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Original article

Synthesis of 1-naphtylpiperazine derivatives as serotoninergic ligands and their evaluation as antiproliferative agents

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ABSTRACT

Serotonin (5-hydroxytryptamine, 5-HT) is one of the most important neuromediator involved in numerous physiological and pathophysiological processes. In addition it is well established that 5-HT acts as a growth factor on several types of non-tumoral and tumoral cells, and recently it was also related to oncogenes. 5-HT_{1A} receptor expression was identified in prostatic tumor cell lines (PC3 cells) and in human hormone refractory prostate cancer tissue. Based on these observations, development of 5-HT_{1A} antagonists could be useful in inhibiting the growth of cancer cells. In order to investigate on potential use of 5-HT_{1A} ligands as antiproliferative agents, we have analyzed a new set of 1-naphtylpiperazine derivatives. In binding studies, several molecules showed affinity in nanomolar and subnanomolar range at 5-HT_{1A} and moderate to no affinity for other relevant receptors (5-HT_{2A}, 5-HT_{2C}, D_1, D_2, α_1 and α_2). All compounds were then evaluated in order to assess their antiproliferative activity using PC3 cells and the most active compounds (**1** and **2**) were fully characterized to define the mechanism responsible for the observed antiproliferative effect.

1. Introduction

Serotonin (5-hydroxytryptamine, 5-HT) has been the subject of intense biological research since its synthesis in 1951 [1]. Among several mediators released by neuroendocrine cells, 5-HT is one of the most important targets for medicinal chemistry because of its involvement in numerous physiological and pathophysiological processes [2–5]. Serotonin receptors (5-HTRs) may be involved in impulsivity and alcoholism [6,7], and in the different phases of sleep [8], sexual behavior, appetite control, thermoregulation, and cardiovascular function [9,10]. In addition it is well established that 5-HT plays a fundamental role in regulation of growth, differentiation, and gene expression [11]. Moreover serotonin is known to act as a growth factor on several types of non-tumoral and tumoral cells, and recently it was also related to oncogenes [11]. 5-HTRs comprise a large and complex family of receptors. Seven classes have been identified, based on functional, structural, and pharmacological characterization. With the exception of 5-HT_3, which is a ligand-gated ion channel, all of these are known to be G-protein coupled receptors (GPCRs) with structural, pharmacological, and transductional characteristics [12]. Besides the already known involvement of 5-HTRs in the regulation of several psychiatric and neurological disorders related to serotoninergic neurotransmission, specific receptor subtypes have been recently associated with tumor growth. In particular, 5-HT_{1A} receptor is the most widely studied subtype even if signal transduction of this receptor is complex and the exact mechanism of action is yet to be determinated. 5-HT_{1A} receptor, as a member of the group of GPCRs [13], shows high similarity with other members of the family; in particular it shows a high degree of homology (45%) with α_1-adrenoreceptor [14]. In addition to its effects on a wide range of psychiatric disorders, 5-HT_{1A}R is also involved in the proliferation of human tumor cells. In fact 5-HTRs expression has been identified in PC3 cells and in human hormone refractory prostate cancer tissue [15]. Based on these observations, as already reported, the development of 5-HT_{1A} antagonists and/or serotonin-uptake inhibitors could be useful to

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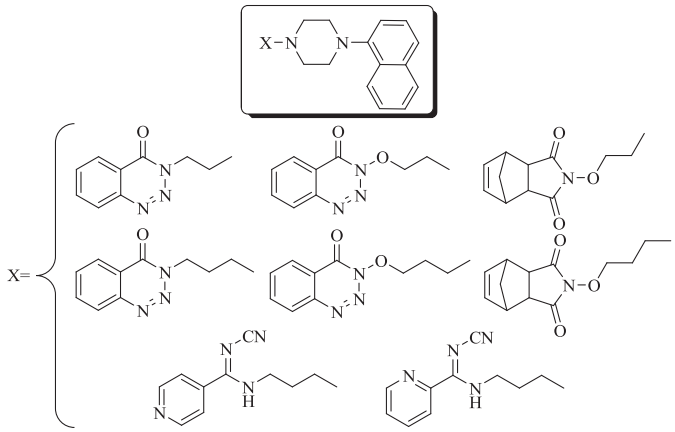


Fig. 1. General structures.

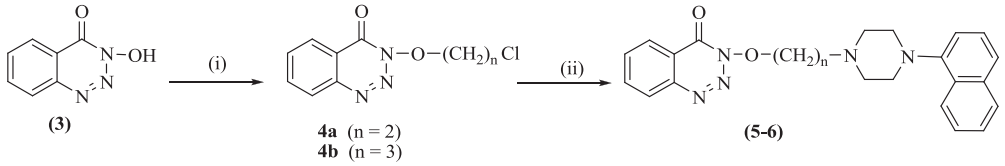
inhibit the growth of prostatic tumor cell lines (PC3) [11,15]. Our research group has been involving for a long time in developing new 5-HT_{1A} ligands [16–23] in order to have novel pharmacological tools that could improve our knowledge of the 5-HT signal transduction mechanism and lead to compounds with high affinity and selectivity. In continuation of our research program and in order to shed more light on potential use of 5-HT_{1A} ligands as antiproliferative agents, we have analyzed a new set of derivatives where a 1-naphtylpiperazine moiety has been linked to a 1,2,3-benzotriazinone, 3-hydroxy-1,2,3-benzotriazione, norbornene, N′-cyanoisonicotinamidine and N′-cyanopicolinamidine fragment respectively. These nuclei were linked via two or three methylene spacing units to the 1-naphtylpiperazine moiety (Fig. 1). The naphtylpiperazine scaffold was selected on the basis of previous investigations demonstrating how 1-naphtylpiperazines, acting as 5-HT_{2A} receptor antagonists, were also able to block skin cancer induction, probably because of an apoptotic mechanism [24]. Instead the choice of the different nuclei was performed on the basis of our previous investigations [16–23], where, as a general trend, different compounds with a 1,2,3-benzotriazinone, 3-hydroxy-1,2,3-benzotriazione, norbornene, N′-cyanoisonicotinamidine fragment, linked to different 4-substituted piperazines through different alkyl chain length, showed good and preferential affinity for the 5-HT_{1A} receptor. All the new compounds were initially tested for their affinity on 5-HT_{1A}, 5-HT_{2A} and 5-HT_{2C} receptors. Moreover, the multireceptor profiles of promising derivatives were also evaluated in terms of binding affinities for dopaminergic (D_1, D_2) and adrenergic (α_1, α_2) receptors. Successively all compounds were evaluated for their antiproliferative activity using prostate cancer derived cell line PC3.

2. Chemistry

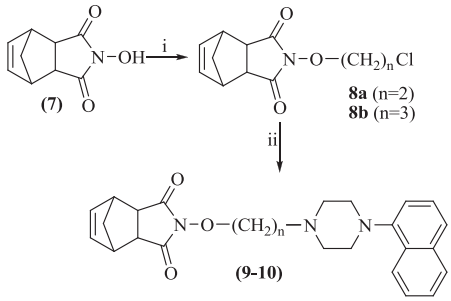
The synthesis of compounds **1** and **2** was already reported in a previous paper [21]. The synthetic strategy employed for the preparation of final compounds **5** and **6** is summarized in Scheme 1. Alkylation of the starting heterocycle 3-hydroxy-1,2,3-benzotriazin-4(3H)-one with 1-bromo-2-chloroethane or 1-bromo-3-chloropropane, in presence of K_2CO_3 in DMF, gave the corresponding chloroalkoxy benzotriazinone derivatives (**4a** and **4b**). The obtained intermediates were condensed with 1-naphtylpiperazine in DMF in presence of K_2CO_3, NaI, under reflux to give the final products **5** and **6**. Compounds **9** and **10** were synthesized following the general procedure summarized in Scheme 2. Alkylation of the starting heterocycle endo-N-hydroxy-5-norbornene-2,3-dicarboximide with 1-bromo-2-chloroethane or 1-bromo-3-chloropropane, in presence of NaOH in absolute ethanol, gave the corresponding chloro-alkyl norbornene derivatives **8a** and **8b.** Subsequent condensation of intermediates **8a** and **8b** with 1-naphtylpiperazine, performed in CH_3CN in presence of K_2CO_3 and NaI, under reflux, provided the final compounds **9** and **10**, respectively.

