d, J = 8.8 Hz, 2H), 5.05 (br s, 1H), 4.53–4.34 (m, 1H), 4.20 (d, J = 12.9 Hz, 1H), 4.01–3.83 (m, 1H), 3.76 (s, 3H), 3.04–2.87 (m, 1H), 2.41–2.08 (m, 2H), 1,19, 1,13 (2d, J = 6.4 Hz, 3H), 1.03, 1.02 (2s, 9H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 182.0, 181.9, 170.4, 170.2, 156.7, 133.8, 127.6, 113.2, 55.2, 51.2, 50.9, 49.2, 45.1, 44.4, 43.6, 43.4, 42.8, 42.2, 40.7, 31.0, 30.9, 29.7, 26.8, 15.2, 14.8. HRMS (*m/z*): calcd for $C_{19}H_{29}N_3O_2SNa$ 386.1873 $[M+Na]^+$; found 386.1870. Anal. Calcd $C_{19}H_{29}N_3O_2S$: C, 62.78; H, 8.04; N, 11.56. Found: C, 62.50; H, 7.81; N, 11.25.

4-(3,3-Dimethylbutanoyl)-2-methyl-1-[(4-methylphenyl)aminothiocarbonyl]piperazine (33). The product was obtained as a solid and purified by column chromatography using hexane-ethyl acetate (3:2) as eluent (197 mg, 76% yield), mp 136–138 ºC. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.19, 9.18 (ds, 1H), 7.16 (d, J = 8.2 Hz, 2H), 7.11 (d, J = 8.2 Hz, 2H), 5.06 (bs, 1H), 4.50–4.34 (m, 1H), 4.21 (d, J = 13.0 Hz, 1H), 4.02–3.82 (m, 1H), 3.04–2.87 (m, 1H), 2.29 (s, 3H), 2.41–2.10 (m, 2H), 1.19, 1.13 (2d, J = 6.4 Hz, 3H), 1.03, 1.02 (2s, 9H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 181.8, 181.6, 170.4, 170.3, 138.3, 133.7, 128.5, 125.8, 51.3, 51.0, 49.2, 48.7, 45.1, 44.4, 43.6, 43.4, 42.9, 42.3, 40.7, 31.0, 30.9, 29.7, 26.8, 20.5, 15.1, 14.8. HRMS (*m/z*): calcd for $C_{19}H_{29}N_3OSNa$ 370.1924 $[M+Na]^+$; found 370.1920. Anal. Calcd $C_{19}H_{29}N_3OS$: C, 65.67; H, 8.41; N, 12.09. Found: C, 65.38; H, 8.25; N, 11.99.

1-[{3,5-Bis(trifluoromethyl)phenyl}aminothiocarbonyl]-4-(3,3-dimethylbutanoyl)-2-methylpiperazine (34). The product was obtained as a solid and purified by column chromatography using hexane-ethyl acetate (2:1) as eluent (239 mg, 68% yield), mp 87–90 ºC. MS (FAB): *m/z* 492 (100%) $[M+Na]^+$. ^{1}H NMR (500 MHz, DMSO-d_6) δ

9.71 (s, 1H), 8.10 (br s, 2H), 7.77 (br s, 1H), 5.12 (br s, 1H), 4.59–4.42 (m, 1H), 4.26 (d, J = 13.3 Hz, 1H), 4.08–3.87 (m, 1H), 3.07–2.92 (m, 1H), 2.45–2.11 (m, 2H), 1.24, 1.18 (2d, J = 6.4 Hz, 3H), 1.04, 1.03 (2s, 9H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 181.0, 180.8, 170.4, 170.3, 143.1, 130.1, 129.9, 129.5, 129.3, 124.8, 124.4, 122.2, 116.7, 51.8, 51.6, 49.1, 45.0, 44.4, 43.6, 43.4, 43.2, 40.6, 31.0, 30.9, 29.7, 15.2, 14.8. HRMS (*m/z*): calcd for $C_{20}H_{25}F_6N_3OSNa$ 492.1515 $[M+Na]^+$; found 492.1500. Anal. Calcd $C_{20}H_{25}F_6N_3OS$: C, 51.17; H, 5.37; N, 8.95. Found: C, 51.10; H, 5.17; N, 8.83.

4-(2-Cyclohexylacetyl)-2-methyl-1-[(4-nitrophenyl)aminothiocarbonyl]piperazine (35). The product was obtained as a solid and purified by column chromatography using hexane-ethyl acetate (2:3) as eluent (230 mg, 76% yield), mp 87–90 ºC. MS (FAB): *m/z* 427 (100%) $[M+Na]^+$. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.77 (s, 1H), 8.18 (d, J = 9.1 Hz, 2H), 7.61 (d, J = 9.1 Hz, 2H), 5.12 (br s, 1H), 4.46–4.34 (m, 1H), 4.30–4.20 (m, 1H), 4.08–3.81 (m, 1H), 3.03–2.85 (m, 1H), 2.37–2.14 (m, 2H), 1.79–1.58 (m, 6H), 1.31–0.90 (m, 9H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 181.2, 181.1, 170.7, 147.8, 142.2, 123.9, 122.9, 59.7, 51.9, 51.8, 48.5, 44.5, 44.4, 43.7, 43.1, 34.4, 34.3, 32.6, 32.5, 25.9, 25.7, 15.1, 14.6. HRMS (*m/z*): calcd for $C_{20}H_{28}N_4O_3SNa$ 427.1774 $[M+Na]^+$; found 427.1768. Anal. Calcd $C_{20}H_{28}N_4O_3S$: C, 59.38; H, 6.98; N, 13.85. Found: C, 59.53; H, 7.09; N, 13.67.

1-[(4-Chlorophenyl)aminothiocarbonyl]-4-(2-cyclohexylacetyl)-2-methylpiperazine (36). The product was obtained as a solid and purified by column chromatography using hexane-ethyl acetate (3:2) as eluent (221 mg, 75% yield), mp 77–80 ºC. MS (FAB): *m/z* 416 (100%) $[M+Na]^+$. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.32 (s, 1H), 7.38–7.31 (m, 4H), 5.09 (br s, 1H), 4.50–4.36 (m, 1H), 4.25–4.16 (m, 1H), 3.95–3.78 (m, 1H), 3.05–2.85 (m, 1H), 2.36–2.15 (m, 2H), 1.81–1.56 (m, 6H), 1.30–0.88 (m, 9H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 181.7, 181.5, 170.7, 140.0, 128.4, 127.8, 127.1, 51.4, 51.2, 44.5,

44.4, 43.1, 42.4, 40.7, 34.4, 34.3, 32.6, 32.5, 25.8, 25.7, 15.1, 14.7. HRMS (*m/z*): calcd for $C_{20}H_{28}ClN_3OSNa$ 416.1534 $[M+Na]^+$; found 416.1527. Anal. Calcd $C_{20}H_{28}ClN_3OS$: C, 60.97; H, 7.16; N, 10.67. Found: C, 60.66; H, 6.97; N, 10.28.

1-[(4-Cyanophenyl)aminothiocarbonyl]-4-(2-cyclohexylacetyl)-2-methylpiperazine (37). The product was obtained as a solid and purified by column chromatography using hexane-ethyl acetate (1:1) as eluent (210 mg, 73% yield), mp 84–87 ºC. MS (FAB): *m/z* 407 (100%) $[M+Na]^+$. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.59 (s, 1H), 7.74 (d, J = 8.4 Hz, 2H), 7.55 (d, J = 8.4 Hz, 2H), 5.11 (br s, 1H), 4.47–4.33 (m, 1H), 4.29–4.18 (m, 1H), 3.98–3.79 (m, 1H), 3.04–2.83 (m, 1H), 2.38–2.15 (m, 2H), 1.82–1.57 (m, 6H), 1.32–0.90 (m, 9H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 181.3, 181.2, 170.7, 145.6, 132.2, 119.1, 105.2, 51.7, 51.6, 48.5, 44.5, 44.4, 43.5, 42.9, 40.7, 34.4, 34.3, 32.6, 32.5, 25.9, 25.7, 15.1, 14.6. HRMS (*m/z*): calcd for $C_{21}H_{28}N_4OSNa$ 407.1876 $[M+Na]^+$; found 407.1866. Anal. Calcd $C_{21}H_{28}N_4OS$: C, 65.59; H, 7.34; N, 14.57. Found: C, 65.52; H, 7.09; N, 14.15.

4-(2-Cyclohexylacetyl)-1-[(4-fluorophenyl)aminothiocarbonyl]-2-methylpiperazine (38). The product was obtained as a solid and purified by column chromatography using hexane-ethyl acetate (1:1) as eluent (218 mg, 77% yield), mp 87–90 ºC. MS (FAB): *m/z* 400 (100%) $[M+Na]^+$. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.23 (s, 1H), 7.33–7.26 (m, 2H), 7.18–7.10 (m, 2H), 5.06 (br s, 1H), 4.52–4.37 (m, 1H), 4.25–4.13 (m, 1H), 3.97–3.75 (m, 1H), 3.06–2.85 (m, 1H), 2.40–2.11 (m, 2H), 1.83–1.53 (m, 6H), 1.33–0.89 (m, 9H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 181.9, 181.8, 170.7, 160.3, 158.3, 137.2, 128.0, 127.9, 114.7, 114.4, 51.3, 51.1, 48.5, 44.5, 44.4, 42.9, 42.2, 40.7, 34.4, 34.3, 32.7, 32.6, 32.5, 25.9, 25.7, 15.1, 14.7. HRMS (*m/z*): calcd for $C_{20}H_{28}FN_3OSNa$ 400.1829

[M+Na]$^+$; found 400.1826. Anal. Calcd $C_{20}H_{28}FN_3OS$: C, 63.63; H, 7.48; N, 11.13. Found: C, 63.38; H, 7.17; N, 10.77.

4-(2-Cyclohexylacetyl)-2-methyl-1-[(4-trifluoromethylphenyl)aminothiocarbonyl] piperazine (39). The product was obtained as a solid and purified by column chromatography using hexane-ethyl acetate (1:1) as eluent (218 mg, 68% yield), mp 153–156 ºC. MS (FAB): *m/z* 450 (100%) [M+Na]$^+$. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.53 (s, 1H), 7.65 (d, J = 8.6 Hz, 2H), 7.55 (d, J = 8.4 Hz, 2H), 5.11 (br s, 1H), 4.50–4.35 (m, 1H), 4.28–4.17 (m, 1H), 3.99–3.78 (m, 1H), 3.05–2.84 (m, 1H), 2.39–2.14 (m, 2H), 1.81–1.56 (m, 6H), 1.34–0.89 (m, 9H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 181.6, 181.5, 170.7, 144.9, 125.1, 125.0, 124.5, 124.0, 123.8, 123.5, 123.3, 51.6, 51.5, 48.5, 44.5, 44.4, 43.4 42.7, 40.7, 34.4, 34.3, 32.7, 32.6, 32.5, 25.9, 25.7, 15.1, 14.7. HRMS (*m/z*): calcd for $C_{21}H_{28}F_3N_3OSNa$ 450.1797 [M+Na]$^+$; found 450.1788. Anal. Calcd $C_{21}H_{28}F_3N_3OS$: C, 59.00; H, 6.60; N, 9.83. Found: C, 59.27; H, 6.35; N, 9.60.

