Bioorganic & Medicinal Chemistry Letters 21 (2011) 7025–7029

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Novel antileukemic agents derived from tamibarotene and nitric oxide donors

Haiyong Bian, Jinhong Feng, Minyong Li *, Wenfang Xu *

Department of Medicinal Chemistry, School of Pharmaceutical Sciences, Shandong University, Ji'nan, Shandong 250012, China

ARTICLE INFO

Article history:
Received 25 June 2011
Revised 23 September 2011
Accepted 27 September 2011
Available online 1 October 2011

Keywords:
Multi-target drugs
Nitric oxide
RAR agonist
Synthesis
Biological evaluation

ABSTRACT

A series of novel nitric oxide-releasing tamibarotene derivatives were synthesized by coupling nitric oxide (NO) donors with tamibarotene via various spacers, and were evaluated for their antiproliferative activities against human leukemic HL-60, NB4 and K562 cell lines in vitro. The test results showed that three compounds (**7g**, **9a** and **9e**) exhibited more potent antileukemic activity than the control tamibarotene. Furthermore, the preliminary structure–activity analysis revealed that amino acids serving as spacers could bring about significantly improved biological activities of NO donor hybrids. These interesting results could provide new insights into the development of NO-based antileukemic agents.

Recently, multi-target drugs, which are designed as single molecules to modulate multiple physiological targets simultaneously, have increasingly attracted the concerns of medicinal chemists,[1] and it represents a very promising way to enhance efficacy and to decrease adverse effects of drugs especially in the treatment of complex diseases such as cancers, cardiovascular diseases, and neurodegenerative diseases.[2,3] Currently, one of the most interesting cases for multi-target drugs is nitric oxide (NO) donor hybrids, which are obtained by combining NO donors with an appropriate reference drug, and several classes of NO-donor hybrids have been extensively studied, including NO-donor nonsteroidal anti-inflammatory drug hybrids (NO-NSAIDs), NO-donor cardiovascular drug hybrids, and NO-donor antioxidant hybrids.[1,4] Nitric oxide is an important cellular messenger molecule in vivo and has been demonstrated to be involved in many physiological processes such as vascular relaxation, neurotransmission and immune responses, and some pathological processes including rheumatoid arthropathies, hypertension and neurodegenerative diseases, etc.[5,6] In particular, a variety of experimental evidences have indicated that nitric oxide, which was found to be a cytotoxic and apoptosis-inducing agent against tumor cells under appropriate conditions of concentration, played a crucial role in the tumoricidal activity of the human immune system, and could prevent cancer cells from metastasis as well as effectively overcome tumor cell resistance to conventional therapeutics.[7] Furoxans (1,2,5-oxadiazole-2-oxides), as an important class of NO donors, have been found to possess a variety of NO-related biological activities such as tumor cell apoptosis-inducing activity, vasodilator capacity, antiaggregant and antibacterial activities. Especially, some furoxans exhibited remarkable antileukemic activities and have been expected as promising lead compounds to develop novel antileukemic agents.[8]

Tamibarotene (AM80), a selective RARα agonist launched in Japan, has proved to be an effective drug for relapsed or refractory APL. Compared to all-trans retinoic acid (ATRA), tamibarotene exhibited higher differentiation-inducing activities for APL cells and lower drug resistance due to its low affinity to cellular retinoic acid binding protein (CRABP). However, the inevitable toxic and side effects, such as hypertriglyceridemia, hypercholesterolemia, rash, bone pain, retinoic acid syndrome and a strong teratogenic effect, would appear to hinder the clinical application of tamibarotene.[9] Therefore, it is interesting to develop tamibarotene derivatives with improved safety features and similar or even better antileukemic activities.