The synthetic strategy employed for the preparation of the N′-cyano-N-(3-(4-(naphthalen-1-yl)piperazin-1-yl)propyl)isonicotinamidine (**14**) is summarized in Scheme 3: isopropyl-N-cyano-4-pyridinecarboximidate **(12)** was prepared starting from 4-cyanopyridine **(11)** by base-promoted reaction with isopropanol followed by treatment with cyanamide in aqueous phosphate buffer ($NaH_2PO_4 \cdot 2H_2O$:Na_2HPO_4 = 4:1). Compound **(12)** was converted to N-(3-bromopropyl)-N′-cyanoisonicotinamidine **(13)** by reaction with 3-bromopropylamine·HBr and NaOMe in methanol. Subsequent condensation of compound **(13)** with 1-naphtylpiperazine, performed in CH_3CN in presence of K_2CO_3 and NaI, under reflux, provided the final compound **14**. Finally the synthesis of the N′-cyano-N-(3-(4-(naphthalen-1-yl) piperazin-1-yl)propyl)picolinamidine (**15**) is summarized in Scheme 4: methyl-N-cyano-2-pyridinecarboximidate **(16)** was prepared starting from 2-cyanopyridine **(15)** by base-promoted reaction with anhydrous methanol followed by treatment with cyanamide in aqueous phosphate buffer ($NaH_2PO_4 \cdot 2H_2O$: Na_2HPO_4 = 4:1). Compound **(16)** was converted to N-(3-bromopropyl)-N′-cyanopicolinamidine **(17)** by reaction with 3-bromopropylamine·HBr and triethylamine in anhydrous methanol. Subsequent condensation of compound **(17)** with 1-naphtylpiperazine, performed in CH_3CN in presence of K_2CO_3 and NaI, under reflux, provided the final compound **18**.

Purification of each final product was performed by chromatography on silica gel column followed by crystallization from the appropriate solvent. All new compounds gave satisfactory elemental analyses and were characterized by ^{1}H-NMR and mass spectrometry (API 2000 Applied Biosystem). ^{1}H-NMR and MS data for all final compounds were consistent with the proposed structures. As already reported in literature, due to tautomeric equilibrium about sp^2 carbon, cyanoamidine derivatives **14** and **18** may exist as a mixture of two tautomers. The NMR spectra of the final compounds **14** and **18**, confirmed that these compounds exist predominantly as the cyanoimino form (I). Moreover, about the configurational determination of geometrical isomers (E/Z) on the amidine bond C=N, the N-cyanoamidine derivatives **14** and **18** exhibited only one set of relevant ^{1}H-NMR signals, implying the existence of either a single geometric form or, more probably, a fast equilibrium of two isomeric forms as already described in literature [23].



Scheme 1. Reagents and conditions: (i) $Br(CH_2)_nCl$, K_2CO_3, DMF; (ii) 1-naphtylpiperazine, K_2CO_3, NaI, DMF.



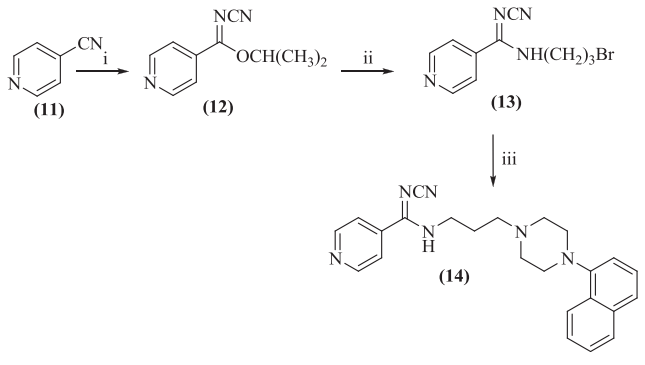
Scheme 2. Reagents and conditions: (i) $Br(CH_2)_nCl$, NaOH, absolute EtOH, 70 °C, 24 h; (ii)1-naphtylpiperazine, K_2CO_3, NaI, CH_3CN, reflux, 24 h.

3. Results and discussion

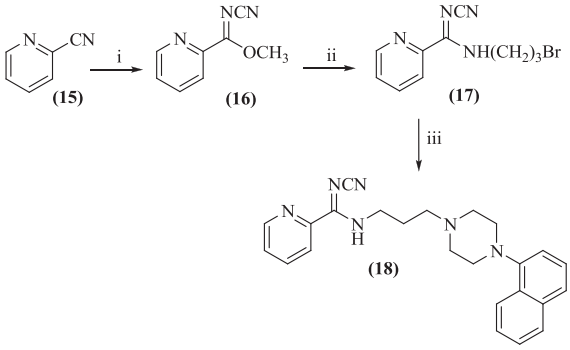
Among the reported naphtylpiperazine derivatives, six compounds (**5**, **6**, **9**, **10**, **14** and **18**) represent new derivatives that were synthesized and fully evaluated for affinity and selectivity on 5-HT_{1A}, 5-HT_{2A} and 5-HT_{2C} receptors and successively tested as antiproliferative agents. Other two derivatives (**1** and **2**) were already reported in our previous paper [21] as potent and selective 5-HT_{1A} receptor ligands and now they were selected in order to investigate the antiproliferative activity on PC3 cell lines. Introduction of different heterocyclic nuclei as terminal part, slight modifications concerning the alkyl or alkoxy spacer chain length (two or three units) are depicted in Fig. 1 and were performed in an effort to obtain compounds with high affinity and selectivity for 5-HT_{1A} over other serotoninergic receptors, as well as dopaminergic and adrenergic receptors. As anticipated on the basis of our previous investigations, several of these compounds were potent 5-HT_{1A} receptor ligands. In fact, they showed nanomolar or even subnanomolar 5-HT_{1A} receptor affinities (Table 1). Besides the already reported [21] 5-HT_{1A} receptor affinity of compound **1** (K_i = 0.000178 nM), most K_i values were clustered in a relatively narrow range from 0.293 nM **(10)** to 7.79 nM **(2)**. Only compounds **5, 6** and **14** were inactive with K_i values $> 10^4$ nM. Therefore the influence of the alkyl chain length, observed for our compounds, does not seem to be decisive in determining a general trend but the affinity/selectivity profile is more influenced by the particular heterocyclic nucleus linked on the naphtylpiperazine moiety.

The 5-HT_{2A} and 5-HT_{2C} receptor affinities of the tested compounds were always lower than those observed for 5-HT_{1A} receptors and ranged from 5.29 **(1)** to 1320 nM **(5)** for 5-HT_{2A} and $>10^4$ nM or no affinity for 5-HT_{2C} receptors.

Additionally, the affinity of all compounds on several other receptors (α_1 and α_2 adrenergic and D_1 and D_2 dopaminergic receptors) was examined in order to verify the selectivity of these



Scheme 3. Reagents and conditions: (i) CH_3ONa, H_2NCN, iPrOH; (ii) $Br(CH_2)_3NH_2$, CH_3ONa, anhydrous MeOH; (iii) 1-naphtylpiperazine, K_2CO_3, NaI, CH_3CN, 70 °C, 4 h.



Scheme 4. Reagents and conditions: (i) CH_3ONa, H_2NCN, anhydrous MeOH; (ii) $Br(CH_2)_3NH_2$, CH_3ONa, anhydrous MeOH; (iii) 1-naphtylpiperazine, K_2CO_3, NaI, CH_3CN, 70 °C, 4 h.

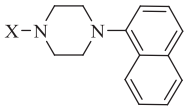
compounds. Results are summarized in Table 2. All the compounds proved highly selective against dopaminergic receptors with K_i values of above 10^4 nM except for compound **6**, which exhibited K_i values of 85.4 nM on D_2 receptor. Regarding α_1 and α_2 adrenergic receptors, only compounds **1** and **10** showed quite moderate affinities (112 and 189 nM respectively). The high selectivity toward α_1 receptors, exhibited by 1-naphtylpiperazine derivatives, is very interesting considering that the amino acid sequence of the transmembrane part of 5-HT_{1A}R is highly homologous to that of the α_1 adrenergic receptor.

