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Sigma Receptor Ligands Carrying a Nitric Oxide Donor Nitrate Moiety: Synthesis, In Silico, and Biological Evaluation

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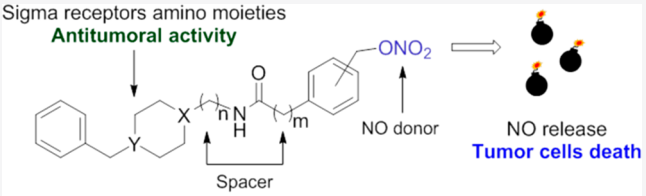
ABSTRACT: We report the development of molecular hybrids in which a nitrate group serving as nitric oxide (NO) donor is covalently joined to σ receptor ligands to give candidates for double-targeted cancer therapy. The compounds have been evaluated in radioligand binding assay at both σ receptors and selected compounds tested for NO release. Compounds **9**, **15**, **18**, **19**, and **21** were subjected to MTT test. Compound **15** produced a significant reduction of MCF-7 and Caco-2 cellular viability with comparable IC_{50} as doxorubicin, being also not toxic for fibroblast HFF-1 cells. Compound **15** has shown a σ_1 receptor antagonist/σ_2 receptor agonist profile. Two derivatives of compound **15** lacking the nitrate group did not induce a reduction of MCF-7 cellular viability, suggesting a potential synergistic effect between the σ receptors and the NO-mediated events. Overall, the combination of NO donor and σ receptors ligands provided compounds with beneficial effects for the treatment of cancer.



Cpd	X	Y	n	m	Substitution
9−16	N	CH	2−5	0	*meta*; *para*
17−20	CH	N	0	0, 1	*meta*; *para*

KEYWORDS: *Sigma receptors, nitric oxide, nitric oxide donors, cancer, bifunctional ligands*

Sigma (σ) receptors represent a unique receptor class involved in various biological and pathological conditions.[1] Two σ receptors subtypes are recognized and termed sigma-1 (σ_1) and sigma-2 (σ_2), having different structures and biological functions.[2,3] The σ_1 receptor is a 25.3 kDa polypeptide that has been cloned in several species and recently crystallized revealing a trimeric architecture.[4,5] The σ_1 receptor has been identified as a ligand-operated chaperone protein localized in the mitochondria-associated endoplasmic reticulum (ER) membranes (MAM). At the MAM, the σ_1 receptor forms a complex with another chaperone, the immunoglobulin heavy-chain binding protein (BiP). The σ_1 receptor-BiP complex is silent and only activated when cells are stimulated by σ_1 receptor agonists like (+)-pentazocine [(+)-PTZ] or undergo prolonged stress.[6] In contrast, haloperidol, methamphetamine, and NE-100 can preserve the σ_1 receptor-BiP complex in a silent state.[7]

The σ_2 receptor is a poorly understood protein whose identification has been controversial. In 2017, Alon and co-workers identified the σ_2 receptor as an endoplasmic reticulum-resident transmembrane protein (TMEM97) playing a role in the cholesterol homeostasis and the sterol transporter Niemann−Pick disease type C1.[8] Despite the challenges in identifying its true identity, σ_2 receptor and its ligands, as determined in radioligand binding assay using [^{3}H]DTG,[9,10] have earned a growing scientific interest due to its involvement in several pathological states.[11,12] The overexpression of the σ_2 receptor has been found in several cancer cells and proliferating tumors such as lung, colorectal, and breast.[13] Due to a 10-fold higher density in proliferating tumor cells than in quiescent tumor cells, the σ_2 receptor also represents a significant clinical biomarker for determining the proliferative status of solid tumors.[14,15] A vast number of data support the use of σ_1 receptor antagonists and σ_2 receptor agonists for the treatment of cancer due to their antiproliferative effects.[16−20] Rimcazole and haloperidol, as well as other σ receptors ligands endowed with opportune functional profile, have been shown to inhibit cell proliferation of several cancer cell lines.[21,22]

Nitric oxide (NO) is a short-lived gas with a dichotomous role in tumor biology. NO operates as a tumor progressor or suppressor based on its concentration, duration of exposure, and cell sensitivity.[23] For instance, μM concentrations of NO result in antitumor effects, while pM−nM concentrations promote cytoprotective effects. In this view, the modulation of