4-(2-Cyclohexylacetyl)-1-[(4-methoxyphenyl)aminothiocarbonyl]-2-methylpiperazine (40). The product was obtained as a solid and purified by column chromatography using hexane-ethyl acetate (3:2) as eluent (204 mg, 70% yield), mp 65–68 ºC. MS (FAB): *m/z* 412 (100%) [M+Na]$^+$. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.14 (s, 1H), 7.17 (d, J = 8.6 Hz, 2H), 6.88 (d, J = 8.6 Hz, 2H), 5.16–4.97 (m, 1H), 4.57–4.36 (m, 1H), 4.29–4.14 (m, 1H), 3.95–3.78 (m, 1H), 3.75 (s, 3H), 3.07–2.83 (m, 1H), 2.37–2.17 (m, 2H), 1.81–1.56 (m, 6H), 1.32–0.89 (m, 9H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 181.9, 181.8, 170.6, 156.6, 133.8, 127.7, 113.2, 55.2, 51.1, 50.9, 48.5, 44.5, 44.4, 42.8, 42.2, 40.7, 34.4, 34.3, 32.7, 32.6, 32.5, 25.8, 25.7, 15.1, 14.7. HRMS (*m/z*): calcd for $C_{21}H_{31}N_3O_2SNa$ 412.2029 [M+Na]$^+$; found 412.2025. Anal. Calcd $C_{21}H_{31}N_3O_2S$: C, 64.75; H, 8.02; N, 10.79. Found: C, 64.65; H, 7.91; N, 10.61.

4-(2-Cyclohexylacetyl)-2-methyl-1-[(4-methylphenyl)aminothiocarbonyl]piperazine (41). The product was obtained as a solid and purified by column chromatography using hexane-ethyl acetate (3:2) as eluent (185 mg, 66% yield), mp 72–75 ºC. MS (FAB): *m/z* 396 (100%) $[M+Na]^+$. ^1H NMR (500 MHz, DMSO-d_6) δ 9.19 (s, 1H), 7.19–7.08 (m, 4H), 5.18–4.97 (m, 1H), 4.55–4.33 (m, 1H), 4.28–4.09 (m, 1H), 3.98–3.72 (m, 1H), 3.06–2.82 (m, 1H), 2.30 (s, 3H), 2.26–2.14 (m, 2H), 1.82–1.54 (m, 6H), 1.34–0.82 (m, 9H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 181.9, 181.7, 170.7, 138.3, 133.7, 128.5, 125.8, 51.2, 51.1, 48.6, 44.5, 44.4, 42.9, 42.3, 40.8, 34.4, 34.3, 32.7, 32.6, 32.5, 25.8, 25.7, 20.5, 15.1, 14.7. HRMS (*m/z*): calcd for $C_{21}H_{31}N_3OSNa$ 396.2080 $[M+Na]^+$; found 396.2075. Anal. Calcd $C_{21}H_{31}N_3OS$: C, 67.52; H, 8.36; N, 11.25. Found: C, 67.24; H, 8.16; N, 10.95.

1-[{3,5-Bis(trifluoromethyl)phenyl}aminothiocarbonyl]-4-(2-cyclohexylacetyl)-2-methylpiperazine (42). The product was obtained as a solid and purified by column chromatography using hexane-ethyl acetate (2:1) as eluent (290 mg, 78% yield), mp 156–159 ºC. MS (FAB): *m/z* 518 (90%) $[M+Na]^+$. ^1H NMR (500 MHz, DMSO-d_6) δ 9.71 (s, 1H), 8.09 (s, 2H), 7.77 (s, 1H), 5.12 (br s, 1H), 4.56–4.39 (m, 1H), 4.31–4.15 (m, 1H), 4.01–3.78 (m, 1H), 3.10–2.87 (m, 1H), 2.39–2.17 (m, 2H), 1.82–1.56 (m, 6H), 1.27–0.91 (m, 9H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 181.0, 180.9, 170.7, 143.1, 130.1, 129.8, 129.5, 129.3, 126.5, 124.7 124.4, 122.2. 120.0, 116.7, 51.7, 51.6, 48.4, 44.4, 44.3, 43.3, 42.7, 40.7, 34.4, 34.3, 32.7, 32.6, 32.5, 25.9, 25.7, 15.1, 14.7. HRMS (*m/z*): calcd for $C_{22}H_{27}F_6N_3OSNa$ 518.1671 $[M+Na]^+$; found 518.1654. Anal. Calcd $C_{22}H_{27}F_6N_3OS$: C, 53.32; H, 5.49; N, 8.48. Found: C, 53.61; H, 5.34; N, 8.40.

2-Methyl-1-[(4-nitrophenyl)aminothiocarbonyl]-4-(2-phenylacetyl)piperazine (43). The product was obtained as a solid and purified by column chromatography using

hexane-ethyl acetate (2:3) as eluent (170 mg, 57% yield), mp 81–84 ºC. MS (FAB): *m/z* 421 (100%) $[M+Na]^+$. ^1H NMR (500 MHz, DMSO-d_6) δ 9.76, 9.74 (2s, 1H), 8.23–8.14 (m, 2H), 7.66–7.56 (m, 2H), 7.40–7.20 (m, 5H), 5.13 (br s, 1H), 4.47–4.32 (m, 1H), 4.29–4.18 (m, 1H), 4.08–3.91 (m, 1H), 3.89–3.70 (m, 2H), 3.26–3.14 (m, 1H), 3.08–2.86 (m, 1H), 1.15–1.09 (m, 3H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 181.2, 181.1, 169.7, 147.8, 142.2, 135.7, 135.6, 129.1, 128.9, 128.4, 128.3, 126.4, 123.9, 122.9, 51.9, 51.8, 48.7, 44.7, 43.4, 42.9, 40.9, 15.0, 14.4. HRMS (*m/z*): calcd for $C_{20}H_{22}N_4O_3SNa$ 421.1305 $[M+Na]^+$; found 421.1298. Anal. Calcd $C_{20}H_{22}N_4O_3S$: C, 60.28; H, 5.56; N, 14.06. Found: C, 59.99; H, 5.70; N, 13.82.

1-[(4-Chlorophenyl)aminothiocarbonyl]-2-methyl-4-(2-phenylacetyl)piperazine (44). The product was obtained as a solid and purified by column chromatography using hexane-ethyl acetate (2:3) as eluent (177mg, 61% yield), mp 168–171 ºC. MS (FAB): *m/z* 410 (100%) $[M+Na]^+$. ^1H NMR (500 MHz, DMSO-d_6) δ 9.31, 9.30 (2s, 1H), 7.43–7.17 (m, 9H), 5.09 (br s, 1H), 4.49–4.33 (m, 1H), 4.27–4.15 (m, 1H), 4.04–3.86 (m, 1H), 3.84–3.71 (m, 2H), 3.28–3.12 (m, 1H), 3.07–2.88 (m, 1H), 1.09 (d, J = 6.9 Hz. 3H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 181.2, 181.1, 169.7, 169.6, 140.0, 135.8, 135.6, 129.1, 128.9, 128.4, 128.3, 128.2, 127.8, 127.1, 126.4, 51.3, 48.7, 44.7, 42.8, 42.3, 40.9, 15.0, 14.5. HRMS (*m/z*): calcd for $C_{20}H_{22}ClN_3OSNa$ 410.1064 $[M+Na]^+$; found 410.1059. Anal. Calcd $C_{20}H_{22}ClN_3OS$: C, 61.92; H, 5.72; N, 10.83. Found: C, 61.53; H, 5.69; N, 10.60.

1-[(4-Cyanophenyl)aminothiocarbonyl]-2-methyl-4-(2-phenylacetyl)piperazine (45). The product was obtained as a solid and purified by column chromatography using hexane-ethyl acetate (1:2) as eluent (190 mg, 67% yield), mp 79–82 ºC. MS (FAB): *m/z* 401 (100%) $[M+Na]^+$. ^1H NMR (500 MHz, DMSO-d_6) δ 9.59, 9.57 (2s, 1H), 7.79–7.72

(m, 2H), 7.61–7.53 (m, 2H), 7.40–7.24 (m, 5H), 5.14 (br s, 1H), 4.47–4.33 (m, 1H), 4.31–4.22 (m, 1H), 4.09–3.71 (m, 4H), 3.32–3.15 (m, 1H), 3.08–2.89 (m, 1H), 1.12 (d, J = 6.6 Hz. 3H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 181.3, 181.2, 169.7, 169.6, 145.6, 135.7, 135.6, 132.2, 129.1, 128.9, 128.4, 128.3, 126.5, 126.4, 123.9, 119.1, 105.2, 51.7, 51.6, 48.7, 44.7, 43.3, 42.8, 40.9, 15.0, 14.5. HRMS (*m/z*): calcd for $C_{21}H_{22}N_4OSNa$ 401.1407 $[M+Na]^+$; found 401.1402. Anal. Calcd $C_{21}H_{22}N_4OS$: C, 66.64; H, 5.86; N, 14.80. Found: C, 66.53; H, 5.88; N, 14.65.

1-[(4-Fluorophenyl)aminothiocarbonyl]-2-methyl-4-(2-phenylacetyl)piperazine (46). The product was obtained as a solid and purified by column chromatography using hexane-ethyl acetate (2:3) as eluent (156 mg, 56% yield), mp 132–136 ºC. MS (FAB): *m/z* 394 (100%) $[M+Na]^+$. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.25, 9.24 (2s, 1H), 7.37–7.10 (m, 9H), 5.07 (br s, 1H), 4.49–4.37 (m, 1H), 4.26–4.16 (m, 1H), 4.04–3.69 (m, 3H), 3.30–3.10 (m, 1H), 3.07–2.89 (m, 1H), 1.09 (d, J = 6.6 Hz. 3H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 181.9, 181.8, 169.7, 169.6, 160.2, 158.3, 137.3, 137.2, 135.8, 135.7, 129.1, 128.9, 128.3, 128.2, 127.9, 127.8, 126.4, 126.3, 114.7, 114.5, 51.1, 48.7, 44.7, 42.7, 42.2, 40.9, 15.0, 14.5. HRMS (*m/z*): calcd for $C_{20}H_{22}FN_3OSNa$ 394.1360 $[M+Na]^+$; found 394.1355. Anal. Calcd $C_{20}H_{22}FN_3OS$: C, 64.67; H, 5.97; N, 11.31. Found: C, 64.45; H, 5.77; N, 10.95.

2-Methyl-4-(2-phenylacetyl)-1-[(4-trifluoromethylphenyl)aminothiocarbonyl]piperazine (47). The product was obtained as a solid and purified by column chromatography using hexane-ethyl acetate (2:3) as eluent (221 mg, 70% yield), mp 161–164 ºC. MS (FAB): *m/z* 444 (100%) $[M+Na]^+$. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.52, 9.51 (2s, 1H), 7.68-7.61 (m, 2H), 7.59–7.51 (m, 2H), 7.39–7.22 (m, 5H), 5.12 (br s, 1H), 4.47–4.33 (m, 1H), 4.29–4.18 (m, 1H), 4.06–3.90 (m, 1H), 3.89–3.69 (m, 2H), 3.28–3.13 (m, 1H), 3.06–2.89 (m, 1H),

1.11 (d, J = 6.4 Hz. 3H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 181.6, 181.5, 169.7, 169.6, 144.9, 135.8, 135.7, 129.1, 128.9, 128.4, 128.3, 126.4, 125.1, 125.0, 124.5, 51.5, 48.7, 44.7, 43.1, 42.7, 42.2, 40.9, 26.82, 15.0, 14.5. HRMS (*m/z*): calcd for $C_{21}H_{22}F_3N_3OSNa$ 444.1328 $[M+Na]^+$; found 444.1316. Anal. Calcd $C_{21}H_{22}F_3N_3OS$: C, 59.84; H, 5.26; N, 9.97. Found: C, 59.56; H, 5.46; N, 9.68.