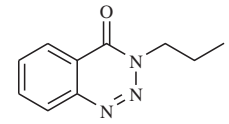
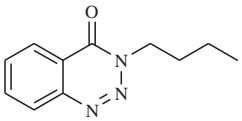
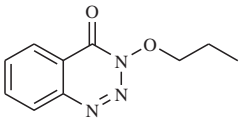
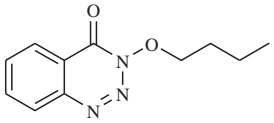
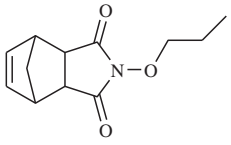
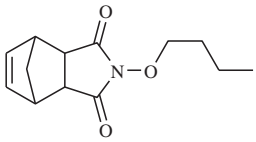
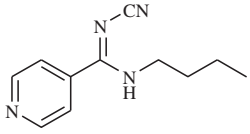
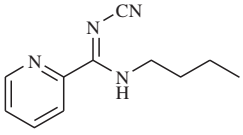
Growth inhibition assay was performed in order to define the biological profile of the synthesized compounds. It is well known that 5-HT_{1A} receptor exhibits a high degree of homology with α_1-ARs (α_{1D}- and α_{1B}-ARs) [14] that are expressed in PC3 prostate cancer cells and are involved in modulation of cell proliferation and apoptosis [35]. Moreover, it has been demonstrated that 5-HT plays a fundamental role in regulation of growth, differentiation and gene expression. On the basis of these observations, all compounds were examined for their capability to inhibit the growth of PC3 prostate cancer cells. Results, depicted in Fig. 2, showed that four of the eight analyzed compounds (**1**, **2**, **6**, **10** and **18**) induced, after 96 h of treatment, a dose-dependent growth inhibition of PC3 cells with IC_{50} ranging between 10.5 and 23.75 μM, as shown in Table 3. Compound **9** induced growth inhibition but with higher IC_{50} value (47.7 μM), while compounds **5** and **14**, but also the 5-HT_{1A} antagonist NAN-190 and the 5-HT_{1A} agonist 8-OH-DPAT (data not shown) did not show antiproliferative effect in these conditions.

Successively, in order to define the mechanism of the antiproliferative effect observed for two of the most active compounds (**1** and **2**), we analyzed their ability to induce apoptosis or cell cycle perturbation. Apoptosis was evaluated by measuring the externalization of phosphatidylserine (PS) on the outer layer of the plasma membrane, one of the first characteristic features of apoptotic cell death, by staining cells with Annexin-V-FITC and performing flow cytometry analysis. As shown in Fig. 3 of neither **1** nor **2** (using for both 15 μM) induced after 24 or 48 h any apoptotic effect in PC3 cells compared with cells treated with the positive control represented by the topoisomerase I inhibitor Topotecan (1 μM).

On the other hand, any significant perturbation of cell cycle was observed when cells were treated with either **1** or **2**. In details both compounds induced only a slight increase of percentage of cells in phase G2-M after 24, 48 and 72 h of treatment (Fig. 4a–c). However, similar results were obtained with NAN-190 and 8-OH-DPAT, two compounds that did not induce any growth inhibition or apoptotic effects in PC3 cells. On the contrary, Topotecan-treatment induced after 24 h an increase of cells in G2-M phase and the appearance of a sub-G1, peak indicating a subpopulation of apoptotic cells, with

Table 1
Affinities of 1-naphtylpiperazine derivatives for 5-HT_{1A}, 5-HT_{2A} and 5-HT_{2C} receptors.



Compd	Substituent	Receptor affinity Ki ± SD (nM)[a]		
	X	5-HT_{1A} [^{3}H]8-OHDPAT	5-HT_{2A} [^{3}H]Ketanserin	5-HT_{2C} [^{3}H]Mesulergine
1		0.000178 ± 0.00003	5.29 ± 0.3	$>10^4$
2		7.79 ± 0.06	127 ± 17	$>10^4$
5		$>10^4$	1320 ± 119	no affinity
6		$>10^4$	721 ± 33.7	no affinity
9		1.58 ± 0.134	113 ± 49.8	no affinity
10		0.293 ± 0.025	91.8 ± 5.1	no affinity
14		$>10^4$	172 ± 11.5	no affinity
18		5.85 ± 0.187	224 ± 12.8	no affinity

[a] For purpose of comparison, 8-OH-DPAT, Ketanserine and Mesulergine binds 5-HT_{1A}, 5-HT_{2A} and 5-HT_{2C} receptors with values of 0.80, 0.85 and 1.90 nM, respectively, under these assay conditions.

a complete disruption of cell cycle kinetic and many cells in sub-G1 after 48 and 72 h of treatment.

It has been previously shown that the intracellular signaling by which serotonin induces mitogenesis is quite complex and involves the stimulation of several signaling pathways that are only partially described. Among these pathways MAPK and AKT pathways are apparently the most relevant to regulate proliferation. On these bases we evaluated in PC3 cells the effects of **1**, **2**, NAN-190, 8-OH-DPAT and topotecan on the activation of MAPK and AKT by western blotting.

We demonstrated that 48 h of treatment with compounds **1**, **2** and topotecan did not reduce basal MAPK phosphorylation while all tree agents induced a complete inhibition of AKT phosphorylation (Fig. 5). The constitutive activation of MAPK signal due to KRAS mutation in PC3 cells might explain the lack of effect on the phosphorylation of MAPK. On the other hand the inhibition of AKT activity, which has been associated with pro-survival signaling in cancer cells, may explain at least in part the growth inhibitory activity induced by both compounds **1** and **2**. In contrast with data

Table 2
Affinities of 1-naphtylpiperazine derivatives for D_1, D_2, α_1 and α_2 receptors.

Compd	Receptor affinity Ki ± SD (nM)			
	D_1	D_2	α_1	α_2
	[^{3}H]SCH-23390	[^{3}H]spiperone	[^{3}H]prazosin	[^{3}H]yohimbine
1	$>10^4$	$>10^4$	112 ± 47	1880 ± 107
2	$>10^4$	$>10^4$	562 ± 170	3600 ± 458
5	$>10^4$	$>10^4$	$>10^4$	$>10^4$
6	$>10^4$	85.4 ± 0.41	800 ± 6.3	$>10^4$
9	$>10^4$	$>10^4$	$>10^4$	1200 ± 175
10	$>10^4$	263 ± 5.8	$>10^4$	189 ± 21.8
14	$>10^4$	1600 ± 137	$>10^4$	878 ± 28.2
18	$>10^4$	$>10^4$	$>10^4$	$>10^4$

Table 3
IC_{50} values computed at 96 h of treatment ($IC_{50}^{96\ h}$) in PC3 cell lines.

Compounds	IC_{50} (μM)
1	10.6 ± 2.06
2	10.5 ± 1.66
5	N.D
6	12.35 ± 1.6
9	47.7 ± 5.9
10	13 ± 1.4
14	N.D
18	23.8 ± 2.47

Note: Mean ± SD of at least three different experiments done in quadruplicate.

reported by Dizeyi et al. [36], we did not observe any change in the phosphorylation of MAPK or AKT in cells treated with NAN-190 and 8-OH-DPAT. This discrepancy can be probably explained by the different timing of the treatment since the authors evaluated the effect on the signaling after few hours of treatment while our experiment was performed at 48 h. Further studies are needed to better define at molecular level the mechanism of the antiproliferative effects we have observed.

4. Conclusion

We have described the synthesis of new series of 1-naphtylpiperazine derivatives, containing different heterocyclic fragments, as serotoninergic ligands with an antiproliferative activity on PC3 cells.

Some of the newly synthesized compounds showed high *in vitro* affinity and selectivity toward 5-HT_{1A} receptor. Moreover, the most interesting derivatives (**1**, **2**, **6**, **10** and **18**) induced, after 96 h of treatment, a dose-dependent growth inhibition of PC3 cells with IC_{50} values ranging between 10.5 and 23.75 μM, as shown in Table 3. In order to define the mechanism of the antiproliferative effects observed for two of the most active compounds (**1** and **2**) we have demonstrated as these derivatives are not able to induce apoptosis or cell cycle perturbation. On these basis we have evaluated in PC3 cells the effects of compounds **1** and **2** on the activation of MAPK and AKT as important signaling pathways, that are apparently the most relevant to regulate cell proliferation and directly connected to the signaling pathways of 5-HT_{1A} receptors. The evidence that compounds **1** and **2** are able to determine inhibition of AKT activity, which has been associated with pro-survival signaling in cancer cells, may explain the growth inhibitory activity induced by these derivatives. Finally, further studies could be really useful to shed more light on the mechanism of action of the

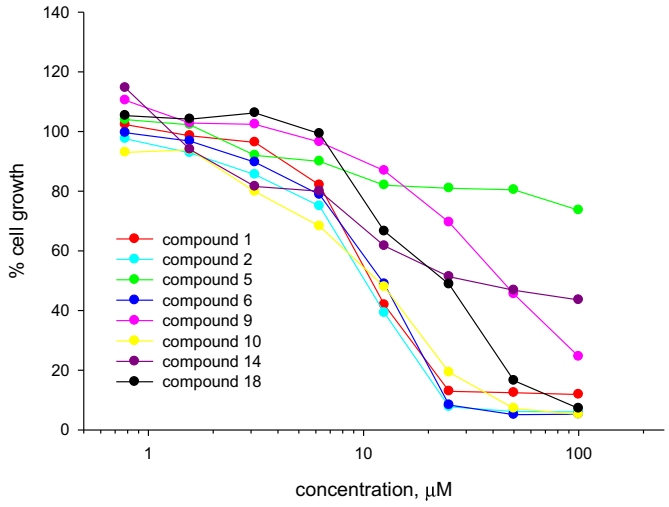


Fig. 2. Antiproliferative effect of compounds **1**, **2**, **5**, **6**, **9**, **10**, **14**, **18** in PC3 cell lines. PC3 cells were seeded at 900 per well in 96-multiwell and 24 h after 10%-FBS RPMI medium was replaced with 1%-FBS RPMI medium and compounds were added at the indicated concentrations. Cell growth assessment was done by sulforhodamine B colorimetric assay after 96 h of treatment (see Materials and Methods). Cell growth is expressed as percentage of control for each time point. Points, mean of quadruplicates. Standard deviation (SD) was always less than 10%.