In the present study, a series of novel Tamibarotene-NO donor hybrids were developed as antileukemic agents by attaching phenyl-substituted furoxans as NO-donors to the reference drug tamibarotene through various spacers such as ethylene glycol, ethanolamine, ethylenediamine, and amino acids. Furoxans as NO-donors might contribute to reduce the side effects of tamibarotene, such as hypertriglyceridemia and hypercholesterolemia, by releasing NO in vivo, as well as effectively avoid the nitrate tolerance from organic nitrates as classical NO donors.[10] Particularly, these hybrids were proposed to release NO and tamibarotene through metabolism in vivo to exert synergistic effects at multiple target sites and to bring about significantly enhanced efficacy. So it is anticipated that more potent antileukemic agents would be found out from these Tamibarotene-NO donor hybrids. In addition, we also attempted to seek a better paradigm for multi-target-di-

* Corresponding authors. Tel./fax: +86 531 88382264 (W.X.); tel./fax: +86 531 88382076 (M.L.).
E-mail addresses: mli@sdu.edu.cn (M. Li), wfxu@yahoo.cn (W. Xu).

0960-894X/$ - see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2011.09.103

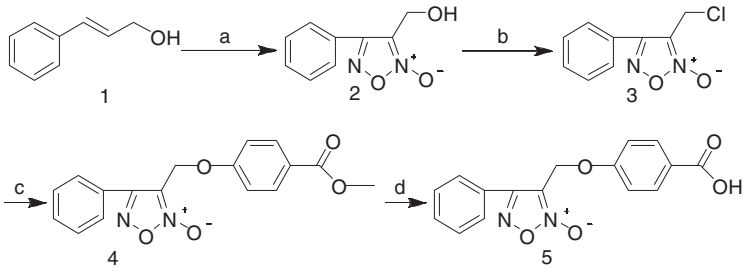
rected drug design strategy by the structure–activity studies of these pharmacodynamic hybrids. In this Letter, we report the synthesis of Tamibarotene-NO donor hybrids, their NO-releasing capacities, and the antiproliferative activities against human leukemic HL-60, NB4 and K562 cell lines.

The synthetic route of several key intermediates (compounds **3**, **5**) was outlined in Scheme 1. Compound **3** was prepared in 48.4% overall yield from cinnamyl alcohol (**1**) by modified procedures described in the literature.[11,12] Compound **4** was synthesized in 83.4% yield by the reaction of compound **3** with 4-(methoxycarbonyl) phenol in DMF in the presence of anhydrous potassium carbonate and potassium iodide at room temperature, and then compound **5** was obtained in 72.9% yield by the selective hydrolysis of compound **4** in DMF–H_2O solution on treatment with lithium hydroxide.