1-[(4-Methoxyphenyl)aminothiocarbonyl]-2-methyl-4-(2-phenylacetyl)piperazine (48). The product was obtained as a solid and purified by column chromatography using hexane-ethyl acetate (2:3) as eluent (158 mg, 55% yield), mp 70–73 ºC. MS (FAB): *m/z* 406 (100%) $[M+Na]^+$. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.14, 9.13 (2s, 1H), 7.38–7.22 (m, 5H), 7.19–7.13 (m, 2H), 6.88 (d, J = 8.0 Hz, 2H), 5.06 (br s, 1H), 4.48–4.36 (m, 1H), 4.26–4.13 (m, 1H), 4.02–3.78 (m, 2H), 3.76 (s, 3H), 3.30–3.09 (m, 2H), 3.06–2.89 (m, 1H), 1.08 (d, J = 6.6 Hz. 3H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 181.9, 181.8, 169.7, 169.6, 156.6, 135.8, 135.7, 133.8, 129.1, 128.9, 128.3, 128.2, 127.6, 126.4, 113.2, 55.2 50.9, 48.7, 44.7, 42.6, 42.0, 40.7, 15.0, 14.5. HRMS (*m/z*): calcd for $C_{21}H_{25}N_3O_2SNa$ 406.1560 $[M+Na]^+$; found 406.1554. Anal. Calcd $C_{21}H_{25}N_3O_2S$: C, 65.77; H, 6.57; N, 10.96. Found: C, 65.52; H, 6.23; N, 10.76.

2-Methyl-1-[(4-methylphenyl)aminothiocarbonyl]-4-(2-phenylacetyl)piperazine (49). The product was obtained as a solid and purified by column chromatography using hexane-ethyl acetate (1:1) as eluent (171mg, 62% yield), mp 66–70 ºC. MS (FAB): *m/z* 390 (100%) $[M+Na]^+$. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.18, 9.17 (2s, 1H), 7.39–7.22 (m, 5H), 7.19–7.08 (m, 4H), 5.08 (br s, 1H), 4.45–4.33 (m, 1H), 4.25–4.15 (m, 1H), 4.04–3.85 (m 1H), 3.83–3.68 (m, 2H), 3.30–3.07 (m, 2H), 3.05–2.87 (m, 1H), 2.29 (s, 3H), 1.08 (d, J = 6.6 Hz. 3H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 181.8, 181.7, 169.7, 169.6, 138.3, 135.8, 135.7, 133.7, 129.1, 128.9, 128.4, 128.3, 128.2, 126.4, 125.7, 51.0,

48.7, 44.7, 42.7, 42.2, 40.9, 20.5, 15.0, 14.5. HRMS (*m/z*): calcd for $C_{21}H_{25}N_3OSNa$ 390.1611 $[M+Na]^+$; found 390.1605. Anal. Calcd $C_{21}H_{25}N_3OS$: C, 68.63; H, 6.86; N, 11.43. Found: C, 68.33; H, 6.69; N, 11.33.

1-[{3,5-Bis(trifluoromethyl)phenyl}aminothiocarbonyl]-2-methyl-4-(2-phenylacetyl)piperazine (50). The product was obtained as a solid and purified by column chromatography using hexane-ethyl acetate (3:2) as eluent (238 mg, 65% yield), mp 178–180 ºC. MS (FAB): *m/z* 512 (100%) $[M+Na]^+$. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.72, 9.69 (2s, 1H), 8.09, 8.07 (2s, 2H), 7.79 (s, 1H), 7.37–7.24 (m, 5H), 5.12 (br s, 1H), 4.52–4.38 (m, 1H), 4.24 (t, J = 12.5 Hz, 1H), 4.07–3.91 (m, 1H), 3.89–3.72 (m, 2H), 3.50–3.43 (m, 1H), 3.29–3.17 (m, 1H), 3.06–2.94 (m, 1H), 1.13, 1.12 (2d, J = 6.5 Hz. 3H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 180.9, 180.8, 169.8, 169.7, 143.0, 135.7, 135.6, 130.0, 129.8, 129.5, 129.3, 129.1, 128.9, 128.4, 128.3, 126.5, 126.4, 124.8, 124.4, 122.2, 116.8, 51.6, 48.6, 44.7, 43.0, 42.6, 40.9, 15.1, 14.6. HRMS (*m/z*): calcd for $C_{22}H_{21}F_6N_3OSNa$ 512.1202 $[M+Na]^+$; found 512.1188. Anal. Calcd $C_{22}H_{21}F_6N_3OS$: C, 53.98; H, 4.32; N, 8.58. Found: C, 53.90; H, 4.29; N, 8.67.

4-(Benzofuran-2-carbonyl)-2-methyl-1-[(4-nitrophenyl)aminothiocarbonyl]piperazine (56). The product was obtained as a solid and purified by column chromatography using hexane-ethyl acetate (1:1) as eluent (292 mg, 92% yield), mp 189–191 ºC. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.83 (s, 1H), 8.19 (d, J = 9.2 Hz, 2H), 7.79 (d, J = 7.7 Hz, 1H), 7.70 (d, J = 8.2 Hz, 1H), 7.64 (d, J = 9.1 Hz, 2H), 7.51–7.47 (m, 2H), 7.37 (t, J = 7.5 Hz, 1H), 5.23 (br s, 1H), 4.55–4.46 (m, 1H), 4.40–4.33 (m, 1H), 4.31–4.25 (m, 1H), 3.61–3.53 (m, 2H), 1.29 (t, J = 6.7 Hz, 3H). ^{13}C RMN (125 MHz, DMSO-d_6) δ 181.3, 159.7, 154.0, 148.0, 147.7, 142.3, 126.7, 123.9, 123.7, 122.9, 122.5, 111.8, 111.4, 51.9, 15.0 HRMS (*m/z*): calcd for

$C_{21}H_{20}N_4O_4SNa$ 447.1077 $[M+Na]^+$; found 447.1094. Anal. Calcd $C_{21}H_{20}N_4O_4S$: C, 59.42; H, 4.75; N, 13.20. Found: C, 59.50; H, 4.92; N, 13.12.

4-(Benzofuran-2-carbonyl)-1-[(4-cyanophenyl)aminothiocarbonyl]-2-methylpiperazine (57). The product was obtained as a solid and purified by column chromatography using hexane-ethyl acetate (1:1) as eluent (218 mg, 72% yield), mp 174–175 ºC. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.66 (s, 1H), 7.79 (d, J = 7.7 Hz, 1H), 7.75 (d, J = 8.3 Hz, 2H), 7.70 (d, J = 8.2 Hz, 1H), 7.59 (d, J = 8.4 Hz, 2H), 7.52–7.46 (m, 2H), 7.37 (t, J = 7.4 Hz, 1H), 5.23 (br s, 1H), 4.55–4.45 (m, 1H), 4.40–4.32 (m, 1H), 4.30–4.23 (m, 1H), 3.60–3.49 (m, 2H), 1.27 (t, J = 6.4 Hz, 3H). ^{13}C RMN (125 MHz, DMSO-d_6) δ 181.3, 159.7, 154.0, 147.9, 145.6, 132.2, 126.7, 123.9, 123.7, 122.5, 119.1, 111.8, 111.4, 105.3, 51.7, 15.1. HRMS (*m/z*): calcd for $C_{22}H_{20}N_4O_2SNa$ 427.1199 $[M+Na]^+$; found 427.1193. Anal. Calcd $C_{22}H_{20}N_4O_2S$: C, 65.33; H, 4.98; N, 13.85. Found: C, 65.42; H, 5.15; N, 13.91.

4-(Benzofuran-2-carbonyl)-1-[(4-fluorophenyl)aminothiocarbonyl]-2-methylpiperazine (58). The product was obtained as a solid and purified by column chromatography using hexane-ethyl acetate (2:1) as eluent (253 mg, 85% yield), mp 171–173 ºC. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.32 (s, 1H), 7.79 (d, J = 7.7 Hz, 1H), 7.70 (d, J = 8.3 Hz, 1H), 7.52–7.46 (m, 2H), 7.37 (t, J = 7.4 Hz, 1H), 7.34–7.29 (m, 2H), 7.15 (t, J = 8.8 Hz, 2H), 5.18 (br s, 1H), 4.58–4.49 (m, 1H), 4.37–4.29 (m, 1H), 4.26–4.19 (m, 1H), 3.57–3.48 (m, 2H), 1.26 (t, J = 6.6 Hz, 3H). ^{13}C RMN (125 MHz, DMSO-d_6) δ 181.9, 160.2, 159.6, 158.4, 154.0, 148.0, 137.2, 128.0, 127.9, 126.7, 123.7, 122.5, 114.7, 114.5, 111.8, 111.4, 51.2, 15.1. HRMS (*m/z*): calcd for $C_{21}H_{20}FN_3O_2SNa$ 420.1152 $[M+Na]^+$; found 420.1148. Anal. Calcd $C_{21}H_{20}FN_3O_2S$: C, 63.46; H, 5.07; N, 10.57. Found: C, 63.78; H, 5.20; N, 10.44.

4-(Benzofuran-2-carbonyl)-2-methyl-1-[(4-trifluoromethyl)aminothiocarbonyl]piperazine (59). The product was obtained as a solid and purified by column chromatography using hexane-ethyl acetate (2:1) as eluent (282 mg, 84% yield), mp 186–188 ºC. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.59 (s, 1H), 7.79 (d, J = 7.7 Hz, 1H), 7.71 (d, J = 8.2 Hz, 1H), 7.67 (d, J = 8.5 Hz, 2H), 7.58 (d, J = 8.2Hz, 2H), 7.52–7.46 (m, 2H), 7.37 (t, J = 7.4 Hz, 1H), 5.22 (br s, 1H), 4.52 (m, 1H), 4.39–4.32 (m, 1H), 4.29–4.23 (m, 1H), 3.60–3.51 (m, 2H), 1.28 (t, J = 6.6 Hz, 3H). ^{13}C RMN (125 MHz, DMSO-d_6) δ 181.6, 159.7, 154.0, 148.0, 144.9, 126.7, 125.0, 123.7, 122.5, 111.8, 111.3, 51.6, 15.1. HRMS (*m/z*): calcd for $C_{22}H_{20}F_3N_3O_2SNa$ 470.1121 $[M+Na]^+$; found 470.1115. Anal. Calcd $C_{22}H_{20}F_3N_3O_2S$: C, 59.05; H, 4.51; N, 9.39. Found: C, 59.20; H, 4.58; N, 9.26.

4-*tert*-Butoxycarbonyl-1-[(4-fluorophenyl)aminothiocarbonyl]-2-phenylpiperazine (60). The product was obtained as a solid and purified by column chromatography using hexane-ethyl acetate (4:1) as eluent (218 mg, 70% yield), mp 145–147 ºC. MS (FAB): *m/z* 438 (100%) $[M+Na]^+$. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.45 (s, 1H), 7.46–7.37 (m, 2H), 7.35–7.28 (m, 5H), 7.15 (t, J = 8.6 Hz, 2H), 6.33 (s, 1H), 4.51 (d, J = 12.7 Hz, 2H), 3.85–3.60 (m, 1H), 3.47 (d, J = 12.7 Hz, 1H), 3.26–3.00 (m, 1H), 1.38 (s, 9H), 1.28 (d, J = 6.8 Hz, 3H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 182.9, 160.3, 158.4, 137.2, 128.4, 128.0, 127.9, 127.0, 126.4, 114.7, 114.5, 79.2, 58.0, 45.5, 43.5, 42.1, 27.9. HRMS (*m/z*): calcd for $C_{22}H_{26}FN_3O_2SNa$ 438.1622 $[M+Na]^+$; found 438.1615. Anal. Calcd $C_{22}H_{26}FN_3O_2S$: C, 63.59; H, 6.31; N, 10.11; S, 7.72. Found: C, 63.42; H, 6.30; N, 9.89; S, 7.53.