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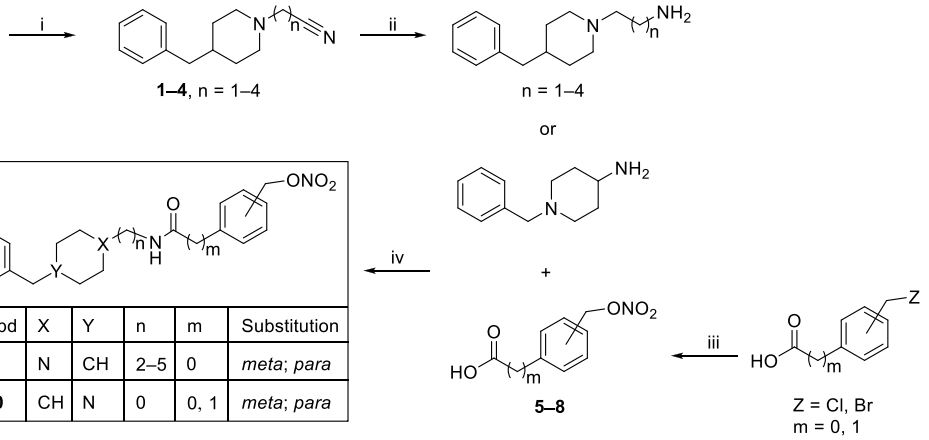
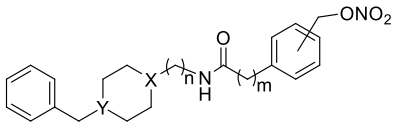
Scheme 1. Synthetic Strategy for the Preparation of Target Compounds[a]



Compd	X	Y	n	m	Substitution
9–16	N	CH	2–5	0	*meta*; *para*
17–20	CH	N	0	0, 1	*meta*; *para*

[a]Reagents and conditions: (i) 4-benzylpiperidine, Br[$(CH_2)_{1-4}$]CN, NaI, K_2CO_3, DMF, 60 °C, overnight; (ii) $LiAlH_4$, THF, rt, N_2; (iii) $AgNO_3$, CH_3CN, rt; (iv) EDC, HOBT, CH_3CN, rt, 5 h.

Table 1. σ_1 and σ_2 Binding Assays for Compounds 9−20



						$K_i \pm SD$[a] (nM)	
compd	X	Y	*n*	*m*	substitution	σ_1	σ_2
9	N	CH	2	0	meta	49 ± 1	330 ± 9
10	N	CH	3	0	meta	64 ± 0.7	93 ± 3
11	N	CH	4	0	meta	148 ± 1	75 ± 2
12	N	CH	5	0	meta	50 ± 0.9	142 ± 13
13	N	CH	2	0	para	89 ± 2	2673 ± 44
14	N	CH	3	0	para	145 ± 12	230 ± 18
15	N	CH	4	0	para	250 ± 9	89 ± 2
16	N	CH	5	0	para	93 ± 2	70 ± 3
17	CH	N	0	0	meta	24 ± 0.6	19 ± 0.2
18	CH	N	0	1	meta	19 ± 1	320 ± 4
19	CH	N	0	0	para	22 ± 0.8	270 ± 5
20	CH	N	0	1	para	170 ± 6	2514 ± 24
haloperidol						2.5 ± 0.4	16 ± 2
(+)-PTZ						4.7 ± 0.7	1465 ± 224
DTG						ND[b]	18 ± 1

[a]Each value is the mean ± SD of at least two experiments performed in triplicate. [b]Not determined.