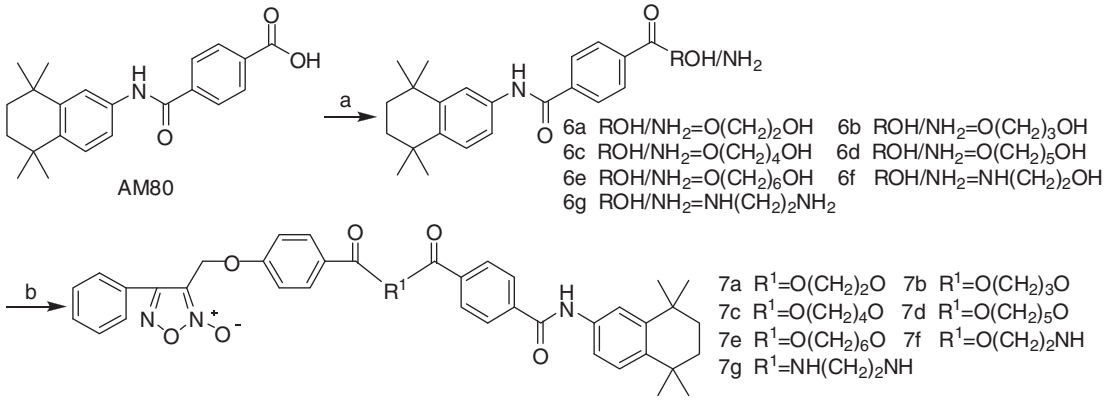
The preparation of target compounds **7a–g** and **9a–e** was shown in Schemes 2 and 3 respectively. Tamibarotene was reacted with short chain alkylene glycols containing from two to five carbon atoms, ethanolamine or ethylenediamine by the catalysis of *N*,*N*′-carbonyldiimidazole (CDI) in dry tetrahydrofuran at 50 °C to give compounds **6a–g** in excellent yield (81.9–91.1%). Compound **3** was reacted with some *N*-tert-butoxycarbonyl protected amino acids (such as N-*t*-Boc-Gly, N-*t*-Boc-L-Ala, N-*t*-Boc-L-Leu, N-*t*-Boc-L-Val, and N-*t*-Boc-L-Phe) in the presence of cesium carbonate and potassium iodide in dry DMF at room temperature, followed by the treatment of trifluoroacetic acid in dichloromethane to afford compounds **8a–e** in moderate yield (50.6–72.2%). Finally, target compounds **7a–g** were obtained in good yield (75.1–88.3%) by esterification of compound **5** with compounds **6a–g** in tetrahydrofuran at room temperature under the catalysis of 1,3-dicyclohexylcarbodiimide (DCC) and 4-dimethylaminopyridine (DMAP), while target compounds **9a–e** were also stereoselectively synthesized in various yield (68.3–87.5%) by amidation of tamibarotene with compounds **8a–e** in tetrahydrofuran under the catalysis of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDCI) and 1-hydroxybenzotrizole (HOBt). All of the target compounds were purified by column chromatography on silica gel and then characterized by IR, ^{1}H NMR and HR-MS.[13]

In order to evaluate the influence of the NO-releasing properties on biological activities of the target compounds, the percentage of NO released in vitro from these Tamibarotene-NO donor hybrids was determined by Griess test (Fig. 1).[14] It has been found that a reduced thiol group from endogenous L-cysteine, glutathione or proteins could mediate the release of NO from furoxan derivatives.[10,15] According to that principle, the evaluation of NO released from furoxan derivatives in vitro is generally performed upon incubation in phosphate buffered saline (PBS) solution with a large excess of L-cysteine. The resulting data showed that all of the target compounds released significant levels of NO in L-cysteine solution, and this evidence also suggested that the antileukemic effects of the target compounds might partly come from NO released from these Tamibarotene-NO donor hybrids.

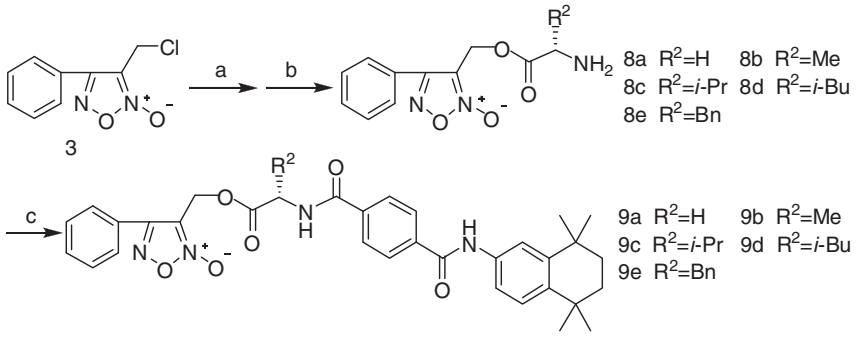
The antiproliferative activities of all target compounds against human leukemic HL-60, NB4 and K562 cell lines were respectively evaluated by MTT cell proliferation assay and the results are summarized in Table 1.[16] The activity data indicated that all the Tamibarotene-NO donor hybrids exhibited higher antiproliferative activity against HL-60 and NB4 cell lines (acute myeloid leukemia) than against K562 cell lines (chronic myeloid leukemia). Three of the target compounds (**7g**, **9a** and **9e**) displayed significantly stronger antiproliferative effects against all of the three human leukemic cell lines than the positive control tamibarotene. In particular, compound **9a** exhibited the best antiproliferative activities against HL-60, NB4 and K562 cells with the IC_{50} of 0.16 μM, 0.19 μM and 7.12 Mm, respectively, which were 56-, 25- and 6-fold higher than that of tamibarotene. Another compound (**9d**) exhib-