1-[(3,5-Bis{trifluoromethyl}phenyl)aminothiocarbonyl]-4-(3,3-dimethylbutanoyl)-2-phenylpiperazine (61). The product was obtained as a solid and purified by column chromatography using dichloromethane-methanol (90:1) as eluent (383 mg, 96% yield).

mp 82-84 MS (FAB): *m/z* 554 (100%) $[M+Na]^+$. ^1H NMR (500 MHz, DMSO-d_6) δ 9.95, 9.84 (2s, 1H), 8.14, 8.08 (2s, 2H), 7.84 (br s, 1H), 7.44–7.25 (m, 5H), 6.42, 6.24 (2s, 1H), 5.02–4.95 (m, 1H), 4.61–4.66 (m, 1H), 3.93–3.73 (m, 1H), 2.40–1.96 (m, 2H), 0.96, 0.88 (2s, 9H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 181.9, 170.3, 170.0, 143.0, 142.9, 137.6, 128.5, 127.2, 126.3, 124.9, 124.4, 122.2,, 116.9, 59.4, 58.6, 48.0, 45.0, 44.3, 44.1, 43.7, 43.5, 42.1, 40.7, 31.0, 30.9, 29.5. HRMS (*m/z*): calcd. for $C_{25}H_{27}F_6N_3OSNa$ 554.1671 $[M+Na]^+$; found 554.1664. Anal. calcd for $C_{25}H_{27}F_6N_3OS$: C, 56.49; H, 5.12; N, 7.91. Found: C, 56.14; H, 4.89; N, 7.54.

1-[(4-Cyanophenyl)aminothiocarbonyl]-4-(2-cyclohexylacetyl)-2-phenylpiperazine (62). The product was obtained as a solid and purified by column chromatography using hexane-ethyl acetate (1:1) as eluent (217 mg, 65% yield), mp 179–181 ºC. MS (CI): *m/z* 447 (100%) $[M+H]^+$. ^1H NMR (500 MHz, DMSO-d_6) δ 9.79, 9.69 (2s, 1H), 7.78–7.71 (m, 2H), 7.63–7.55 (m, 2H), 7.41–7.25 (m, 5H), 6.36, 6.24 (2s, 1H), 4.89 (d, J = 13.5 Hz, 1H), 4.57–4.42 (m, 1H), 4.36 (dd, J = 2.8 Hz, J = 14.1 Hz, 1H), 3.89–3.75 (m, 2H), 3.46–3.39 (m, 1H), 2.23–2.02 (m, 2H), 1.67–1.46 (m, 5H), 1.22–0.74 (m, 6H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 182.1, 170.7, 170.6, 145.6, 145.4, 138.6, 137.8, 132.2, 128.5, 128.4, 127.1, 126.3, 124.3, 119.1, 105.5, 59.2, 58.6, 47.5, 44.4, 42.3, 41.0, 34.6, 34.1, 32.6, 32.5, 32.3, 25.8, 25.7, 25.6. HRMS (*m/z*): calcd for $C_{26}H_{31}N_4OS$ 447.2213 $[M+H]^+$; found 447.2206. Anal. Calcd $C_{26}H_{30}N_4OS$: C, 69.92; H, 6.77; N, 12.55. Found: C, 69.92; H, 6.92; N, 12.22.

4-(2-Cyclohexylacetyl)-1-[(4-fluorophenyl)aminothiocarbonyl]-2-phenylpiperazine (63). The product was obtained as a solid and purified by column chromatography using hexane-ethyl acetate (1:1) as eluent (198 mg, 60% yield), mp 158–160 ºC. MS (CI): *m/z* 440 (100%) $[M+H]^+$. ^1H NMR (500 MHz, DMSO-d_6) δ 9.45, 9.33 (2s, 1H), 7.42–7.24

(m, 7H), 7.18–7.11 (m, 2H), 6.33, 6.21 (2s, 1H), 4.85 (d, J = 12.8 Hz, 1H), 4.57–4.42 (m, 1H), 4.35 (dd, J = 3.1 Hz, J = 14.1 Hz, 1H), 3.91–3.76 (m, 2H), 3.46–3.39 (m, 1H), 2.24–2.00 (m, 2H), 1.67–1.42 (m, 5H), 1.27–0.73 (m, 6H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 182.6, 170.7, 170.6, 160.3, 158.4, 138.9, 138.1, 137.2, 137.1, 128.4, 128.3, 128.1, 128.0, 127.9, 127.0, 126.3, 114.7, 114.5, 58.8, 58.3, 47.6, 44.3, 43.9, 43.8, 42.4, 40.8, 34.6, 34.1, 32.6, 32.5, 32.4, 25.8, 25.7, 25.6. HRMS (*m/z*): calcd for $C_{25}H_{31}FN_3OS$ 440.2166 $[M+H]^+$; found 440.2160. Anal. Calcd $C_{25}H_{30}FN_3OS$: C, 68.31; H, 6.88; N, 9.56. Found: C, 68.19; H, 6.96; N, 9.38.

4-(2-Cyclohexylacetyl)-1-[(4-methylphenyl)aminothiocarbonyl]-2-phenylpiperazine (64). The product was obtained as a solid and purified by column chromatography using hexane-ethyl acetate (2:1) as eluent (228 mg, 70% yield). MS (FAB): *m/z* 458 (100%) $[M+Na]^+$. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.37, 9.25 (2s, 1H), 7.43–7.25 (m, 7H), 7.22–7.08 (m, 2H), 6.33, 6.23 (2s, 1H), 4.84 (d, J = 13.0 Hz, 1H), 4.57–4.42 (m, 1H), 4.34 (dd, J = 2.9 Hz, J = 14.0 Hz, 1H), 3.82–3.72 (m, 2H), 3.44–3.37 (m, 1H), 2.30, 2.28 (2s, 3H), 2.23–2.00 (m, 2H), 1.68–1.43 (m, 5H), 1.22–0.73 (m, 6H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 182.5, 170.7, 170.5, 138.3, 138.2, 128.5, 128.4, 128.3, 127.0, 126.3, 125.9, 58.7, 58.1, 47.6, 44.4, 43.9, 43.8, 42.4, 40.8, 34.6, 34.1, 32.6, 32.5, 32.4, 25.9, 25.8, 25.7, 25.6, 20.5. HRMS (*m/z*): calcd for $C_{26}H_{33}N_3OSNa$ 458.2237 $[M+Na]^+$; found 458.2229. Anal. Calcd $C_{26}H_{33}N_3OS$: C, 71.69; H, 7.64; N, 9.65. Found: C, 71.42; H, 7.71; N, 9.26

1-[(4-Nitrophenyl)aminothiocarbonyl]-2-phenyl-4-(2-phenylacetyl)piperazine (65). The product was obtained as a solid and purified by column chromatography using hexane-ethyl acetate (1:1.5) as eluent (224 mg, 65% yield), mp 168–170 ºC. MS (FAB): *m/z* 483 (100%) $[M+Na]^+$. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.94, 9.87 (2s, 1H), 8.21–8.16 (m, 2H), 7.70–7.62 (m, 2H), 7.42–7.00 (m, 10H), 6.42, 6.23 (2s, 1H), 4.97–4.91

(m, 1H), 4.55–4.38 (m, 1H), 3.96–3.56 (m, 5H), 3.23–3.12 (m, 1H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 182.1, 169.7, 169.4, 147.7,147.5, 142.2, 138.4, 137.6, 135.4, 135.3, 129.2, 128.6, 128.5, 128.2, 127.2, 126.4, 126.3, 126.2, 123.8, 123.3, 59.3, 58.6, 44.7, 44.3, 44.2, 42.4, 41.3, 40.7. HRMS (*m/z*): calcd for $C_{25}H_{24}N_4O_3SNa$ 483.1461 $[M+Na]^+$; found 483.1454. Anal. Calcd $C_{25}H_{24}N_4O_3S$: C, 65.20; H, 5.25; N, 12.17. Found: C, 64.85; H, 5.50; N, 11.86.

1-[(4-Cyanophenyl)aminothiocarbonyl]-2-phenyl-4-(2-phenylacetyl)piperazine (66). The product was obtained as a solid and purified by column chromatography using hexane-ethyl acetate (1:1) as eluent (238 mg, 72% yield), mp 181–183 ºC. MS (FAB): *m/z* 463 (60%) $[M+Na]^+$. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.76, 9.69 (2s, 1H), 7.76–7.72 (m, 2H), 7.61–7.55 (m, 2H), 7.41–7.13 (m, 8H), 7.06–7.00 (m, 2H), 6.40, 6.22 (2s, 1H), 4.92 (d, J = 13.6 Hz, 1H), 4.52–4.39 (m, 1H), 3.96–3.56 (m, 4H), 3.22–3.13 (m, 1H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 182.1, 169.7, 169.4, 145.5, 145.2, 138.8, 135.4, 132.2, 129.2, 128.6, 128.5, 128.2, 127.2, 126.3, 126.2 124.7, 124.2, 119.0, 105.5, 59.2, 58.4, 44.7, 44.0, 42.4, 41.2. Anal. Calcd $C_{26}H_{24}N_4OS$: C, 70.88; H, 5.49; N, 12.72; S, 7.28. Found: C, 70.81; H, 5.52; N, 12.81.

1-[{3,5-Bis(trifluoromethyl)phenyl}aminothiocarbonyl]-2-phenyl-4-(2-phenylacetyl)piperazine (67). The product was obtained as a solid and purified by column chromatography using dichloromethane-methanol (90:1) as eluent (384mg, 93% yield). mp 88–90 ºC. MS (FAB): *m/z* 574 (100%) $[M+Na]^+$. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.88, (br s, 1H), 8.11, 8.08 (2s, 2H), 7.79 (s, 1H), 7.41–7.02 (m, 10H), 6.43, 6.23 (2s, 1H), 4.99–4.92 (m, 1H), 4.53–4.39 (m, 1H), 3.97–3.53 (m, 4H), 3.22–3.14 (m, 1H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 181.9, 169.4, 135.6, 129.8, 129.6, 129.3, 128.6, 128.5, 128.2, 127.2, 126.3, 126.2, 124.8, 124.3, 58.5, 44.6, 43.8, 42.3.

HRMS (*m/z*): calcd for $C_{27}H_{23}F_6N_3OSNa$ 574.1358 $[M+Na]^+$; found 574.1347. Anal. Calcd $C_{27}H_{23}F_6N_3OS$: C, 58.80; H, 4.20; N, 7.62. Found: C, 58.65; H, 3.96; N, 7.35.

4-(3,3-Dimethylbutanoil)-1-[(4-nitrophenyl)aminocarbonyl]-2-phenylpiperazine (68). The product was obtained as a solid and purified by column chromatography using dichloromethane-methanol (50:1) as eluent (286 mg, 90% yield), mp 101–103 ºC. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.40, 9.29 (2s, 1H), 8.24–8.15 (m, 2H), 7.79–7.61 (m, 2H), 7.37–7.25 (m, 5H), 5.58, 5.45 (2s, 1H), 4.33–3.47 (m, 3H), 3.25–3.05 (m, 2H), 2.31–2.09 (m, 2H), 1.06–0.97 (m 1H), 0.92–0.87 (2s, 9H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 181.9, 181.8, 170.4, 170.2, 156.6, 133.8, 127.6, 113.2, 55.2, 51.2, 50.9, 49.2, 45.1, 44.4, 43.6, 43.4, 42.8, 42.2, 40.7, 29.7, 15.2, 14.8. HRMS (*m/z*): calcd for $C_{23}H_{28}N_4O_4Na$ 447.2003 $[M+Na]^+$; found 447.1999. Anal. Calcd $C_{23}H_{28}N_4O_4$: C, 65.08; H, 6.65; N, 13.20. Found: C, 64.86; H, 6.60; N, 12.90.