NO levels seems to have benefits in the treatment of cancer. However, NO is a reactive and unstable gas; thus, the use of NO donors (NODs) is preferred since these agents are stable chemical compounds able to release NO under specific chemical or enzymatical conditions. In order to ensure a high and effective release of cytotoxic amounts of NO, the choice of the releasing agent is crucial. Among the different classes of donors, organic nitrates have been reported to release adequate amounts of NO.[16,24]

The cytotoxic activity may be carried out by NODs alone, or they may be conjugated or embedded in chemical compounds that possess additional antitumoral action for double-targeted cytotoxic activity.[25,26] On the basis of the aforementioned, the purpose of the present work is the development, biological evaluation, and molecular modeling studies of a series of molecular hybrids where the cytotoxic activity of the NOD is conjugated with that of σ receptors for optimal effect.[16]

Compounds **9−20** were synthesized according to the steps illustrated in Scheme 1. Starting from commercially available 4-benzylpiperidine, intermediates **1−4** were obtained with opportune nitrile compounds and then converted into the corresponding amine derivatives by reaction with $LiAlH_4$.[6,16] The amine intermediates underwent condensation with [(nitrooxy)methyl]benzoic acid (**5**, **6**) or *para*- or *meta*-[(nitrooxy)methyl]phenylacetic acid (**7**, **8**) previously prepared via nitration with $AgNO_3$ to give the final compounds **9−16**. Compounds **17−20** were obtained through condensation of 1-benzylpiperidin-4-amine with the same acids **5−8**.

The synthesized compounds were evaluated for affinity at both σ_1 and σ_2 receptors through radioligand binding assay (Table 1). Within the series bearing a 4-benzylpiperidine (**9−16**), the *meta*-substituted compound **9** has shown a preferential σ_1 receptor affinity with K_i value of 49 nM, and 330 nM for the σ_2 receptor. The elongation to three-carbon chain (**10**)

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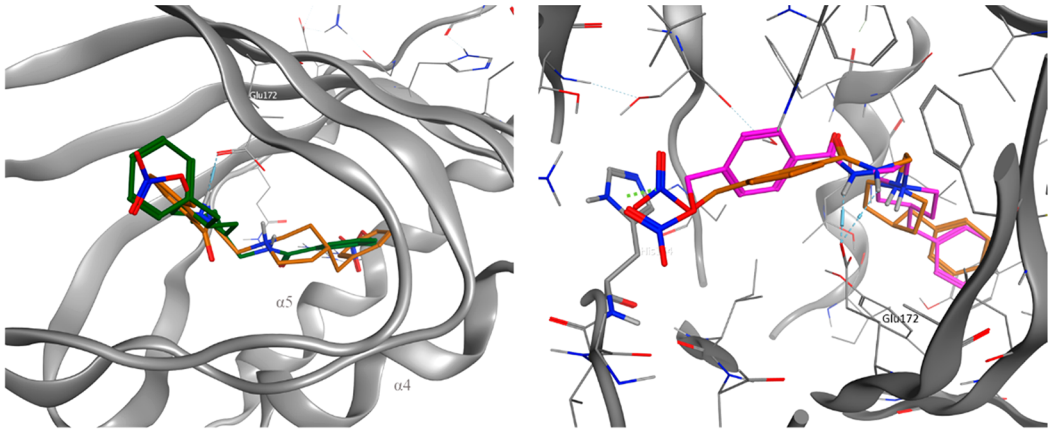

a b

Figure 1. (a) 3D superposition of the best-docked pose for **9** (orange) and **11** (green) bound to the σ_1 receptor orthosteric site. The different lengths of the aliphatic linker reverse the orientation of the ligand inside the receptor. (b) Top-scored docking poses for **9** (orange) and **13** (magenta) bound to the σ_1 receptor orthosteric site. The different position of the nitrate group allows better interaction with His154 (green dotted line).

increased the σ_2 receptor affinity by more than 3-fold with $K_i\sigma_1$ value of 64 nM and $K_i\sigma_2$ of 93 nM. The increased σ_2 receptor affinity has also been maintained in compound **11** with a K_i value of 75 nM, although in the four-carbon chain, the σ_1 receptor affinity (148 nM) is reduced. Further elongation of the chain to five carbons takes back σ_1 receptor preferential affinity with a K_i value of 50 nM over the σ_2 receptor (K_i value of 142 nM).