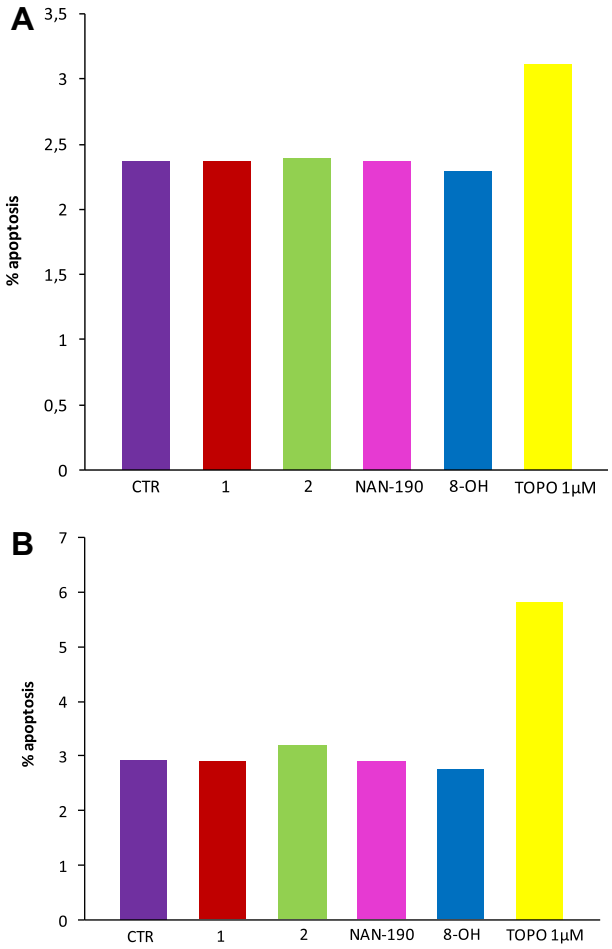


Fig. 3. Apoptosis was evaluated by flow cytometry analysis with Annexin-V-FITC. 24 h after seeding of PC3 cells, 10%-FBS RPMI medium was replaced with 1%-FBS RPMI medium and cells were untreated or treated for 24 h (A) or 48 h (B) with compounds **1**, **2**, NAN-190, 8-OH-DPAT (15 μM) and Topotecan (2 μM).

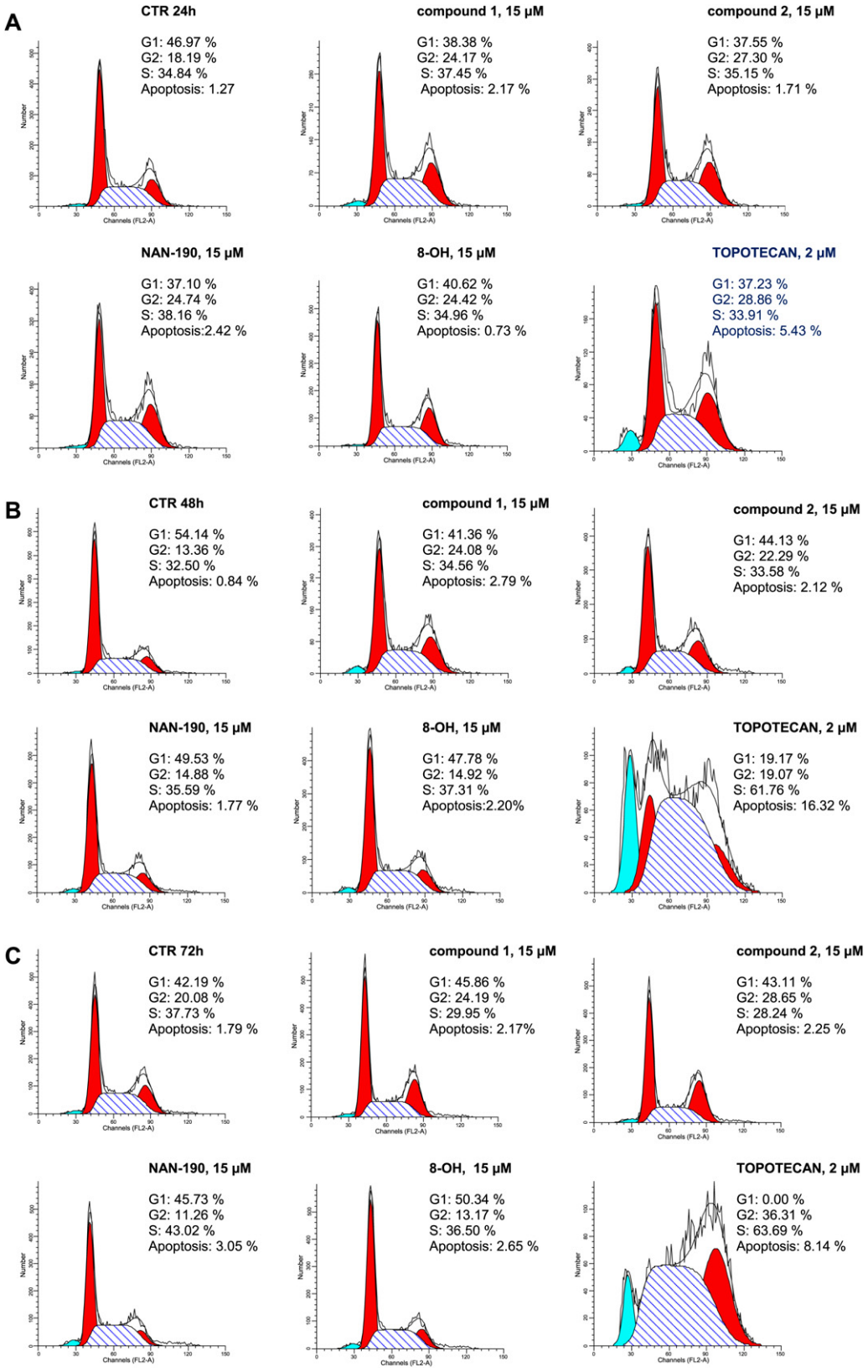


Fig. 4. Cell cycle analysis was performed after PI staining. 24 h after seeding of PC3 cells, 10%-FBS RPMI medium was replaced with 1%-FBS RPMI medium and cells were untreated or treated with compounds **1**, **2**, NAN-190, 8-OH-DPAT (15 µM) and Topotecan (2 µM) for 24 h (A), 48 h (B) or 72 h (C).

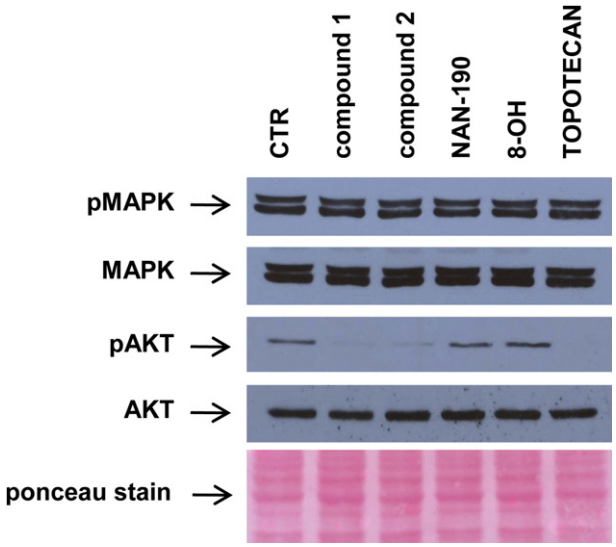


Fig. 5. MAPK and AKT activations were determined by western blotting. 24 h after seeding of PC3 cells, 10%-FBS RPMI medium was replaced with 1%-FBS RPMI medium and cells were untreated or treated with compounds **1**, **2**, NAN-190, 8-OH-DPAT (15 μM) and Topotecan (2 μM) for 48 h 70 μg of whole cell lysates were resolved by 10%-SDS-PAGE, and then immuoblotted with pMAPK, MAPK, pAKT and AKT antibodies. Ponceau staining ensured the equal loading of samples in each lane.

analyzed compounds that could represent new interesting leads as antiproliferative agents on prostatic cancer.