4-(3,3-Dimethylbutanoyl)-1-[(2-nitrophenyl)aminocarbonyl]-2-phenylpiperazine (69). The product was obtained as a solid and purified by column chromatography using dichloromethane-methanol (60:1) as eluent (280 mg, 88% yield), mp 93–96 ºC. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.44, 9.39 (2s, 1H), 7.99–7.63 (m, 3H), 7.43–7.20 (m, 6H), 5.46, 5.36 (2s, 1H), 4.30–3.52 (m, 3H), 3.28–3.10 (m, 2H), 2.29–2.09 (m, 2H), 1.31–1.00 (m 1H), 0.92–0.87 (2s, 9H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 170.2, 170.0, 154.4, 140.0, 139.8, 139.2, 138.4, 134.6, 134.4, 134.3, 134.1, 128.5, 128.4, 127.2, 127.1, 126.5, 126.4, 125.0, 123.9, 123.5, 123.1, 122.9, 55.2, 54.1, 48.6, 45.5, 43.6, 43.4, 42.4, 41.2, 30.9, 29.5, 13.8. HRMS (*m/z*): calcd for $C_{23}H_{28}N_4O_4Na$ 447.2003 $[M+Na]^+$; found 447.1999. Anal. Calcd $C_{23}H_{28}N_4O_4$: C, 65.08; H, 6.65; N, 13.20. Found: C, 64.99; H, 6.57; N, 12.82

4-(2-Cyclohexylacetyl)-1-[(4-nitrophenyl)aminocarbonyl]-2-phenylpiperazine (70). The product was obtained as a solid and purified by column chromatography using dichloromethane-methanol (60:1) as eluent (280 mg, 83% yield), mp 159–161 ºC. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.40, 9.32 (ds, 1H), 8.24–8.14 (m, 2H), 7.78–7.71 (m, 2H), 7.37–7.25 (m, 5H), 5.55, 5.47 (2s, 1H), 4.86–4.83 (m, 1H), 4.28–3.46 (m, 3H), 3.29–3.10 (m, 2H), 2.24–1.94 (m, 2H), 1.62–1.44 (m, 6H), 1.19–0.99 (m, 3H), 0.92–0.71 (m, 2H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 170.5, 170.4, 154.4, 154.2, 147.2, 147.1, 141.0, 139.7, 138.7, 128.5, 128.4, 127.0, 126.4, 126.3, 125.0, 124.7, 118.5, 54.5, 53.8, 48.0, 44.8, 42.8, 41.2, 34.6, 34.1, 32.5, 32.4, 25.8, 25.7, 25.6, 25.5. HRMS (*m/z*): calcd for $C_{25}H_{30}N_4O_4Na$ 473.2159 $[M+Na]^+$; found 473.2147. Anal. Calcd $C_{25}H_{30}N_4O_4$: C, 66.65; H, 6.71; N, 12.44. Found: C, 66.51; H, 6.79; N, 12.22.

4-(2-Cyclohexylacetyl)-1-[(2-nitrophenyl)aminocarbonyl]-2-phenylpiperazine (71) The product was obtained as a solid and purified by column chromatography using dichloromethane-methanol (70:1) as eluent (297 mg, 88% yield), mp 139–141 ºC. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.42, 9.38 (ds, 1H), 7.98–7.96 (m, 1H), 7.84–7.64 (m, 2H), 7.43–7.21 (m, 6H), 5.45, 5.36 (2s, 1H), 4.82–4.79 (m, 1H), 4.08–3.51 (m, 3H), 3.28–3.18 (m, 2H), 2.22–1.92 (m, 2H), 1.62–1.46 (m, 6H), 1.18–1.06 (m 3H), 0.91–0.73 (m, 2H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 170.6, 170.5, 154.4, 140.6,140.0, 139.3, 138.5, 134.6, 134.4, 134.3, 134.1, 128.5, 128.3, 127.1, 127.0, 126.4, 126.3, 125.0, 123.8, 123.5, 123.1, 122.9, 55.0, 54.2, 48.1, 44.7, 42.7, 41.3, 34.5, 32.5, 32.4, 32.3, 25.8, 25.7, 25.6, 25.5. HRMS (*m/z*): calcd for $C_{25}H_{30}N_4O_4Na$ 473.2159 $[M+Na]^+$; found 473.2153. Anal. Calcd $C_{25}H_{30}N_4O_4$: C, 66.65; H, 6.71; N, 12.44. Found: C, 66.28; H, 6.66; N, 12.12.

1-[(4-Nitrophenyl)aminocarbonyl]-2-phenyl-4-(2-phenylacetyl)piperazine (72) The product was obtained as a solid and purified by column chromatography using

dichloromethane-methanol (60:1) as eluent (326 mg, 98% yield), mp 98–100 ºC. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.34, 9.31 (ds, 1H), 8.24–8.15 (m, 2H), 7.77–7.72 (m, 2H), 7.36–7.03 (m, 10H), 5.57–5.44 (2s, 1H), 4.90–4.87 (m, 1H), 4.36–4.10 (m, 1H), 4.00–3.94 (m, 1H), 3.83–3.45 (m, 3H), 3.31–2.89 (m, 2H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 169.6, 169.4, 154.3, 154.2, 147.2, 147.1, 141.0, 139.5, 135.5, 135.4, 129.1, 128.6, 128.5, 128.4, 128.2, 128.1, 127.0, 126.4, 126.2, 124.6, 118.5, 54.5, 53.6, 48.0, 45.0, 42.7, 41.6, 28.4, 26.8. HRMS (*m/z*): calcd for $C_{24}H_{24}N_4O_4Na$ 467.1690 $[M+Na]^+$; found 467.1677.

1-[(2-Nitrophenyl)aminocarbonyl]-2-phenyl-4-(2-phenylacetyl)piperazine (73). The product was obtained as a solid and purified by column chromatography using dichloromethane-methanol (70:1) as eluent (320 mg, 96% yield), mp 55–57 ºC. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.41 (s, 1H), 8.00–7.64 (m, 3H), 7.40–7.04 (m, 11H), 5.46–5.29 (2s, 1H), 4.87–4.84 (m, 1H), 4.29–4.09 (m, 1H), 3.96–3.81 (m, 1H), 3.76–3.49 (m, 3H), 3.33–3.30 (m, 1H), 3.02–2.97 (m, 1H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 169.7, 169.4, 154.4, 147.2, 140.6, 139.7, 139.1, 138.4, 135.5, 135.4, 134.6, 134.4, 134.1, 129.1, 128.6, 128.5, 128.4, 128.2, 127.2, 127.1, 126.5, 126.3, 126.2, 125.1, 125.0, 123.9, 123.4, 123.1, 122.9, 55.2, 54.0, 48.0, 45.1, 42.9, 41.7, 28.4, 28.0, 26.8. HRMS (*m/z*): calcd for $C_{24}H_{24}N_4O_4Na$ 467.1690 $[M+Na]^+$; found 467.1678.

1-[(2-Chloro-5-trifluoromethylphenyl)aminocarbonyl]-2-phenyl-4-(2-phenylacetyl)piperazine (74). The product was obtained as a solid and purified by column chromatography using dichloromethane-methanol (70:1) as eluent (360 mg, 96% yield), mp 58–61 ºC. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.37 (s, 1H), 8.11–8.02 (m, 2H), 4.00–3.81 (m, 2H), 7.70–7.05 (m, 11H), 5.46, 5.30 (2s, 1H), 4.73–4.70 (m, 1H), 4.22–4.09 (m, 1H), 3.76–3.55 (m, 3H), 3.49–3.39 (m, 2H), 3.13-3.07 (m, 1H). ^{13}C NMR

(125 MHz, DMSO-d_6) δ 169.6, 169.5, 154.5, 154.4, 139.3, 138.8, 137.5 137.2, 135.5, 135.4, 130.4, 130.3, 129.1, 128.7, 128.5, 128.2, 126.5, 126.4, 126.3, 126.2, 55.6, 54.4, 48.0, 45.0, 42.9, 41.9. HRMS (*m/z*): calcd for $C_{26}H_{23}N_3ClF_3O_2Na$ 524.1323 $[M+Na]^+$; found 524.1316.

4-(Benzofuran-2-carbonyl)-2,6-dimethyl-1-[(4-nitrophenyl)aminocarbonyl]piperazine (79). The product was obtained as a solid and purified by column chromatography using dichloromethane-methanol (50:1) as eluent (237 mg, 75% yield), mp 169–172 ºC. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.10 (s, 1H), 7.79 (d, J = 7.6 Hz, 1H), 7.70 (d, J = 8.4 Hz, 1H), 7.56–7.46 (m, 4H), 7.40–7.28 (m, 3H), 4.46–4.37 (m, 2H), 4.36–4.27 (m, 2H), 1.25 (d, J = 6.8 Hz, 6H). ^{13}C RMN (125 MHz, DMSO-d_6) δ 160.0, 154.0, 153.7, 148.1, 147.3, 141.0, 126.7, 124.6, 123.7, 122.5, 118.9, 111.8, 111.4, 46.3. HRMS (*m/z*): calcd for $C_{22}H_{22}N_4O_5Na$ 445.1482 $[M+Na]^+$; found 445.1476. Anal. Calcd $C_{22}H_{22}N_4O_5$: C, 62.55; H, 5.25; N, 13.26. Found: C, 62.68; H, 5.38; N, 13.20.

4-(Benzofuran-2-carbonyl)-1-[(4-chlorophenyl)aminocarbonyl]-2,6-dimethylpiperazine (80). The product was obtained as a solid and purified by column chromatography using hexane-ethyl acetate (3:1) as eluent (216 mg, 70% yield), mp 154–155 ºC. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.53 (s, 1H), 8.21–8.15 (m, 1H), 7.83–7.67 (m, 4H), 7.54–7.34 (m, 3H), 4.51–4.41 (m, 2H), 4.39–4.30 (m, 2H), 1.27 (d, J = 6.9 Hz, 6H). ^{13}C RMN (125 MHz, DMSO-d_6) δ 160.0, 154.1, 153.9, 148.2, 139.4, 128.1, 126.6, 125.6, 122.5, 121.8, 118.9, 111.3, 46.0, 26.8, 20.1. HRMS (*m/z*): calcd for $C_{22}H_{22}ClN_3O_3Na$ 434.1242 $[M+Na]^+$; found 434.1234. Anal. Calcd $C_{22}H_{22}ClN_3O_3$: C, 64.15; H, 5.38; N, 10.20. Found: C, 64.48; H, 5.69; N, 9.86.

4-(Benzofuran-2-carbonyl)-1-[(4-cyanophenyl)aminocarbonyl]-2,6-dimethylpiperazine (81). The product was obtained as a solid and purified by column chromatography using hexane-ethyl acetate (1:1) as eluent (256 mg, 85% yield), mp 240–242 ºC. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.53 (s, 1H), 8.21–8.15 (m, 1H), 7.83–7.67 (m, 4H), 7.54–7.34 (m, 3H), 4.51–4.41 (m, 2H), 4.39–4.30 (m, 2H), 1.27 (d, J = 6.9 Hz, 6H). ^{13}C RMN (125 MHz, DMSO-d_6) δ 160.0, 154.1, 153.9, 148.2, 139.4, 128.1, 126.6, 125.6, 122.5, 121.8, 118.9, 111.3, 46.0, 26.8, 20.1. HRMS (*m/z*): calcd for $C_{23}H_{22}N_4O_3Na$ 425.1584 $[M+Na]^+$; found 425.1578. Anal. Calcd $C_{23}H_{22}N_4O_3$: C, 68.64; H, 5.51; N, 13.92. Found: C, 68.77; H, 5.26; N, 14.05.