Scheme 1. Reagents and conditions: (a) $NaNO_2$, HOAc, 62.4%; (b) $SOCl_2$, Py, CH_2Cl_2, 77.6%; (c) 4-(methoxycarbonyl) phenol, K_2CO_3, KI, DMF, 83.4%; (d) LiOH, DMF, H_2O, 72.9%.



Scheme 2. Reagents and conditions: (a) $HO(CH_2)_nOH$ (n = 2–6), $H_2N(CH_2)_2OH$ and $H_2N(CH_2)_2NH_2$, CDI, THF, 50 °C, 81.9–91.1%; (b) compound **5**, DCC, DMAP, THF, 75.1–88.3%.



Scheme 3. Reagents and conditions: (a) N-Boc amino acid, Cs_2CO_3, KI, DMF; (b) TFA, CH_2Cl_2, 50.6–72.2%; (c) tamibarotene, EDCI, HOBt, THF, 68.3–87.5%.

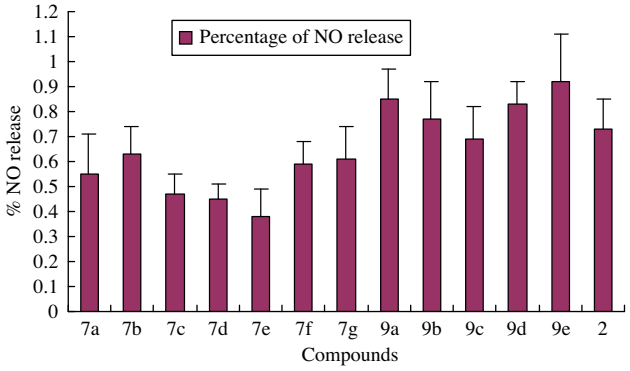


Figure 1. NO releasing properties of compounds **2**, **7a–g** and **9a–e** in vitro (all values are mean ± SD; $n = 3$).

Table 1
Antiproliferative activity of compounds **7a–g**, **9a–e** and Tamibarotene against human leukemic cell lines in vitro

Compound	IC_{50}[a] (μM)		
	HL-60	NB4	K562
7a	394.84 ± 29.15	118.92 ± 15.61	>1000
7b	184.70 ± 26.37	139.72 ± 13.19	>1000
7c	650.89 ± 59.95	951.95 ± 87.55	>1000
7d	281.74 ± 25.82	239.68 ± 38.24	>1000
7e	486.18 ± 62.13	>1000	>1000
7f	12.31 ± 2.39	10.41 ± 3.65	>1000
7g	1.14 ± 0.28	1.93 ± 0.51	13.32 ± 1.91
9a	0.16 ± 0.06	0.19 ± 0.09	7.12 ± 0.96
9b	6.97 ± 1.14	5.10 ± 0.95	60.40 ± 8.25
9c	5.85 ± 1.61	4.39 ± 1.91	191.80 ± 28.26
9d	0.74 ± 0.17	0.97 ± 0.25	64.04 ± 12.32
9e	0.50 ± 0.19	0.75 ± 0.13	22.59 ± 3.67
Tamibarotene[b]	8.94 ± 3.16	4.81 ± 2.03	42.37 ± 5.62

[a] All values are mean ± SD ($n = 3$).
[b] Tamibarotene was used as a positive control drug in MTT assay.

ited remarkably stronger antiproliferative effects against HL-60 and NB4 cells than tamibarotene, while exhibited similar biological activity against K562 cells with tamibarotene. Moreover, two compounds (**9b–c**) exhibited similar antiproliferative activity against HL-60 and NB4 cells with tamibarotene as well. In contrast, five of the target compounds (**7a–e**) showed a significant decrease in antiproliferative activity against HL-60, NB4 and K562 cell lines. Obviously, the different biological activities of the tested compounds should be attributed to the different spacers between NO-donor and tamibarotene moieties.