5. Experimental

5.1. Synthesis

5.1.1. General procedures

All reagents and substituted piperazines were commercial products purchased from Aldrich. Melting points, determined using a Buchi Melting Point B-540 instrument, are uncorrected and represent values obtained on recrystallized or chromatographically purified material. ^{1}H-NMR spectra were recorded on Varian Mercury Plus 400 MHz instrument. Unless otherwise stated, all spectra were recorded in $CDCl_3$. Chemical shifts are reported in ppm using Me_4Si as internal standard. The following abbreviations are used to describe peak patterns when appropriate: s (singlet), d (doublet), t (triplet), m (multiplet), q (quartet), qt (quintet), dd (double doublet), ddd (double double doublet), bs (broad singlet), m (multiplet). Mass spectra of the final products were performed on API 2000 Applied Biosystem mass spectrometer. Elemental analyses were carried out on a Carlo Erba model 1106; analyses indicated by the symbols of the elements were within ±0.4% of the theoretical values. All reactions were followed by TLC, carried out on Merck silica gel 60 F_{254} plates with fluorescent indicator and the plates were visualized with UV light (254 nm). Preparative chromatographic purifications were performed using silica gel column (Kieselgel 60). Solutions were dried over Na_2SO_4 and concentrated with a Buchi R-114 rotary evaporator at low pressure.

5.1.2. General procedure for preparation of 3-(2 or 3 chloroalkoxy)-1,2,3-benzotriazin-4(3H)-one (*4a* and *4b*)

A mixture of 1-bromo-2-chloroethane or 1-bromo-3-chloropropane (0.09 mol), 3-hydroxy-1,2,3-benzotriazin-4H-one (**3**) (0.03 mol), NaOH (0.05 mol) in DMF (70 mL) was stirred at 70 °C for 24 h. After cooling, the mixture was concentrated to dryness and the residue was dissolved in water (50 mL); the solution was extracted several times with CH_2Cl_2. The organic phase was dried, concentrated and chromatographed on silica gel column (diethyl ether/methanol, 9:1 v/v) to give compounds **4a** and **4b** as solids (obtained yields: **4a** 80%; **4b** 94%). ^{1}H-NMR spectra for all intermediates were consistent with the proposed structures.

5.1.3. General procedure for preparation of 3-[2 or 3-(4-naphthalen-piperazin-1-yl)-alkoxy]-1,2,3-benzotriazin-4(3H)-one (*5*–*6*)

A mixture of appropriate 3-(*n*-chloroalkoxy)-1,2,3-benzotriazin-4(3H)-one **4a** or **4b** (0.03 mol) and NaI (0.05 mol) in DMF (50 mL) was stirred under reflux for 30 min. Then 1-naphtylpiperazine (0.03 mol) and K_2CO_3 (0.05 mol) were added. The reaction mixture was stirred under reflux for 24 h. After cooling, the mixture was concentrated to dryness and the residue was dissolved in water (50 mL). The solution was extracted several times with CH_2Cl_2. The organic phase was dried on anhydrous Na_2SO_4, concentrated and chromatographed on silica gel column (diethyl ether/ethanol 9:1 v/v) to give the final compounds **(5–6)** as white crystalline solids.

5.1.4. 3-(2-(4-(naphthalen-1-yl)piperazin-1-yl)ethoxy)benzo[d][1,2,3]triazin-4(3H)-one (*5*)

Yield: 30%. Mp: 85–87 °C. ^{1}H-NMR ($CDCl_3$) δ 2.85 (m, 4H), 3.00 (m, 4H), 3.06 (t, 2H, J = 6.8 Hz, CH_2-N-pip), 4.67 (t, 2H, J = 6.9 Hz, CH_2-N-benz), 6.93(d,1H, J = 8.1 Hz, ArH), 7.34 (t, 1H, J = 7.6 Hz, ArH), 7.45 (m, 2H, ArH), 7.51(d,1H, J = 8.1 Hz, ArH), 7.79 (m, 2H, ArH), 7.95 (t, 1H, J = 7.6 Hz, ArH), 8.17 (m, 2H, ArH), 8.38 (d,1H, J = 8.1 Hz, ArH). ESI-MS: 402.1 $[M + H]^+$.

Anal. ($C_{23}H_{23}N_5$), C, H, N.

5.1.5. 3-(3-(4-(naphthalen-1-yl)piperazin-1-yl)propoxy)benzo[d][1,2,3]triazin-4(3H)-one (*6*)

Yield: 45%. Mp: 90–92 °C ^{1}H-NMR ($CDCl_3$) δ 2.14 (q, 2H, J = 6.9 Hz, $-CH_2-$), 2.78 (t, 2H, J = 6.8 Hz, CH_2-N-pip), 2.90 (m, 4H), 3.15 (m, 4H), 4.56 (t, 2H, J = 6.9 Hz, CH_2-N-benz), 7.07 (d, 1H, J = 8.1 Hz, ArH), 7.37 (t, 1H, J = 7.6 Hz, ArH), 7.45 (m, 2H, ArH), 7.46 (d,1H, J = 8.1 Hz, ArH), 7.80 (m, 2H, ArH), 7.96 (t, 1H, J = 7.6 Hz, ArH), 8.20 (m, 2H, ArH), 8.39 (d,1H, J = 8.1 Hz, ArH). ESI-MS: 416.9 $[M + H]^+$.

Anal. ($C_{24}H_{25}N_5$), C, H, N.

5.1.6. General procedure for preparation of 4-(2 or 3 chloroalkoxy)-4-aza-tricyclo[5.2.1.02,6]dec-8-ene-3,5-dione (*8a* and *8b*)

A solution of absolute ethanol (50 mL) and sodium hydroxide 0.72 g (0.018 mol) was reacted with 3.22 g (0.018 mol) of commercially available endo-N-hydroxy-5-norbornene-2,3-dicarboximide **(7)** and 2.58 g (0.018 mol) of 1-bromo-2-chloroethane or 1-bromo-3-chloropropane at 70 °C for 24 h. Afterward the mixture was cooled to room temperature, concentrated to dryness and the residue diluted in water (40 mL). The solution was extracted several times with CH_2Cl_2. The combined organic layers were dried on anhydrous Na_2SO_4 and concentrated *in vacuo*. The residue was purified by column chromatography (diethyl ether/ethanol 9:1 (v/v)). The combined and evaporated product fractions were crystallized from diethyl ether/hexane, to give the final compounds **8a** and **8b** as white solid (obtained yields **8a** 80%; **8b** 94%). ^{1}H-NMR spectra for all intermediates were consistent with the proposed structures.

5.1.7. General procedure for the preparation of 4-[2 or 3-[4-(naphthalen)piperazin-1-yl]alkoxy]-4-aza-tricyclo[5.2.1.02,6]dec-8-ene-3,5-dione (*9*–*10*)

A mixture of 4-(2-chloroethoxy)-4-aza-tricyclo[5.2.1.02,6]dec-8-ene-3,5-dione (**8a**) or 4-(3-chloro-propoxy)-4-aza-tricyclo [5.2.1.02,6]dec-8-ene-3,5-dione (**8b**) (0.006 mol), and NaI (0.009 mol) in acetonitrile was stirred under reflux for 30 min. Then 1-naphtylpiperazine (0.03 mol) and anhydrous K_2CO_3

(0.009 mol) were added. The reaction mixture was stirred under reflux for 24 h. After cooling, the mixture was filtered, concentrated to dryness and the residue was dissolved in water (50 mL). The solution was extracted several times with CH_2Cl_2. The combined organic layers were dried on anhydrous Na_2SO_4 and the solvent removed *in vacuo*. The crude mixtures were purified by silica gel column chromatography using diethyl ether/methanol 8:2 (v/v) as eluent. The crude products were recrystallized from diethyl ether.