4-(Benzofuran-2-carbonyl)-2,6-dimethyl-1-[(2-nitrophenyl)aminocarbonyl]piperazine (82). The product was obtained as a solid and purified by column chromatography using hexane-ethyl acetate (3:1) as eluent (269 mg, 85% yield), mp 128–130 ºC. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.36 (s, 1H), 7.99 (dd, J = 8.3 Hz, J = 1.4 Hz, 1H), 7.84–7.76 (m, 2H), 7.73–7.63 (m, 2H), 7.54–7.44 (m, 2H), 7.41–7.33 (m, 1H), 7.28–7.21 (m, 1H), 4.51–4.24 (m, 4H), 1.31 (d, J = 6.7 Hz, 6H). ^{13}C RMN (125 MHz, DMSO-d_6) δ 160.0, 154.0, 153.7, 148.2, 140.5, 134.7, 134.1, 126.6, 125.0, 123.8, 122.9, 122.5, 111.8, 111.4, 40.5, 19.9. HRMS (*m/z*): calcd for $C_{22}H_{22}N_4O_5Na$ 445.1482 $[M+Na]^+$; found 445.1474. Anal. Calcd $C_{22}H_{22}N_4O_5$: C, 62.55; H, 5.25; N, 13.26. Found: C, 62.23; H, 5.46; N, 12.96.

4-(Benzofuran-2-carbonyl)-2,6-dimethyl-1-[(4-methylphenyl)aminocarbonyl]piperazine (83). The product was obtained as a solid and purified by column using chromatography hexane-ethyl acetate (2:1) as eluent (185 mg, 63% yield), mp 217–220 ºC. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.30 (s, 1H), 7.79 (d, J = 7.6 Hz, 1H), 7.70 (d, J = 8.3 Hz, 1H), 7.51–7.45 (m, 2H), 7.40–7.31 (m, 3H),

7.11–7.04 (m, 2H), 4.44–4.36 (m, 2H), 4.34–4.26 (m, 2H), 2.25 (s, 3H), 1.24 (d, *J* = 6.7 Hz, 6H). ^{13}C RMN (125 MHz, DMSO-*d*$_6$) δ 160.0, 154.0, 153.9, 148.2, 137.7, 130.8, 129.1, 128.6, 126.6, 123.8, 123.7, 122.5, 120.6, 118.2, 111.8, 111.4, 45.9, 20.3, 20.1. HRMS (*m/z*): calcd for $C_{23}H_{25}N_3O_3Na$ 414.1788 $[M+Na]^+$; found 414.1782. Anal. Calcd $C_{23}H_{25}N_3O_3$: C, 70.57; H, 6.44; N, 10.73. Found: C, 70.28; H, 6.63; N, 10.76.

4-(Benzofuran-2-carbonyl)-1-[(2-chloro-5-trifluoromethylphenyl)aminocarbonyl]-2,6-dimethylpiperazine (84). The product was obtained as a solid and purified by column using chromatography hexane-ethyl acetate (4:1) as eluent (249 mg, 69% yield), mp 178–180 ºC. ^{1}H NMR (500 MHz, DMSO-*d*$_6$) δ 8.38 (s, 1H), 7.93 (d, *J* = 1.3 Hz, 1H), 7.79 (d, *J* = 7.8 Hz, 1H), 7.74 (d, *J* = 8.5 Hz, 1H), 7.71 (d, *J* = 8.5 Hz, 1H), 7.54–7.45 (m, 3H), 7.41–7.34 (m, 1H), 4.49–4.27 (m, 4H), 1.30 (d, *J* = 6.7 Hz, 6H). ^{13}C RMN (125 MHz, DMSO-*d*$_6$) δ 160.0, 154.0, 153.9, 148.2, 137.7, 132.1, 130.5, 129.1, 126.6, 123.8, 122.8, 122.7, 122.5, 121.8, 111.8, 111.4, 46.6, 19.9. HRMS (*m/z*): calcd for $C_{23}H_{21}ClF_3N_3O_3Na$ 502.1116 $[M+Na]^+$; found 502.1110. Anal. Calcd $C_{23}H_{21}ClF_3N_3O_3$: C, 57.57; H, 4.41; N, 8.76. Found: C, 57.54; H, 4.45; N, 8.76.

4-(Benzofuran-2-carbonyl)-1-[(4-chloro-3-trifluoromethylphenyl)aminocarbonyl]-2,6-dimethylpiperazine (85). The product was obtained as a solid and purified by column using chromatography hexane-ethyl acetate (2:1) as eluent (252 mg, 70% yield), mp 218–220 ºC. ^{1}H NMR (500 MHz, DMSO-*d*$_6$) δ 8.84 (s, 1H), 8.10 (d, *J* = 2.2 Hz, 1H), 7.86 (d, *J* = 8.8 Hz, *J* = 2.2 Hz, 1H), 7.79 (d, *J* = 7.8 Hz, 1H), 7.70 (d, *J* = 8.3 Hz, 1H), 7.60 (d, *J* = 8.8 Hz, 1H), 7.53–7.45 (m, 2H), 7.40–7.34 (m, 1H), 4.49–4.38 (m, 2H), 4.37–4.28 (m, 2H), 1.27 (d, *J* = 6.7 Hz, 6H). ^{13}C RMN (125 MHz, DMSO-*d*$_6$) δ 160.0, 154.0, 153.9, 148.2, 140.1, 131.5, 126.6, 124.5, 123.7, 122.5, 121.8, 118.8, 111.8, 111.3, 46.1, 20.1. HRMS (*m/z*): calcd for $C_{23}H_{21}ClF_3N_3O_3Na$ 502.1116

[M+Na]$^+$; found 502.1111. Anal. Calcd $C_{23}H_{21}ClF_3N_3O_3$: C, 57.57; H, 4.41; N, 8.76. Found: C, 57.59; H, 4.65; N, 8.54.

1-*tert*-Butoxycarbonyl-4-[(4-nitrophenyl)aminocarbonyl]piperazine (87). The product was obtained as a solid and purified by column using chromatography dichloromethane-methanol (100:1) as eluent (247 mg, 94% yield), mp 190–192 ºC. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.27 (s, 1H), 8.17 (d, J = 9.5 Hz, 2H), 7.37 (d, J = 9.5 Hz, 2H), 3.50–3.48 (m, 4H), 3.40–3.38 (m, 4H), 1.43 (m, 9H). ^{13}C RMN (125 MHz, DMSO-d_6) δ 154.0, 153.8, 147.3, 140.9, 124.7 118.3, 79.1, 43.6, 28.0. HRMS (*m/z*): calcd for $C_{16}H_{22}N_4O_5Na$ 373.1482 [M+Na]$^+$; found 373.1476. Anal. Calcd $C_{16}H_{22}N_4O_5$: C, 54.85; H, 6.33; N, 15.99. Found: C, 54.79; H, 6.21; N, 15.87.

1-*tert*-Butoxycarbonyl-4-[(2-nitrophenyl)aminocarbonyl]piperazine (88). The product was obtained as a solid and purified by column using chromatography dichloromethane-methanol (100:1) as eluent (234 mg, 89% yield), mp 91–94 ºC. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.34 (s, 1H), 7.96 (dd, J = 8.2 Hz, J = 1.2 Hz, 1H), 7.71–7.63 (m, 2H), 7.26–7.22 (m, 1H), 3.48–3.46 (m, 4H), 3.40–3.38 (m, 4H), 1.44 (m, 9H). ^{13}C RMN (125 MHz, DMSO-d_6) δ 154,2, 153,9, 140,6, 134,5, 134,0, 125,0, 123,8, 123,0. 79.2, 43.6, 28,0. HRMS (*m/z*): calcd for $C_{16}H_{22}N_4O_5Na$ 373.1482 [M+Na]$^+$; found 373.1476. Anal. Calcd $C_{16}H_{22}N_4O_5$: C, 54.85; H, 6.33; N, 15.99. Found: C, 55.11 H, 6.46; N, 15.94.

4-*tert*-Butoxycarbonyl-1-[(2-chloro-5-trifluoromethylphenyl)aminocarbonyl]piperazine (89). The product was obtained as a solid and purified by column using chromatography dichloromethane-methanol (150:1) as eluent (285 mg, 92% yield), mp 153–155 ºC. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.45 (s, 1H), 7.96 (dd, J = 1.9 Hz, 1H), 7.71 (d, J = 8.1 Hz, 1H), 7.49 (dd, J = 1.9 Hz,

J = 8.3 Hz, 1H), 3.48–3.46 (m, 4H), 3.41–3.39 (m, 4H), 1.44 (m, 9H). ^{13}C RMN (125 MHz, DMSO-*d*$_6$) δ 154.5, 153.9, 137.6, 131.3, 130.4, 122.3, 121.5 79.1, 43.6, 28.0. HRMS (*m/z*): calcd for $C_{17}H_{21}ClF_3N_3O_3Na$ 430.1116 $[M+Na]^+$; found 430.1110. Anal. Calcd $C_{17}H_{21}ClF_3N_3O_3$: C, 50.07; H, 5.19; N, 10.30. Found: C, 50.16, H, 5.06; N, 10.27.

1,4-Bis[(2-chloro-5-trifluoromethylphenyl)aminocarbonyl]-2-phenylpiperazine (76). The product was obtained as a solid and purified by column using chromatography hexane-ethyl acetate (4:1) as eluent (408 mg, 90% yield), mp 134–137 ºC. ^{1}H NMR (500 MHz, DMSO-*d*$_6$) δ 8.35 (s, 1H), 8.27 (s, 2H), 8.06 (dd, *J* = 1.9 Hz, 1H), 7.84 (m, 1H), 7.70 (t, *J* = 7.5 Hz, 2H), 7.50–7.45 (m, 4H), 7.40 (t, *J* = 7.5 Hz, 2H) 7.30 (t, *J* = 7.2 Hz, 1H), 5.45 (t, *J* = 4.0 Hz, 1H), 4.36 (dd, *J* = 4.1 Hz, *J* = 13.9 Hz, 1H), 4.18–4.12 (m, 1H), 3.93–3.88 (m, 1H), 3.75 (dd, *J* = 4.4 Hz, *J* = 13.9 Hz, 1H), 3.54–3.40 (m, 2H). ^{13}C RMN (125 MHz, DMSO-*d*$_6$) δ 154.5, 154.4, 139.2, 137.4, 137.3, 131.3, 130.4, 128.6, 127.3, 126.6, 122.6, 122.2, 121.6, 121.3, 54.9, 45.4, 43.5. HRMS (*m/z*): calcd for $C_{26}H_{20}Cl_2F_6N_4O_2Na$ 627.0760 $[M+Na]^+$; found 627.0752. Anal. Calcd $C_{26}H_{20}Cl_2F_6N_4O_2$: C, 51.59; H, 3.33; N, 9.26. Found: C, 51.61, H, 3.56; N, 9.06.