By comparing compounds **7f–g** and **7a–e**, it was found that the types of chemical bonds associated with spacers make a strong impact on the biological activity of these Tamibarotene-NO donor hybrids, and the amide bonds, formed between spacers and tamibarotene moieties, might give rise to a significant increase in antileukemic activities of the target compounds. That might partially due to the fact that the amide groups, as excellent hydrogen bond donors and acceptors, generally exhibit much stronger interaction with biomacromolecules than ester groups, which can only act as hydrogen bond acceptors. Particularly, by comparing compounds **9a–e** and **7a–e**, we concluded that those target compounds with amino acids as spacers usually exhibited more potent antiproliferative activity against human leukemic cell lines than the other compounds. This occurrence might be partially due to the short and chiral structure of amino acid spacers, which can help facilitate stereoselective enzyme-catalyzed metabolic reactions for these final derivatives to produce NO and tamibarotene to exert synergistic antileukemic effects. However, the precise mechanisms underlying the differential antileukemic activity of these compounds still remain unclear. Furthermore, the statistical significance test was performed to compare compounds **9a–e** group with **7a–e** group on the average percentage of NO released in vitro using a dual-tailed *t*-test by the SPSS software. The results showed that there were significant differences in the average percentage of NO released between two groups of compounds ($P < 0.01$), which was in accordance with the results of MTT cell proliferation assay (Fig. 2). Altogether, it was suggested that amino acids acting as spacers might bring about markedly enhanced biological activities for NO-donor hybrids.

Biological activities of drug molecules are mainly dependent on their physicochemical properties including electronic, steric, and hydrophobic profiles. To help design more potent NO donor hy-

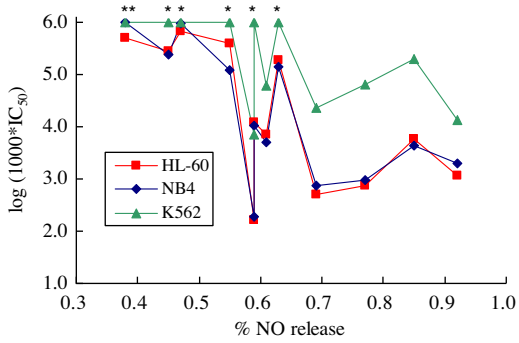


Figure 2. The correlations between the percentages of NO released in vitro from the target compounds and the antiproliferative activities against three human leukemic cell lines. (*)The response IC_{50} values of these compounds are more than 1000 μM.

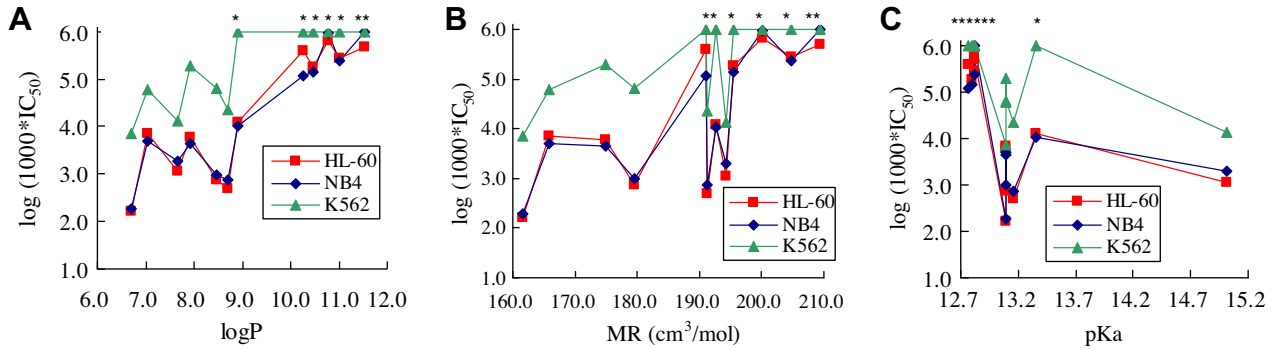


Figure 3. The correlations between the physicochemical properties of the target compounds and the antiproliferative activities against three human leukemic cell lines. (*)The response IC_{50} values of these compounds are more than 1000 μM.