*5.1.8. 4-[2-[4-(naphthalen)piperazin-1-yl]ethoxy]-4-aza-tricyclo [5.2.1.02,6]dec-8-ene-3,5-dione (**9**)*

Yield: 50%; mp 126–128 °C; ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 1.49 (d, 1H, $J = 8.9$); 1.76 (d, 1H, $J = 8.9$); 2.80 (bs, 4H, $2CH_2$ pip.); 2.82 (t, 2H, N^1-CH_2, $J = 7.3$); 3.16 (bs, 4H, $2CH_2$ pip.); 3.19 (s, 2H); 3.43 (s, 2H); 4.15 (t, 2H, $O-CH_2$, $J = 6.5$); 6.17 (s, 2H); 7.07(d, 1H, $J = 8.1$ Hz, ArH), 7.37 (t, 1H, $J = 7.6$ Hz, ArH), 7.44 (m, 2H, ArH), 7.53(d, 1H, $J = 8.1$ Hz, ArH), 7.80 (m, 1H, ArH), 8.17 (m, 1H, ArH). ESI-MS: 418.0 $[M + H]^+$.

Anal. ($C_{25}H_{27}N_3$), C, H, N.

*5.1.9. 4-[3-[4-(naphthalen)piperazin-1-yl]propoxy]-4-aza-tricyclo [5.2.1.02,6]dec-8-ene-3,5-dione **(10)***

Yield: 58%; mp 125–127 °C; ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 1.49 (d, 1H, $J = 8.9$); 1.75 (d, 1H, $J = 8.9$); 1.89 (q, 2H, $-CH_2-$, $J = 7.3$); 2.62 (t, 2H, N^1-CH_2, $J = 7.3$); 2.74 (bs, 4H, $2CH_2$ pip.); 3.13 (bs, 4H, $2CH_2$ pip.); 3.18 (s, 2H); 3.43 (s, 2H); 4.05 (t, 2H, $O-CH_2$, $J = 6.5$); 6.18 (s, 2H); 7.07(d, 1H, $J = 8.1$ Hz, ArH), 7.37 (t, 1H, $J = 7.6$ Hz, ArH), 7.44 (m, 2H, ArH), 7.52 (d, 1H, $J = 8.1$ Hz, ArH), 7.80 (m, 1H, ArH), 8.17 (m, 1H, ArH). ESI-MS: 432.9 $[M + H]^+$.

Anal. ($C_{26}H_{29}N_3$), C, H, N.

*5.1.10. Isopropyl-N-cyano-4-pyridinecarboximidate **(12)***

A mixture of 4-cyanopyridine **(11)** (15 g, 0.144 mol) and NaOMe (0.24 g, 0.0044 mol) in anhydrous isopropanol (180 mL) was stirred overnight at 0 °C. AcOH (0.25 g, 0.0042 mol) was then added with stirring and the solution was evaporated *in vacuo*. *n*-Hexane (150 mL) was added to the residue and the resultant precipitate was filtered off. The filtrate was evaporated to give crude isopropyl-4-pyridinecarboximidate (18.6 g) as an oil. Crude compound was then added to a mixture of NH_2CN (8.83 g, 0.21 mol), $NaH_2PO_4 \cdot 2H_2O$ (65.55 g) and Na_2HPO_4 (14.9 g) in water (112.5 mL). After vigorous stirring for 6 h at room temperature, the reaction mixture was extracted several times with CH_2Cl_2. The combined organic layers were dried on anhydrous Na_2SO_4 and concentrated *in vacuo* to yield crude isopropyl-N-cyano-4-pyridinecarboximidate **(12)** (22 g), which was used directly in the following reaction: ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 1.50 (d, 6H $J = 6.1$); 5.42 (m, 1H); 8.0–7.8 (m, 2H); 8.9–8.7 (m, 2H).

*5.1.11. N-(3-bromopropyl)-N′-cyanoisonicotinamidine **(13)***

To a solution of isopropyl-N-cyano-4-pyridinecarboximidate (21.89 g, 0.116 mol) in anhydrous MeOH (120 mL), 3-bromopropylamine·HBr (28.24 g, 0.129 mol) and NaOMe (6.77 g, 0.125 mol) were successively added and the reaction mixture was stirred at room temperature for 18 h. After evaporation, the residue was dissolved in $CHCl_3$. The solution was washed with water, dried on anhydrous Na_2SO_4 and concentrated *in vacuo*. The residue was purified by column chromatography (diethyl ether/ethanol 9:1 (v/v)). The combined and evaporated product fractions were crystallized from diethyl ether/hexane, yielding 12.5 g (40%) of the desired product as a white solid: mp: 201–203 °C; ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 2.30 (qt, 2H, $J = 6.2$); 3.51 (t, 2H $J = 6.2$); 3.72 (q, 2H, $J = 6.2$); 7.49 (d, 2H, $J = 5.5$); 8.83 (d, 2H, $J = 5.5$); 9.60 (s, 1H).

*5.1.12. N′-cyano-N-(3-(4-(naphthalen-1-yl)piperazin-1-yl)propyl) isonicotinamidine **(14)***

A mixture of N-(3-bromopropyl)-N′-cyanoisonicotinamidine **(13)** (0.006 mol), and NaI (0.009 mol) in acetonitrile was stirred under reflux for 30 min. Then 1-naphtylpiperazine (0.03 mol) and anhydrous K_2CO_3 (0.009 mol) were added. The reaction mixture was stirred under reflux for 4 h. After cooling, the mixture was filtered, concentrated to dryness and the residue was dissolved in water (50 mL). The solution was extracted several times with CH_2Cl_2. The combined organic layers were dried on anhydrous Na_2SO_4 and the solvent removed *in vacuo*. The crude mixture was purified by silica gel column chromatography using diethyl ether/methanol 8:2 (v/v) as eluent. The crude product was recrystallized from diethyl ether.

*5.1.13. N′-cyano-N-(3-(4-(naphthalen-1-yl)piperazin-1-yl)propyl) isonicotinamidine **(14)***

Yield: 25%; mp 158–160 °C; ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 1.63 (m, 2H); 1.89 (bs, 4H, $2CH_2$ pip.); 2.70 (t, 2H, N^1-CH_2, J=*5.1*); 2.76 (bs, 4H, $2CH_2$ pip.); 3.67 (bs, 2H, $N-CH_2$); 6.77 (d, 1H, $J = 8.1$ Hz, ArH); 7.37 (t, 1H, $J = 7.6$ Hz, ArH); 7.45 (m, 2H, ArH); 7.54 (m, 1H, ArH); 7.56 (d, 2H, J=*5.4*); 7.81 (bs, 1H, ArH); 8.09 (bs, 1H, ArH); 8.82 (d, 2H, $J = 5.4$); 9.74 (s, 1H). ESI-MS: 399.1 $[M + H]^+$; 421.2 $[M + Na]^+$; 437.1$[M + K]^+$.

Anal. ($C_{24}H_{26}N_6$), C, H, N.

*5.1.14. Methyl-N-cyano-2-pyridinecarboximidate **(16)***

A mixture of 2-cyanopyridine **(15)** (15 g, 0.192 mol) and NaOMe (0.24 g, 0.0096 mol) in anhydrous methanol (180 mL) was stirred overnight at room temperature. AcOH (0.0106 mol) was then added with stirring and the solution was evaporated *in vacuo*. *n*-Hexane (150 mL) was added to the residue and the resultant precipitate was filtered off. The filtrate was evaporated to give crude methyl-2-pyridinecarboximidate (26.3 g; 0.193 mol) as an oil. Crude compound was then added to a mixture of NH_2CN (0.384 mol), $NaH_2PO_4 \cdot 2H_2O$ (68.60 g) and Na_2HPO_4 (15.62 g) in water (140 mL). After vigorous stirring for 6 h at room temperature, the reaction mixture was extracted several times with CH_2Cl_2. The combined organic layers were dried on anhydrous Na_2SO_4 and concentrated *in vacuo* to yield crude methyl-N-cyano-2-pyridinecarboximidate **(16)** (19 g), which was used directly in the following reaction: ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 4.16 (s, 3H); 7.63 (dd, 1H, $J = 9.4, 7.3$); 7.94 (d, 1H, $J = 3.4$); 7.98 (dd, 1H, $J = 7.3, 2.4$); 8.83 (ddd, 1H, $J = 9.4, 3.4, 2.4$).

*5.1.15. N-(3-bromopropyl)-N′-cyanopicolinamidine **(17)***

To a solution of methyl-N-cyano-2-pyridinecarboximidate **(16)** (0.118 mol) in anhydrous MeOH (120 mL), 3-bromopropylamine·HBr (28.24 g, 0.129 mol) and triethylamine (0.129 mol) were successively added and the reaction mixture was stirred at room temperature for 18 h. After evaporation, the residue was dissolved in $CHCl_3$. The solution was washed with water, dried on anhydrous Na_2SO_4 and concentrated *in vacuo*. The residue was purified by column chromatography (diethyl ether/ethanol 9:1 (v/v)). The combined and evaporated product fractions were crystallized from diethyl ether/hexane, yielding 14.2 g (45%) of the desired product as a white solid: mp: 230–231 °C; ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 2.10 (qt, 2H, $J = 6.2$); 2.73 (t, 2H, $J = 6.2$); 3.89 (q, 2H, $J = 6.2$); 7.47 (t, 1H, $J = 7.3$); 7.86 (t, 1H, $J = 7.3$); 8.5 (d, 1H, $J = 7.3$); 8.64 (d, 1H, $J = 7.3$); 9.80 (s, 1H).