At the same time, the *para* substitution of the nitrate group gives compounds with generally lower affinity with respect to both receptor subtypes. It is observed an improvement of the σ_2 receptor affinity with the linker elongation. Indeed, compound **13** has a $K_i\sigma_2$ of 2673 nM, while compound **16** has a $K_i\sigma_2$ of 70 nM. In terms of σ_1 receptor affinity, compounds **9**−**16** show a mixed behavior with affinity ranging from 89 nM for compound **13** to 249 nM for compound **15**. Compounds bearing the 1-benzylpiperidin-4-amine (**17**−**20**) have shown worthy σ_1 receptor affinities and a general lower affinity at the σ_2 receptor. In particular, compounds **17**−**19** have a σ_1 receptor affinity in the low double digits nM range ($K_i\sigma_1$ of 24, 19, and 22 nM, respectively), while only compound **17** shows σ_2 receptor affinity in the same range ($K_i\sigma_2$ of 19 nM). The other compounds show a lower affinity to the σ_2 receptor, which leads to a >10 times selectivity with respect to the σ_1 receptor.

A docking study was conducted to identify and evaluate the key molecular interactions involved in the receptor/ligand recognition. The crystal structure of the σ_1 receptor PD144418 (PDB code 5HK1)[4] reveals an occluded and elongated binding cavity in a similar β-barrel fashion, with the highly conserved Glu172 amino acid residue located near the center of the cavity, forming a salt bridge with the ligands. Since all compounds possess a tertiary amine, we performed the study considering the *N*-protonated structures (pH = 7.4). Therefore, the formation of the salt bridge was used as a filter for the docking poses. The calculated free binding energies (ΔG) and K_i values to the catalytic site of the σ_1 receptor for compounds **9**−**20** and haloperidol are reported in Table S1.

Active analysis of the site showed that the orientation changes according to the length of the ligands and the protonated *N*-position. Moreover, the position of the protonated piperidine ring is rather characteristic, being close to the carboxyl function of Glu172. The docking studies conducted upon the σ_2 receptor were performed by using its homology model previously built by us.[27] In this case, the salt bridge interactions between the ligands and the residues Asp29 and Asp56 were considered. In the 4-benzylpiperidine series with the nitrate group in the *meta* position (**9**−**12**), compound **9** showed a preferential affinity for the σ_1 receptor with a K_i value of 49 nM.

Compound **9** is placed inside the receptor site with the piperidinium proton engaged in a salt bridge interaction with Glu172 and a hydrogen bond with N−H amide. The nitrate group is positioned on the opposite side helices α4 and α5, showing polar interactions with the residues Asp126 and His154. The optimal position of the piperidine ring favors the $\pi-\pi$ stacking of the hydrogen in position 4 of the piperidine system with the residue Tyr103. The benzyl group is involved in hydrophobic interactions with the Leu182 and Met93 residues. The elongation to the four-carbon chain (**11**) decreases the affinity for the σ_1 receptor while increasing the affinity for the σ_2 receptor. Indeed, derivative **11** has an inverted orientation with respect to derivative **9**, with the nitrate group toward the helix α5 to allow the accommodation of the aliphatic chain within the receptor pocket (Figure 1a).

The *para* substitution of the nitrate group (**13**−**16**) decreases the affinity with respect to the ligands substituted in the *meta* position. This is probably due to a lower cation−π interaction with the His154 residue (Figure 1b). An elongation of the aliphatic portion of the ligands leads to a better affinity for the σ_2 receptor. The most similar compounds in the series (**11** and **16**) show comparable interactions within the σ_2 receptor pocket. Both form the salt bridge with the residue Asp56 while the benzyl portion in position 4 to the piperidine ring shows aliphatic interactions with the residues Leu70, Met108, Pro113, and Val146. The nitrate group establishes electrostatic interactions with the Arg36 residue, discriminating against the different affinity between the different isomers. A detailed description of the interactions with σ_2 receptor is reported in the Molecular Docking section in the Supporting Information.

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Compound **18** showed the preeminent affinity to the σ_1 receptor. On this compound, a molecular dynamics (MD) simulation was performed (Supporting Information). An elongation of the aliphatic portion of the ligands leads to a better affinity for σ_2 receptor.

Once the affinity profiles of the synthesized compounds at σ receptors were evaluated, we determined the ability of these compounds to release NO. Nitrite content was measured by the Griess method incubating the compounds (100 μM), at 37 °C, in Tris-HCl buffer for 30 min (Figure 2).