brids as antileukemic agents, the correlations between antileukemic activity of these target compounds and their physicochemical parameters including log*P*, molar refraction (MR) and pKa (calculated by ACD/Labs software), were well examined (Fig. 3). The results showed that there was no simple linear relationship between the biological activity and the corresponding physicochemical parameter values. However, in the overall trend, a significant decrease in antileukemic activity of these final compounds was depicted with the increase of log*P* or MR values, while a remarkable increase in the antileukemic activity was showed with the increase of pKa values. These results suggested that small-sized, hydrophilic and alkalinous spacers might bring about enhanced biological activity for NO donor hybrids. Since amino acids are common small-sized and hydrophilic molecules and also can react with amines or carboxylic acids to give weak alkalinous amide groups, it could be expected that amino acids serving as spacers in NO donor hybrids might afford more potent antileukemic agents. This finding has also been confirmed by the present research.

In conclusion, a series of novel Tamibarotene-NO donor hybrids were developed in the current study. Three target compounds (**7g**, **9a** and **9e**) exhibited more potent antiproliferative activity against three human leukemic cell lines than the control tamibarotene. Particularly, compound **9a** was the most potent antileukemic agent with a low micromolar IC_{50} value in vitro. Furthermore, the preliminary SAR analysis of these derivatives revealed that amino acids serving as spacers might give rise to a remarkable enhance in the biological activity of NO donor hybrids. These interesting results provide a new insight for the development of NO-based multi-target antileukemic agents. In addition, a further pharmacological evaluation for these derivatives is in progress and will be reported subsequently.

Acknowledgments

We are grateful to the National Nature Science Foundation of China (Grant No. 90713041) and National Science and Technology Major Project of the Ministry of Science and Technology of China (Grant No. 2009ZX09103-118) for financial support for this research.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.09.103.