1,4-Bis[(2-chloro-5-trifluoromethylphenyl)aminocarbonyl]piperazine (93). The product was obtained as a solid and purified by column using chromatography hexane-ethyl acetate (3:1) as eluent (388 mg, 98% yield), mp 249–250 ºC. ^{1}H NMR (500 MHz, DMSO-*d*$_6$) δ 8.49 (s, 2H), 7.98 (d, *J* = 1.6 Hz, 2H), 7.73 (d, *J* = 8.3 Hz, 2H), 7.50 (dd, *J* = 8.4 Hz, *J* = 1.7 Hz, 2H), 3.56–3.59 (m, 8H). ^{13}C RMN (125 MHz, DMSO-*d*$_6$) δ 154.6, 137.6, 131.4, 130.5, 128.3, 128.0, 127.8, 124.8, 122.6, 122.4, 121.7, 43.6. Anal. Calcd $C_{20}H_{16}Cl_2F_6N_4O_2$: C, 46.43; H, 3.34; N, 10.31 Found: C, 46.74, H, 3.33; N, 10.45.

General Procedure 3. *N,N'*-diacylation reaction of 2-phenylpiperazine or piperazine (75 and **92).** To a solution of 2-phenylpiperazine or piperazine (1.0 mmol)

in CH_2Cl_2 (30 mL), benzofurane-2-carbonyl choride (2.4 mmol) and pyridine (3.0 mmol) were added. The reaction mixture was stirring at room temperature until TLC showed that all the starting material had reacted. The reaction mixture was evaporated to dryness to obtain the corresponding diacylderivative. Column chromatography gave the pure compound in high yield.

1,4-Bis(benzofurane-2-carbonyl)-2-phenylpiperazine (75). The product was obtained as a solid and purified by column using chromatography dichloromethane-methanol (60:1) as eluent (374 mg, 83% yield), mp 159–162 ºC. ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.79–7.74 (m, 2H), 7.69–7.64 (m, 2H), 7.54–7.24 (m, 11H), 5.98–5.74 (m, 1H), 5.01–4.75 (m, 1H), 4.55–4.38 (m, 1H), 4.36–4.08 (m, 2H), 3.88–3.42 (m, 2H). ^{13}C RMN (125 MHz, DMSO-d_6) δ 160.0, 159.3, 154.0, 153.9, 147.8, 128.6, 127.3, 126.7, 126.6, 126.5, 123.7, 122.5, 111.8, 111.3. HRMS (*m/z*): calcd for $C_{28}H_{22}N_2O_4Na$ 473.1472 $[M+Na]^+$; found 473.1464.

1,4-Bis(benzofurane-2-carbonyl)piperazine (92). The product was obtained as a solid and purified by column using chromatography hexane-ethyl acetate (1:1) as eluent (388 mg, 91% yield), mp 240–241 ºC. ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.78 (d, J = 7.8 Hz, 2H), 7.69 (d, J = 8.4 Hz, 2H), 7.50–7.46 (m, 4H), 7.36 (d, J = 7.6 Hz, 2H), 3.98–3.79 (m, 8H). ^{13}C RMN (125 MHz, DMSO-d_6) δ 159.1, 154.0, 148.0, 126.7, 126.6, 123.7, 122.5, 118.8, 111.2. HRMS (*m/z*): calcd for $C_{22}H_{18}N_4O_2Na$ 397.1159 $[M+Na]^+$; found 397.1153. Anal. Calcd $C_{22}H_{18}N_4O_2$: C, 70.58; H, 4.85; N, 7.48. Found: C, 70.71, H, 5.09; N, 7.19.

General Procedure 4. Synthesis of compounds 90 and 91 [44]. CF_3COOH (10 mmol) was added to a solution of **87** and **88** (1 mmol) in CH_2Cl_2 (20 mL) at 0 C. The reaction mixture was warmed to room temperature and stirred at the same temperature. Upon

completion, the reaction mixture was concentrated under vacuum, the residue was dissolved in CH_2Cl_2, washed with saturated $NaHCO_3$, brine, dried over anhydrous Na_2SO4 and concentrated under vacuum to afford *N*-deprotected compounds which were used in the next reaction without further purification. They were dissolved in dry dichloromethane (30 mL) and cooled to 0 ºC, then benzofurane-2-carbonyl chloride (1 mmol) and pyridine (2.5 mmol) were added. The reaction mixture was kept into an ice-water bath with stirring 12 hours and left at room temperature until TLC showed that all the starting material had reacted. The reaction mixture was evaporated to dryness to obtain the corresponding acylderivatives. Column chromatography gave the pure compounds in high yield.

1-(Benzofurane-2-carbonyl)-4-[(4-nitrophenyl)aminocarbonyl]piperazine (90). The product was obtained as a solid and purified by column using chromatography dichloromethane-methanol (80:1) (315 mg, 80% yield), mp 165–167 ºC. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.34 (s, 1H), 8.19–8.15 (m, 2H), 7.81–7.65 (m, 4H), 7.52–7.45 (m, 2H), 7.38–7.33 (m, 1H), 3.93–3.75 (m, 4H), 3.52–3.22 (m, 4H). ^{13}C RMN (125 MHz, DMSO-d_6) δ 159.1, 154.1, 153.9, 148.9, 147.3, 141.0, 126.7, 126.5, 124.7, 123.7, 122.5, 118.4, 111.7, 111.1, 43.8. HRMS (*m/z*): calcd for $C_{20}H_{18}N_4O_5Na$ 417.1169 $[M+Na]^+$; found 417.1163. Anal. Calcd $C_{20}H_{18}N_4O_5$: C, 60.91; H, 4.60; N, 14.21. Found: C, 60.77, H, 4.73 N, 13.83.

1-(Benzofurane-2-carbonyl)-4-[(2-nitrophenyl)aminocarbonyl]piperazine (91). The product was obtained as a solid and purified by column using chromatography hexane-ethyl acetate (1:1) (346 mg, 88% yield), mp 105–108 ºC. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.41 (s, 1H), 8.00–7.94 (m, 1H), 7.85–7.64 (m, 4H), 7.57–7.21 (m, 4H), 3.89–3.73 (m, 4H), 3.69–3.58 (m, 4H). ^{13}C RMN (125 MHz, DMSO-d_6) δ 159.1, 154.1, 153.9, 137.6, 148.1, 147.3, 141.1, 126.7, 126.6, 124.7, 123.7, 122.5, 118.4, 111.8, 111.1, 43.8.

HRMS (*m/z*): calcd for $C_{20}H_{18}N_4O_5Na$ 417.1169 $[M+Na]^+$; found 417.1164 Anal. Calcd $C_{20}H_{18}N_4O_5$: C, 60.91; H, 4.60; N, 14.21. Found:C, 60.99, H, 4.50; N, 13.97.

4.2. Biological Evaluation: **Cells and Virus.** Human A549, 293 and MRC-5 cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The 293β5 stable cell line overexpressing the human β5 integrin subunit was kindly provided by Dr. Glen Nemerow [45]. The cell lines were propagated in Dulbecco's modified Eagle medium (DMEM, Life Technologies/Thermo Fisher) supplemented with 10% fetal bovine serum (FBS) (Omega Scientific, Tarzana, CA), 10 mM HEPES, 4 mM L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, and 0.1 mM non-essential amino acids (complete DMEM).

Wild-type HAdV-5, and HCMV (AD169) were obtained from the ATCC. The HAdV-5-GFP used in this study is a replication-defective virus containing a CMV promoter-driven enhanced green fluorescent protein (eGFP) reporter gene cassette in place of the E1/E3 regions [46]. HAdV were propagated in 293β5 cells and isolated from the cellular lysate by cesium chloride density centrifugation. Virus concentration, in mg/ml, was calculated with the Bio-Rad Protein Assay (Bio-Rad Laboratories) and converted to virus particles/ml (vp/ml) using 4×10^{12} vp/mg.

4.3 Cytotoxicity assay. The cytotoxicity of the compounds was evaluated using the AlamarBlue Cell Viability Assay (Invitrogen) according to the manufacturer's instructions. Actively dividing A549 cells were incubated with the thiourea /urea derivatives for 48 h. After the incubation the AlamarBlue reagent was added to the cells (1/10th Alamar Blue reagent in culture medium) for an extra 4 h. The 50% cytotoxic concentration (CC_{50}) of the molecules was calculated according to Cheng *et al.* [47].

The selectivity index (SI) was evaluated as the ratio of CC_{50} to IC_{50}, where the IC_{50} was defined as the concentration of compound that inhibits HAdV infection by 50%.

4.4. Plaque assay. Compounds were tested in a dose-response assay using 0.06 vp/cell with concentrations ranging from 10 to 0.62 μM. Briefly, 293β5 cells were seeded in 6-well plates at a density of 4 × 105 cells per well in duplicates for each condition. When cells reached 80−90% confluency, they were infected with HAdV5-GFP (0.06 vp/cell) and rocked for 2 h at 37°C. After the incubation the inoculum was removed, and the cells were washed once with PBS. The cells were then carefully overlaid with 2 mL/well of equal parts of 1.6% (water/vol) Difco Agar Noble (Becton, Dickinson & Co., Sparks, MD) and 2× EMEM (Minimum Essential Medium Eagle, BioWhittaker) supplemented with 2×penicillin/streptomycin, 2× L-glutamine, and 10% FBS. The mixture also contained the compounds in concentrations ranging from 10 to 0.62 μM. Following incubation for 7 days at 37°C, plates were scanned in a Typhoon 9410 imager (GE Healthcare Life Sciences), and plaques were quantified with ImageJ [48].

4.5. Nuclear-associated HAdV genomes. Nuclear delivery of the HAdV genomes was assessed with real-time PCR following nuclear isolation from infected cells using a previously described protocol with a few modifications [50]. Briefly, 1×106 A549 cells in 6-well plates were infected with HAdV5 wild-type at an MOI of 2,000 vp/cell in the presence of 50 μM of the selected derivatives or the same volume of DMSO. Forty-five min after the infection, A549 cells were trypsinized and collected, and then washed twice with PBS. The cell pellet was resuspended in 500 μL of 1×hypotonic buffer (20 mM Tris-HCl pH 7.4, 10 mM NaCl, 3 mM MgCl2) and incubated for 15 min at 4°C. Then, 25 μL of NP-40 was added, and the samples were vortexed. The homogenates were centrifuged for 10 min at 835g at 4°C. Following the removal of the cytoplasmic fraction (supernatant), DNA was isolated from the nuclear fraction (pellet) using the

QIAamp DNA Mini Kit (QIAGEN, Valencia, CA). We also measured the DNA copy number of the cellular housekeeping gene GAPDH in both samples the nucleus and the cytoplasm as a control for the purity of nuclear isolation.

4.6. HAdV yield reduction. The effect of the selected derivatives on virus production was evaluated in a burst assay. A549 cells were infected with wildtype HAdV-5, in the presence or absence of 50 μM of the compounds. After 48 h, cells were harvested and subjected to three rounds of freeze/thaw. Serial dilutions of clarified lysates were titrated on A549 cells, and the $TCID_{50}$ values were calculated using an end-point dilution method [49].

4.7. DNA and mRNA quantification by real-time PCR. For DNA quantification, A549 cells (1.5 x 105 cells/well in a 24-well plate) were infected with wild-type HAdV5 (100 vp/cell) and incubated for 2 h at 37°C in complete DMEM. After the incubation, the excess virus was removed, and the medium was replaced with 500 μL of complete DMEM containing 50 μM of either compounds or the same volume of DMSO (positive control). All samples were done in triplicates. After 24 h of incubation at 37°C and 5% CO2, DNA was purified from the cell lysate with the QIAamp DNA Mini Kit (QIAGEN, Valencia, CA) following the manufacturer's instructions. TaqMan primers and probes were designed for a region of the HAdV5 hexon with the GenScript Real-Time PCR (TaqMan) Primer Design software (GenScript). Oligonucleotides sequences were AdF, 5′-GACATGACTTTTGAGGTGGA-3′; AdR, 5′-GTGGCGTTGCCGGCCGAGAA-3′; and AdProbe, 5′-TCCATGGGATCCACCTCAAA-3′. Real-time PCR mixtures consisted of 2 μL of the purified DNA, AdF, and AdR at a concentration of 200 nM each and AdProbe at a concentration of 50 nM in a total volume of 12.5 μL mixed with 12.5 μL of KAPA

PROBE FAST qPCR Master Mix (KAPA Biosystems, MA). The PCR cycling protocol was 95°C for 3 min followed by 40 cycles of 95°C for 10 s and 60°C for 30 s.