*5.1.16. N′-cyano-N-(3-(4-(naphthalen-1-yl)piperazin-1-yl)propyl) picolinamidine **(18)***

A mixture of N-(3-bromopropyl)-N′-cyanoisonicotinamidine **(17)** (0.006 mol), and NaI (0.009 mol) in acetonitrile was stirred

under reflux for 30 min. Then 1-naphtylpiperazine (0.03 mol) and anhydrous K_2CO_3 (0.009 mol) were added. The reaction mixture was stirred under reflux for 4 h. After cooling, the mixture was filtered, concentrated to dryness and the residue was dissolved in water (50 mL). The solution was extracted several times with CH_2Cl_2. The combined organic layers were dried on anhydrous Na_2SO_4 and the solvent removed *in vacuo*. The crude mixture was purified by silica gel column chromatography using diethyl ether/methanol 8:2 (v/v) as eluent. The crude product was recrystallized from diethyl ether.

5.1.17. *N′-cyano-N-(3-(4-(naphthalen-1-yl)piperazin-1-yl)propyl) picolinamidine* ***(18)***

Yield: 45%; mp 148–150 °C; ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 1.94 (qt, 2H, $J = 6.2$); 2.71 (t, 2H, $J = 6.2$); 2.82 (bs, 4H, $2CH_2$ pip.); 3.21 (bs, 4H, $2CH_2$ pip.); 3.89 (q, 2H, $J = 6.2$); 7.04 (d, 1H, $J = 8.1$); 7.41 (t, 1H, $J = 8.1$); 7.46 (m,3H, ArH); 7.56 (d,1H, $J = 8.1$ Hz, ArH); 7.82 (m,1H, ArH); 7.86 (t, 1H, $J = 7.3$); 8.16 (m,1H, ArH); 8.52 (d, 1H, $J = 7.3$); 8.61 (d, 1H, $J = 7.3$); 9.95 (s, 1H). ESI-MS: 399.1 $[M + H]^+$.

Anal. ($C_{24}H_{26}N_6$), C, H, N.

5.2. *In vitro receptor binding*

5.2.1. *General procedures*

The newly synthesized compounds were tested for *in vitro* affinity for serotonin 5-HT_{1A}, 5-HT_{2A} and 5-HT_{2C} receptors by radioligand binding assays. All the compounds were dissolved in ethanol or in 5% DMSO. The following specific radioligands and tissue sources were used: (a) serotonin 5-HT_{1A} receptor, [^{3}H]-8-OH-DPAT, rat brain cortex; (b) serotonin 5-HT_{2A} receptor, [^{3}H]ketanserin, rat brain cortex; (c) serotonin 5-HT_{2C} receptor, [^{3}H]mesulergine, rat brain cortex; (d) dopamine D_1 receptor [^{3}H]SCH-23390, rat striatum; (e) dopamine D_2 receptor [^{3}H]spiperone, rat striatum; (f) α_1 adrenergic receptor [^{3}H]prazosin, rat brain cortex; (g) α_2 adrenergic receptor [^{3}H]yohimbine, rat brain cortex.

Non-specific binding was determined as described in the experimental section, and specific binding as the difference between total and non-specific binding. Blank experiments were carried out to determine the effect of 5% DMSO on the binding and no effects were observed. Competition experiments were analyzed by the "Easy Fit" program [25] to obtain the concentration of unlabeled drug that caused 50% inhibition of ligand binding (IC_{50}), with six concentrations of test compounds, each performed in triplicate. The IC_{50} values obtained were used to calculate apparent inhibition constants (K_i) by the method of Cheng and Prussoff [26], from the following equation: $K_i = IC_{50}/(1 + S/K_D)$ where S represents the concentration of the hot ligand used and K_D its receptor dissociation constant (K_D values, obtained by Scatchard analysis [27], were calculated for each labeled ligand).

5.2.2. *5-HT_{1A} binding assay*

Radioligand binding assays were performed following a published procedure [28]. Cerebral cortex from male Sprague–Dawley rats (180–220 g) was homogenized in 20 volumes of ice-cold Tris–HCl buffer (50 mM, pH 7.7 at 22 °C) with a Polytron PT10, Brinkmann Instruments (setting 5 for 15 s), and the homogenate was centrifuged at 50000 *g* for 10 min. The resulting pellet was then resuspended in the same buffer, incubated for 10 min at 37 °C, and centrifuged at 50000 *g* for 10 min. The final pellet was resuspended in 80 volumes of the Tris–HCl buffer containing 10 μM pargyline, 4 mM $CaCl_2$, and 0.1% ascorbate. To each assay tube was added the following: 0.1 mL of the drug dilution (0.1 mL of distilled water if no competing drug was added), 0.1 mL of [^{3}H]-8-hydroxy-2-(di-n-propylamino)tetralin ([^{3}H]-8-OH-DPAT) (170.0 Ci/mmol, Perkin Elmer Life Sciences, Boston, MA, USA) in the same buffer as above to achieve a final assay concentration of 0.1 nM, and 0.8 mL of resuspended membranes. The tubes were incubated for 30 min at 37 °C, and the incubations were terminated by *vacuum* filtration through Whatman GF/B filters (Brandel Biomedical Research and Laboratories Inc., Gaithersburg, MD, USA). The filters were washed twice with 5 mL of ice-cold Tris–HCl buffer, and the radioactivity bound to the filters was measured by liquid scintillation spectrometer (Packard TRI-CARB® 2000CA – Packard BioScience s.r.l., Pero, Milan, Italy). Specific [^{3}H]-8-OH-DPAT binding was defined as the difference between binding in the absence and presence of 5-HT (10 μM).

5.2.3. *5-HT_{2A} and 5-HT_{2C} binding assays*

Radioligand binding assays were performed as previously reported by Herndon et al. [29]. Briefly, frontal cortical regions of male Sprague–Dawley rats (180–220 g) were dissected on ice and homogenized (1:10 w/v) in ice-cold buffer solution (50 mM Tris HCl, 0.5 mM EDTA, and 10 mM $MgCl_2$ at pH 7.4) with a Polytron PT10 (setting 5 for 15 s) and centrifuged at 3000 *g* for 15 min. The pellet was resuspended in buffer (1:30 w/v), incubated at 37 °C for 15 min and then centrifuged twice more at 3000 *g* for 10 min (with resuspension between centrifugations). The final pellet was resuspended in buffer that also contained 0.1% ascorbate and 10^{-5} M pargyline.

Assays were performed in triplicate in a 2.0 mL volume containing 5 mg wet weight of tissue and 0.4 nM [^{3}H]ketanserin hydrochloride (88.0 Ci/mmol; Perkin Elmer Life Sciences, Boston, MA, USA) for 5-HT_{2A} receptor assays, and 10 mg wet weight of tissue and 1 nM [^{3}H]mesulergine (87.0 Ci/mmol; Amersham Biosciences Europe GmbH) for 5-HT_{2C} receptor assays. Cinanserin (1.0 μM) was used to define nonspecific binding in the 5-HT_{2A} assay. In the 5-HT_{2C} assays, mianserin (1.0 μM) was used to define nonspecific binding, and 100 nM spiperone was added to all tubes to block binding to 5-HT_{2A} receptors. Tubes were incubated for 15 min at 37 °C, filtered on Schliecher and Schuell (Keene, NH, USA) glass fiber filters presoaked in polyethylene imine, and washed with 10 mL of ice-cold buffer. Filters were counted at an efficiency of 50%.

5.2.4. *D_1 dopaminergic binding assay*

The binding assay for D_1 dopaminergic receptors was that described by Billard et al. [30]. Corpora striata were homogenized in 30 vol. (w/v) ice-cold 50 mM Tris–HCl buffer (pH 7.7 at 25 °C) using a Polytron PT10 (setting 5 for 20 s). Homogenates were centrifuged twice for 10 min at 50000 *g* with resuspension of the pellet in fresh buffer. The final pellet was resuspended in 50 mM ice-cold Tris–HCl containing 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 0.1% ascorbic acid and 10 μM pargyline (pH 7.1 at 37 °C). Each assay tube contained 50 μL [^{3}H]SCH-23390 (85.0 Ci/mmol, Perkin Elmer Life Sciences, Boston, MA, USA) to achieve a final concentration of 0.4 nM, and 900 μL resuspended membranes (3 mg fresh tissue). The tubes were incubated for 15 min at 37 °C and the incubation was terminated by rapid filtration under *vacuum* through Whatman GF/B glass fiber filters. The filters were washed three times with 5 mL ice-cold 50 mM Tris–HCl buffer (pH 7.7 at 25 C). The radioactivity bound to the filters was measured by a liquid scintillation counter. Specific [^{3}H] SCH-23390 binding was defined as the difference between binding in the absence or in the presence of 0.1 μM piflutixol.