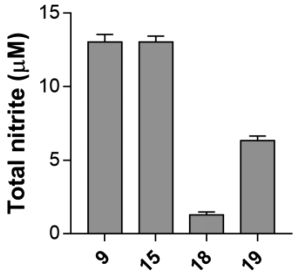

Figure 2. Total nitrite content measured using Griess reagent.

For this assay, we selected the compounds based on their affinity profile against σ receptors. In particular, we evaluated compounds **9**, **11**, **15**, and **17**−**19**, having shown good affinity at both receptors or prevalence for one receptor subtype. Compounds **9** and **15** were able to release a significant amount of NO in the μM range (**9**, 13.0 ± 0.5 μM; **15**, 13.0 ± 0.4 μM). The amount of NO released by compound **18** was 1.3 ± 0.2 μM, while compound **19** produced 6.3 ± 0.3 μM NO. Negligible NO amounts were detected for compounds **11** and **17** (data not shown).

After having obtained the desired chemical tools, we evaluated their activity in the appropriate cell lines. We evaluated those compounds that, in previous experiments, have been demonstrated to possess the desired profile (compounds **9**, **15**, **18**, and **19**). Two cancer cell lines were selected, Caco-2 and MCF-7 cells, for their expression of both σ receptors. Although MCF-7 cells are reported to exclusively express the σ_2 subtypes, a Western blot analysis has shown the presence of the σ_1 isoform in our in-house cell line (Figure S4).[28] The toxicity against human fibroblast HFF-1 cells was also evaluated. Doxorubicin (**21**) was used as the standard cytotoxic compound. Results showed a reduction of cellular viability on both Caco-2 and MCF-7 cell lines for compounds **9** and **15** (Table 2). These compounds were those with a higher rate of NO release. Measured IC_{50} values were better with respect to compound **21** for MCF-7 cells, with IC_{50} of 36 μM for compound **9** and 26 μM for compound **15**. None of the synthesized compounds, nor compound **21**, were demonstrated to be toxic for the human fibroblasts HFF-1 at the maximal tested concentration. Compounds **18** and **19** have been shown to be nontoxic for the three evaluated cell lines (IC_{50} > 100 μM), probably due to the lower rate of NO release and to an unsuitable functional profile at σ receptors.

Table 2. MTT Test on MCF-7, Caco-2, and HFF-1 for Compounds 21, 9, 15, 18, and 19

	IC_{50} ± SD (μM)[a]		
compd	MCF-7	Caco-2	HFF-1
21	44 ± 0.3	21 ± 0.3	>100[b]
9	36 ± 0.2	59 ± 0.5	>100[b]
15	26 ± 0.4	28 ± 0.2	>100[b]
18	>100[b]	>100[b]	>100[b]
19	>100[b]	>100[b]	>100[b]

[a]Each value is the mean ± SD of at least two experiments performed in quadruplicate. [b]Cell viability reduction lower than 50% at 100 μM.

In order to determinate the precise mechanism for the reduction of cellular viability, compound **15** (IC_{50} concentration) was evaluated in combination with the σ_1 receptor agonist (+)-PTZ (1 μM) and the σ_2 receptor antagonist 1-phenethylpiperidine (AC927, 1 μM) by MTT.[29]

Incubation of compound **15** with (+)-PTZ or AC927 wholly restored the loss of cell viability induced by **15** alone (Figure 3). This outlines a σ_1 and σ_2 receptors involvement in the observed cellular events and a σ_1 receptor antagonist/σ_2 receptor agonist functional profile for compound **15**.

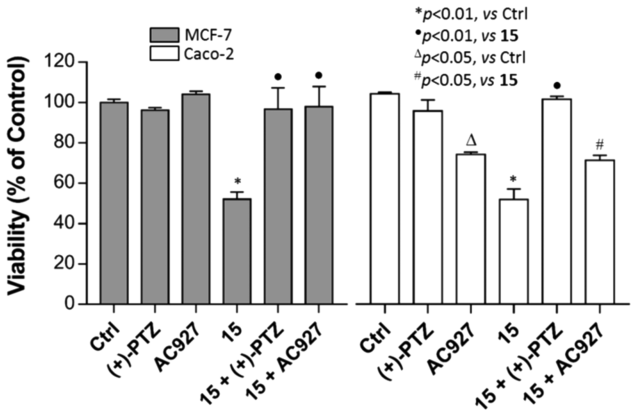

Figure 3. Effects of compound **15** in combination with the selective σ_1 receptor agonist (+)-PTZ and σ_2 receptor antagonist AC927 on MCF-7 and Caco-2 viability by MTT test.