References and notes

1. Alberto, G.; Donatella, B.; Konstantin, C.; Clara, C.; Antonella, D. S.; Roberta, F.; Loretta, L.; Barbara, R.; Paolo, T. *Pure Appl. Chem.* **2008**, *80*, 1693.
2. Cavalli, A.; Bolognesi, M. L.; Minarini, A.; Rosini, M.; Tumiatti, V.; Recanatini, M.; Melchiorre, C. *J. Med. Chem.* **2008**, *51*, 347.
3. Morphy, R.; Kay, C.; Rankovic, Z. *Drug Discovery Today* **2004**, *9*, 641.
4. Martelli, A.; Rapposelli, S.; Calderone, V. *Curr. Med. Chem.* **2006**, *13*, 609.
5. Burgaud, J. L.; Riffaud, J. P.; Del Soldato, P. *Curr. Pharm. Des.* **2002**, *8*, 201.
6. (a) Pacher, P.; Beckman, J. S.; Liaudet, L. *Physiol. Rev.* **2007**, *87*, 315; (b) Wimalawansa, S. J. *Expert. Opin. Pharmacother.* **2008**, *9*, 1935.
7. (a) Hirst, D.; Robson, T. *Curr. Pharm. Des.* **2010**, *16*, 411; (b) Wink, D. A.; Vodovotz, Y.; Laval, J.; Laval, F.; Dewhirst, M. W.; Mitchell, J. B. *Carcinogenesis* **1998**, *19*, 711; (c) Huerta, S.; Chilka, S.; Bonavida, B. *Int. J. Oncol.* **2008**, *33*, 909; (d) Ushmorov, A.; Ratter, F.; Lehmann, V.; Dröge, W.; Schirrmacher, V.; Umansky, V. *Blood* **1999**, *93*, 2342.
8. (a) Cerecetto, H.; Porcal, W. *Mini-Rev. Med. Chem.* **2005**, *5*, 57; (b) Wang, P. G.; Xian, M.; Tang, X.; Wu, X.; Wen, Z.; Cai, T.; Janczuk, A. J. *Chem. Rev.* **2002**, *102*, 1091.
9. Miwako, I.; Kagechika, H. *Drugs Today* **2007**, *43*, 563.
10. (a) Feelisch, M.; Schönafinger, K.; Noack, E. *Biochem. Pharmacol.* **1992**, *44*, 1149; (b) Turnbull, C. M.; Cena, C.; Fruttero, R.; Gasco, A.; Rossi, A. G.; Megson, I. L. *Br. J. Pharmacol.* **2006**, *148*, 517; (c) Schulz, R.; Rassaf, T.; Massion, P. B.; Kelm, M.; Balligand, J. L. *Pharmacol. Ther.* **2005**, *108*, 225.
11. Gasco, A. M.; Fruttero, R.; Sorba, G.; Gasco, A. *Liebigs Ann. Chem.* **1991**, *1991*, 1211.
12. Monge, A.; López de Ceráin, A.; Ezpeleta, O.; Cerecetto, H.; Dias, E.; Di Maio, R.; González, M.; Onetto, S.; Seoane, G.; Suescun, L.; Mariezcurrena, R. *Pharmazie* **1998**, *53*, 758.
13. All the target compounds **7a**–**g** and **9a**–**e** provided acceptable IR, ^{1}H NMR and HR-MS spectra that exhibit no discernible impurities. The HPLC analysis data of representative compounds **7b**, **7g** and **9a** were reported in Supplementary data. Compound **7b**: mp 103–105 °C; IR (KBr, cm^{-1}): 3331.41, 2959.45, 2926.47, 2860.44, 1717.15, 1654.17, 1606.24, 1580.25, 1507.49, 1457.15; ^{1}H NMR (300 MHz, DMSO-d_6) *δ*: 1.225 (s, 6H, 2×CH_3), 1.234 (s, 6H, 2×CH_3), 1.632 (s, 4H, 2×CH_2), 2.156–2.240 (quint, *J* = 6.3 Hz, 2H, C–CH_2), 4.400–4.491 (m, 4H, 2×O–CH_2), 5.296 (s, 2H, O–CH_2), 7.067–7.097 (m, 2H, 2×Ph-H), 7.260–7.289 (d, *J* = 8.7 Hz, 1H, Ph-H), 7.545–7.643 (m, 4H, 4×Ph-H), 7.675–7.681 (d, *J* = 1.8 Hz, 1H, Ph-H), 7.777–7.808 (m, 2H, 2×Ph-H), 7.905–7.935 (m, 2H, 2×Ph-H), 8.027–8.100 (m, 4H, 4×Ph-H), 10.269 (s, 1H, NH); HR-MS (ESI-TOF) (*m*/*z*): 704.2949 $[M+H]^+$ (calcd for $[C_{41}H_{41}N_3O_8+H]^+$: 704.2967). Compound **7g**: mp 193–194 °C; IR(KBr, cm^{-1}): 3340.87, 2959.40, 2929.70, 2858.63, 1644.17, 1608.76, 1579.53, 1501.89, 1457.2; ^{1}H NMR (300 MHz, DMSO-d_6) *δ*: 1.238 (s, 6H, 2×CH_3), 1.252 (s, 6H, 2×CH_3), 1.648 (s, 4H, 2×CH_2), 3.455 (br s, 4H, 2×N–CH_2), 5.287 (s, 2H, O–CH_2), 7.054–7.084 (m, 2H, 2×Ph-H), 7.278–7.307 (d, *J* = 8.7 Hz, 1H, Ph-H), 7.574–7.650 (m, 4H, 4×Ph-H), 7.680–7.688 (m, 1H, Ph-H), 7.806–7.857 (m, 4H, 4×Ph-H), 7.956–7.985 (d, *J* = 8.7 Hz, 2H, 2×Ph-H), 8.020–8.049 (d, *J* = 8.7 Hz, 2H, 2×Ph-H), 8.544 (br s, 1H, NH), 8.767 (br s, 1H, NH), 10.197 (s, 1H, NH); HR-MS (ESI-TOF) (*m*/*z*): 688.3107 $[M+H]^+$ (calcd for $[C_{40}H_{41}N_5O_6+H]^+$: 688.3130). Compound **9a**: mp 183–184 °C; IR(KBr, cm^{-1}): 3283.25, 2957.70, 2922.81, 2858.11, 1764.25, 1642.12, 1611.50, 1580.67, 1499.43, 1457.91; ^{1}H NMR (300 MHz, DMSO-d_6) *δ*: 1.239 (s, 6H, 2×CH_3), 1.253 (s, 6H, 2×CH_3), 1.649 (s, 4H, 2×CH_2), 4.074–4.093 (d, *J* = 5.7 Hz, 2H, N–CH_2), 5.220 (s, 2H, O–CH_2), 7.054–7.084 (m, 2H, 2×PhH), 7.279–7.308 (d, *J* = 8.7 Hz, 1H, PhH), 7.558–7.642 (m, 4H, 4×PhH), 7.657–7.686 (m, 1H, PhH), 7.778–7.810 (m, 2H, 2×PhH), 7.954–8.059 (q, *J* = 8.4 Hz, 4H, 4×PhH), 9.155–9.192 (t, *J* = 5.7 Hz, 1H, NH), 10.210 (s, 1H, NH); HR-MS (ESI-TOF) (*m*/*z*): 583.2511 $[M+H]^+$ (calcd for $[C_{33}H_{34}N_4O_6+H]^+$: 583.2551).
14. In vitro assays for NO released: A solution of the test compound in DMSO (5.0 mM) was added to 100 mM phosphate buffer solution (pH 7.4) containing of 3.4 mM L-cysteine, and the terminal concentration of target compounds was 0.4 mM. The mixture was incubated at 37 °C for 3 h, and then 1.5 mL of the reaction mixture was treated with 0.5 mL of freshly prepared Griess reagent. After 10 min at room temperature, the absorbance was measured at 540 nm by a Simadzu UV-2550 UV–VIS scanning spectrophotometer. Potassium nitrite standard solutions (1–25 μM) were used to prepare the calibration curve under the same experimental conditions. The percentage of NO released ($n = 3$), which is relative to a theoretical maximum release of 1 mol NO per mol of test compound, was calculated according to the calibration curve.