5.2.5. *D_2 dopaminergic binding assay*

The procedure used in the radioligand binding assay was reported in detail by Creese et al. [31]. Corpora striata were homogenized in 30 vol. (w/v) ice-cold 50 mM Tris–HCl buffer (pH 7.7 at 25 °C) using a Polytron PT10 (setting 5 for 20 s).

Homogenates were centrifuged twice for 10 min at 50000 *g* with resuspension of the pellet in fresh buffer. The final pellet was resuspended in 50 mM ice-cold Tris–HCl containing 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 0.1% ascorbic acid and 10 μM pargyline (pH 7.1 at 37 °C). Each assay tube contained 50 μL [^{3}H] spiperone (15.7 Ci/mmol, Perkin Elmer Life Sciences, Boston, MA, USA) to achieve a final concentration of 0.4 nM, and 900 μL resuspended membranes (3 mg fresh tissue). The tubes were incubated for 15 min at 37 °C and the incubation was terminated by rapid filtration under *vacuum* through Whatman GF/B glass fiber filters. The filters were washed three times with 5 mL ice-cold 50 mM Tris–HCl buffer (pH 7.7 at 25 °C). The radioactivity bound to the filters was measured by a liquid scintillation counter. Specific [^{3}H]spiperone binding was defined as the difference between binding in the absence or in the presence of 1 μM (+)–butaclamol.

5.2.6. α_1 adrenergic binding assay

The procedure used in the radioligand binding assay has been reported in detail by Greengrass and Brenner [32]. Brain cortex was homogenized in 30 vol. (w/v) ice-cold 50 mM Tris–HCl buffer, (pH 7.2 at 25 °C) using a Polytron PT10 (setting 5 for 20 s). Homogenates were centrifuged twice for 10 min at 50000 *g* with resuspension of the pellet in fresh buffer. The final pellet was resuspended in 50 mM ice-cold Tris–HCl, (pH 7.4 at 25 °C). Each assay tube contained 50 μL drug solution, 50 μL [^{3}H]prazosin (80.5 Ci/mmol, Perkin Elmer Life Sciences, Boston, MA, USA) to achieve a final concentration of 0.4 nM, and 900 μL resuspended membranes (10 mg fresh tissue). The tubes were incubated for 30 min at 25 °C and the incubation was terminated by rapid filtration under *vacuum* through Whatman GF/B glass fiber filters. The filters were washed three times with 5 mL ice-cold 50 mM Tris–HCl, buffer (pH 7.2 at 25 °C). The radioactivity bound to the filters was measured by a liquid scintillation counter. Specific [^{3}H]prazosin binding was defined as the difference between binding in the absence or in the presence of 10 μM phentolamine.

5.2.7. α_2 adrenergic binding assay

The procedure used in the radioligand binding assay was reported in detail by Perry and U'Prichard [33]. Brain cortex was homogenized in 30 vol. (w/v) ice-cold 5 mM tris–HCl, 5 mM EDTA buffer (pH 7.3 at 25 °C) using a polytron PT10 (setting 5 for 20 s). Homogenates were centrifuged three times for 10 min at 50000 *g* with resuspension of the pellet in fresh buffer. The final pellet was resuspended in 50 mM ice-cold Tris–HCl, 0.5 mM EDTA (pH 7.5 at 25 °C). Each assay tube contained 50 μL drug solution, 50 μL [^{3}H] yohimbine (80.5 Ci/mmol, Perkin Elmer Life Sciences, Boston, MA, USA) to achieve a final concentration of 1 nM, and 900 μL resuspended membranes (10 mg fresh tissue). The tubes were incubated for 30 min at 25 °C and the incubation was terminated by rapid filtration under *vacuum* through Whatman GF/B glass fiber filters. The filters were washed three times with 5 mL ice-cold 50 mM Tris–HCl, 0.5 mM EDTA buffer (pH 7.5 at 25 °C). The radioactivity bound to the filters was measured by a liquid scintillation counter. Specific [^{3}H]yohimbine binding was defined as the difference between binding in the absence or in the presence of 10 μM phentolamine.

5.3. Biology

5.3.1. Reagents

All media, serum, antibiotics and glutamine were purchased from Cambrex Bio Science (Verviers, Belgium). NAN-190 hydrobromide (NAN-190) and S(−)-8-hydroxy-DPAT hydrobromide (8-OH-DPAT) were from Sigma–Aldrich Co (St. Louis, MO, USA), Topotecan (Hycamtin®) was from GlaxoSmithKline S.p.A. – Brentford, UK. Sulforhodamine B (SRB) was from ICN Biomedicals (Irvine, CA, USA). Primary antibodies pMAPK, MAPK, pAKT, AKT were from Cell Signaling Technology, Inc. (Boston, MA, USA); γH2AX was from Upstate (Lake Placid, NY, USA). Secondary horseradish peroxidase-linked antibodies were from Bio-Rad (Richmond, CA, USA). Enhanced chemiluminescence (ECL) immunodetection reagents were from GE Healthcare (Buckinghamshire, UK). Annexin-V-FITC was from Becton Dickinson (San Jose, CA, USA).

5.3.2. Cell culture and cell proliferation assay

PC3 cell line was grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 50 units/mL penicillin, 500 μg/ml streptomycin, and 4 mmol/L glutamine in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. For cell growth assays, 1000 cells were seeded, in medium containing 10%-FBS, in quadruplicate in 96-multiwell plates (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ, USA), after 24 h medium was replace with a medium containing 10%-FBS or 1%-FBS as indicated and cells were treated with increasing concentrations of the indicated compounds. Cell survival/proliferation was measured by a spectrophotometric dye incorporation assay using sulforhodamine B (SRB) after 96 h from treatment as described before [34].

5.3.3. Protein extraction and western blotting

Cells grown and treated for 48 h with **1**, **2**, NAN-190, 8-OH-DPAT and Topotecan were washed once with ice-cold phosphate-buffered saline (PBS) and scraped in ice-cold PBS, and the cell pellets were lysed for 1 h at 4 °C in NP-40 lysis buffer (0.5% NP-40, 50 mM HEPES pH 7, 250 mM NaCl, 5 mM EDTA, 0.5 mM Na pyrophosphate, 0.5 mM Na orthovanadate, 50 mM NaF, 1 mM DTT, 0.5 mM PMSF, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 5 μg/ml benzamidine, 1 μg/ml pepstatin) and clarified by centrifugation. Equal amounts of proteins were separated by SDS-PAGE. Proteins were then transferred to nitrocellulose paper, immunoblotted with the appropriate antibody probed with the appropriate horseradish peroxidase-linked IgG and immunoreactive bands were detected by ECL.

5.3.4. Analysis of cell cycle kinetics

Analysis of cell cycle kinetics was performed on PC3 cells treated for 24 and 48 h with **1**, **2**, NAN-190, 8-OH-DPAT and Topotecan. Briefly, cells were harvested, fixed in 70% ethanol and stored at −20 °C until analysis. After nuclear DNA staining with propidium iodide (PI), flow cytometry was performed in duplicate by a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA). For each sample, 20,000 events were stored and cell cycle analysis was performed by the ModFit LT software (Verity Software House, Inc., Topsham, ME, USA). FL2 area versus FL2 width gating was done to exclude doublets from the G2-M region. The percentage of apoptotic cells was calculated in the sub-diploid region of the DNA content, registered as FL2 signals in linear scale. To avoid cell debris contamination due to necrotic cell death, cells were selected by side-scatter (SSC) versus DNA signals (FL2) gating.

5.3.5. Annexin-V assay

Annexin-V binding was identified by flow cytometry using Annexin-V-FITC staining as described previously [35]. Briefly, cells were harvested, centrifuged and washed twice with PBS, then resuspended in 1X binding buffer at a concentration of 10^6 cells/ml. The cell suspension was incubated with 5 μL of Annexin-V-FITC for 10 min at room temperature (20–25 °C) in the dark and then analyzed by Cellquest™ data analysis software (Becton Dickinson, San Jose, CA, USA).

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