For the evaluation of RNA expression, same conditions of infection applied for the DNA quantification were used. Six hours after infection, RNA was purified with the miRCURY RNA Isolation Kit (Exiqon Inc., MA) following the manufacturer's instructions. Quantification of RNA copy numbers was performed using primers and conditions previously reported for E1A [50]. The internal control was human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene. Oligonucleotides sequences for GAPDH and conditions applied were those previously reported by Rivera *et al.* [50]. For the quantification, gene fragments from hexon, and GAPDH were cloned into the pGEM-T Easy vector (Promega). Known concentrations of the template were used to generate a standard curve in parallel for each experiment. All assays were performed in a LightCycler® 96 System (Roche).

4.8. Antiviral activity of compound combinations. To assess the compound concentrations required in combination to generate a given effect to the derivative concentration that would be needed individually to achieve that same effect the software packet Calcusyn (BioSoft, Ferguson, MO, USA) was used. A plaque dose-response assay was carried out using all the possible combination of the three piperazine derivatives starting from twice the IC_{50} obtained previously for each compound and the ratio of those concentrations. CalcuSyn software interpolates the compound concentrations needed in combination at the selected ratio to generate effects of 50%, 75% and 90% inhibition and compares these combinations with the concentrations from the three compound's individual dose-response curves required to achieve the same inhibition. The combination effect of the three compounds is reported by the combination index (CI) value, a pharmacological interaction estimation which uses the

IC_{50} and the dose-response curve's shape of each individual compound and their combinations. The CI value was interpreted in accordance with Matthews *et al*. [37].

4.9. HCMV Infectivity Assay by Quantitative PCR. To test the sensitivity of HCMV to our selection of compounds, MRC-5 cells (1.75 × 105 cells/well in a 6-well plate) were infected with HCMV at an MOI of 0.05 vp/cell. The samples were incubated in complete DMEM supplemented with 50 μM of the compounds or the same volume of DMSO in triplicates. After 72 h of incubation at 37 °C, the DNA was purified from the cell lysate with the QIAamp DNA Mini Kit (Qiagen, Valencia, CA) following the manufacturer's instructions. TaqMan primers and probes were designed for a region of the US28 gene with the GenScript Real-time PCR (TaqMan) Primer Design software (GenScript). Oligonucleotides sequences were: CMV-F, 5′-TCTACGTGGCTATGTTTGCC-3′; CMV-R, 5′- GGCCGATATCTCATGTAAACAA-3′; and CMVProbe, 5′-CACGGAGATTGCACTCGATCGC-3′. Real-time PCR mixtures consisted of 10 μL of the purified DNA, CMV-F at a concentration of 100 nM, CMV-R at a concentration of 300 nM, and CMV-Probe at a concentration of 50 nM in a total volume of 12.5 μL mixed with 12.5 μL of KAPA PROBE FAST qPCR Master Mix (KAPABiosystems, MA). The PCR cycling protocol was 95°C for 10 min followed by 40 cycles of 95°C for 30 s and 58°C for 60 s. Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was the internal control. Oligonucleotides sequences for GAPDH, and conditions applied were those previously reported by Rivera *et al* [52]. For quantification, gene fragment from US28 and GAPDH were cloned into the pGEM-T Easy vector (Promega), and known concentrations of template were used to generate a standard curve in parallel for each experiment. All assays were performed in a LightCycler® 96 System (Roche).

4.10. Phi29 DNA polymerase amplification efficiency assay. Amplification of a BAC vector containing HAdV DNA genome was performed in the presence of the compounds at a concentration of 50 μM or the same amount of DMSO in triplicates, using the RCA DNA Amplification Kit (MCLAB) following the manufacturer's instructions. To quantify the amplified products, a quantitative real-time PCR was performed using TaqMan primers and probes designed for a region of the HAdV5-wt hexon with GenScript Real-time PCR (TaqMan) First Design software (GenScript). The oligonucleotide sequences were AQ1:5' -GCC ACG GTG GGG TTT CTA AAC TT -3'; AQ2:5' -GCC CCA GTG GTG TTC TTA CAT GCA CAT -3'; and AP: 6-FAM-5' -TGC ACC AGA CCC CGG CTC AGG TAC TCC GA-3' –TAM. Real-time PCR mixtures consisted of 2 μL of the purified DNA, AdF, and AdR at a concentration of 200 nM each and AdProbe at a concentration of 50 nM in a total volume of 12.5 μL mixed with 12.5 μL of KAPA PROBE FAST qPCR Master Mix (KAPABiosystems, MA). The PCR cycling protocol was 95°C for 3 min followed by 40 cycles of 95°C for 10 s and 60°C for 30 s. All assays were conducted on a C1000 ThermalCycler thermal cycler (BioRad). The results represent the mean ± SD of the samples in triplicates from three independent experiments.

4.11. Hamster serum stability assay. The test compound solution (1 μM, 0.25% final DMSO concentration) was incubated with Syrian hamster serum (IGHMS-SER, Innovative Grade US Origin Hamster Serum- Syrian Gold) at 37ºC. Serial samples were taken at 0, 15, 30, 60 and 120 min. All samples were added immediately to 3 volumes of methanol in a microtiter plate cooled in dry-ice to halt chemical degradation. All the samples were analyzed by LC-MS/MS. The percentage of parent compound remaining at each time point relative to the 0 min sample was calculated from peak area. The chemical stability assay returns the percent parent compound remaining at each time

point for thiourea derivatives. LC/MS/MS conditions were as follow: Analytes were detected by electrospray ionization (ESI) mass spectrometry in positive mode for **616** and **628**, and negative mode for **585**. Identification was obtained using multiple reaction monitoring (MRM) mode of the transitions at m/z 352.074/252.100 for **585**, m/z 378.285/143.200 for **616**, and m/z 399.116/143.100 for **688**. The chromatographic separation was accomplished on a Discovery® C18 column (50 × 2.1 mm, 3 μm) coupled with a Discovery® HS C18 (20 × 2.1 mm, 3 μm) guard column. The mobile phase consisting of water with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B). The flow rate was 0.5 ml/min and the run time was 5 minutes using a linear gradient. The volume injection was 5 μl.

4.12. Statistical Analyses Statistical analyses were performed with the GraphPad Prism 5 suite. Unless otherwise indicated, data are presented as the mean of triplicate samples ± standard deviation (SD). P-values are indicated when statistically significant.

ASSOCIATED CONTENT

Supporting Information. Copies of NMR spectra, dose-response graph of the inhibitory activity of the compounds, nuclear-association assay of HAdV5 genomes, Syrian Hamster serum stability data of the selected compounds and calculated physical chemical properties, can be found in the Supporting Information. “This material is available free of charge via the Internet at http://pubs.acs.org.”

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Notes

The authors declare no competing financial interest. J.S.C., J.P., J.M.V.P., F.I.G., M. V.H. and S.M. are co-inventors of the European patent EP16382073.1 (Name of the Invention: Piperazine derivatives as antiviral agents with increased therapeutic activity; year of application: 2016).

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ABBREVIATIONS USED

ATCC, American Type Culture Collecton; CC_{50}, 50% cell cytotoxic concentration; COSY, correlation spectroscopy; DEPT, distortionless enhancement by polarization transfer; DMEM, Dulbecco/Vogt Modified Eagle’s minimal essential medium; DMSO, dimethyl sulfoxide; EMEM, minimum essential medium Eagle; GAPDH, Gliceraldehído-3-fosfato deshidrogenasa; HAdV, human adenovirus; HCMV, human cytomegalovirus; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HSCT, human stem cell transplant; HSQC, heteronuclear single quantum correlation; IC_{50}, 50% inhibitory concentration; MOI, multiplicity of infection (viral particles/cell); NMR, nuclear magnetic resonance spectroscopy; NOESY, nuclear Overhauser effect spectroscopy; NP-40, nonyl phenoxypolyethoxylethanol; NPC1, endosomal membrane protein Niemann-PickC1; PCR, polymerase chain reaction; SD, standard deviation; SI,

Selectivity Index; SOT, solid organ transplant; $TCID_{50}$, 50% tissue culture infective dose; TLC, thin layer chromatography

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List of Captions

Figure 1. Cidofovir and its lipidic conjugates

Figure 2. General structures of 5-amino uracil (**4**) and 3-hidroxy-quinazoline derivatives (**5**)

Figure 3. Structures of the prototypes **6**–**11**

Figure 4. Three routes for modifying the general structure of the prototypes

Figure 5. From the active but cytotoxic compounds **12** and **13** to the general structure of route **A.**

Figure 6. Structures of the diamide and diurea derivatives **75**, **76**, **92** and **93**

Figure 7. Effect of the selected compounds on HAdV and HCMV DNA replication. Coompounds **41, 43**, **45, 50**, and **87** significantly reduced *de novo* production of HAdV DNA copies cmpared to the positive control 24-hour post-infection in a quantitative PCR assay (A). Compounds **50** and **87** also significantly reduced the expression of the immediate early gene E1A compared to the positive control 6-hour post-infection in a quantitative PCR assay (B). Furthermore, compounds **41**, **43**, **45**, **50**, **71**, **73**, **87,** and **89** reduced *de novo* production of HCMV DNA copies of the 72-hour post-infection in a quantitative PCR assay **(C)**. Impact on the amplification efficiency of the Phi29 DNA polymerase **(D)**. The results are expressed as the relative copy number of HAdV/HCMV DNA and E1A mRNA normalized to GAPDH copy number, and they are presented as the mean ± SD of triplicate assays. *P<0.05.

Scheme 1. Synthesis of 2-substituted piperazine thiourea derivatives

Scheme 2. Synthesis of 4-(2-substituted acetyl)-2-phenylpiperazine urea derivatives

Scheme 3. Synthesis of 4-(benzofurane-2-carbonyl)-2,6-dimethylpiperazine urea derivatives

Scheme 4. Synthesis of piperazine urea derivatives

Table 1. 4-Acyl-2-methylpiperazine thiourea derivatives **12**, **20–50**. Inhibition of HAdV infection in the plaque assays and effects on cellular viability

Table 2. 4-Acyl-2-substituted piperazine thiourea derivatives **56–67**. Inhibition of HAdV infection in the plaque assays and effects on cellular viability.

Table 3. 4-(2-substituted acetyl)-2-phenylpiperazine urea derivatives **68–74,** diamide **75** and diurea **76** derivatives. Inhibition of HAdV infection in the plaque assays and effects on cellular viability.

Table 4. Core modified urea derivatives **79–85**, **87–93**. Inhibition of HAdV infection in the plaque assays and effects on cellular viability for compounds

Table 5. IC_{50}, CC_{50}, SI and yield reduction values for the selected compounds and the prototypes **6**–**11**

Table 6. CalcuSyn output for the different combinations of the three anti-HAdV compounds. The combinatory index values are shown for the combinations at the IC_{50}, IC_{75}, and IC_{90} levels of inhibition. The r value for each combination is also reported to indicate the correlation coefficient of the data to the mass-action law.