To dig into the dual mechanism of the prepared compounds, we tested fragments **5** and **22a**,**b** and synthesized two derivatives of compound **15** lacking the nitrate function, compounds **23a** and **23b**, as negative control (Table 3 and Scheme S1).[30,31]

The fragments **5** and **22a**,**b** have shown no significant viability reduction on MCF-7 and no affinity for both σ receptors. Compound **23a** showed a loss of affinity at both σ receptors, while compound **23b** retained a similar affinity profile at both σ receptors with respect to compound **9** or **15**. When evaluated on the MCF-7 cell line, compound **23a** induced lower than 50% viability reduction at 100 μM, while compound **23b** had an IC_{50} of 87 μM. Overall compound **23b**, while maintaining a similar profile against σ receptors, showed a lower ability in reducing MCF-7 viability, thus sustaining a possible synergistic effect between σ receptors and the NO-mediated events.

In conclusion, this contribution reports the development of novel hybrid compounds able to release NO and to bind σ receptors as candidates for double-targeted cancer therapy. The compounds have been evaluated for their affinity at σ receptors and ability to release NO. Four compounds showed the desired profile with compounds **9** and **15** also able to induce a marked loss of viability in MCF-7 and Caco-2 cell lines while not being toxic for healthy human fibroblast HFF-1.

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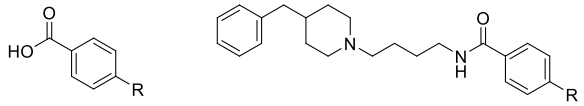
Table 3. Binding Assays and MTT Viability Test on MCF-7 for Compounds 22a,b, 23a,b, and 5



5, R = CH_2ONO_2
22a, R = CH_2OH
22b, R = CH_3

23a, R = CH_2OH
23b, R = CH_3

	$K_i \pm SD^a$ (nM)		$IC_{50} \pm SD$ (μM)^b
compd	σ_1	σ_2	MCF-7
5	>10000[c]	>10000[c]	>100[d]
22a	>10000[c]	>10000[c]	>100[d]
22b	>10000[c]	>10000[c]	>100[d]
23a	711 ± 127	2600 ± 730	>100[d]
23b	62 ± 3	127 ± 16	87 ± 0.3

[a]Each value is the mean ± SD of at least two experiments performed in triplicate. [b]Each value is the mean ± SD of at least two experiments performed in quadruplicate. [c]Radioligand displacement lower than 50% at 10 μM. [d]Cell viability reduction lower than 50% at 100 μM.

In cellular experiments involving the use of selective σ receptors agonist and antagonist, compound **15** has shown a σ_1 receptor antagonist/σ_2 receptor agonist functional profile. The elimination of the nitrate function of compound **15** as in compounds **23a,b** determined the loss of its ability to reduce MCF-7 viability sustaining a possible synergistic effect between the σ receptors and the NO-mediated events. Molecular docking studies have shown that the length of the aliphatic linker has an essential role in the orientation of the ligands inside the receptor pocket and for σ_1/σ_2 selectivity. The nitrate group stabilizes the ligand/receptor complex to cation–π interaction with His154 residue in the σ_1 receptor, also confirmed by the MD simulation, and with Phe71 and Pro113 residues in the σ_2 receptor. Overall, the combination of NO donor and σ receptors ligands provided compounds with potential beneficial effects in the treatment of neoplastic disorders.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsmedchemlett.9b00661.

General synthetic methods and spectral data of final compounds, procedures for in vitro biological assays, and computational methods (PDF)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

σ, sigma; σ_1, sigma-1; σ_2, sigma-2; ER, endoplasmic reticulum; [^{3}H]-DTG, [^{3}H]-1,3 di-*o*-tolylguanidine; NO, nitric oxide; NOD, nitric oxide donor; AC927, 1-phenetylpiperidine

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