15. (a) Medana, C.; Ermondi, G.; Fruttero, R.; Di Stilo, A.; Ferretti, C.; Gasco, A. *J. Med. Chem.* **1994**, *37*, 4412; (b) Civelli, M.; Giossi, M.; Caruso, P.; Razzetti, R.; Bergamaschi, M.; Bongrani, S.; Gasco, A. *Br. J. Pharmacol.* **1996**, *118*, 923.
16. MTT cell proliferation assay: HL-60, NB4 and K562 cells were cultured in RPMI-1640 medium with 10% fetal bovine serum at 37 °C in a humidified incubator with atmosphere of 5% CO_2, then cells were planted at 10,000 cells per 100 μL per well in 96-well cell culture plates and incubated for 4 h at 37 °C, 5% CO_2 in a humidified incubator, after that, test compounds within medium were added to triplicate wells at various concentrations (final concentration at 0.01, 0.1, 1, 10, 100 μM) and then incubated for 48 h. Following this, 20 μL of MTT solution (5 mg mL^{-1} within complete medium) was added to each well and incubated for additional 4 h. After centrifugal separation, the medium was gently removed from the wells and the formazan crystals were dissolved in 100 μL of DMSO. The optical density was measured at 570 nm using an enzyme-linked immunosorbent assay (ELISA) reader.