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Nitric Oxide Releasing Derivatives of [(2-Chloroethyl)ureido] Benzoic Acid Esters as Potential Antineoplastic Agents

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New series of [(2-chloroethyl)ureido] benzoic acids (compounds 3-8) and nitric oxide releasing derivatives of [(2-chloroethyl)ureido] benzoic acid esters (compounds 9-14) were synthesized as potential anti-cancer agents. These compounds were screened for their anti-proliferative activities on A549 (human lung carcinoma) cells and for their cytotoxic effects on L929 (mouse fibroblast) cells. The compounds 3-8 exhibited mild cytotoxic effects on L929 cells (cell viability from 100% to 85% – 90% only) whereas they had very little anti-proliferative activities towards A549 lung carcinoma cells. On the other hand, compounds 10, 11, and 13 had some growth inhibition activity in A549 (human lung carcinoma) cells. Among them, compounds 11 and 13 exhibited better anti-proliferative activities towards A549 cells, but also appeared to be cytotoxic towards L929 cells. In this group, the compound 10 with (1-(2-nitrooxyethyl)-3-[(2-chloroethyl)ureido]benzoate) structure appeared to have very weak anti-neoplastic activity towards A549 cells, with very little cytotoxic activity towards fibroblasts at physiological concentrations. Therefore, this compound is a better candidate for a potential anti-cancer therapy for non-small cell lung carcinomas. However, further studies are required in order to show applicability and effectiveness in animal models for this type of cancer.

Key Words: Nitric oxide, anti-cancer agent, arylchloroethylureas.

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Introduction

Cancer is a malignant disease responsible for high levels of mortality and morbidity throughout the world. Numerous chemotherapeutic agents developed to date often failed as drug-resistant tumour cells became predominant and tumours recurred, but drug-based approaches for the cancer therapy are still among the most widely used methods. Among them, microtubule-stabilizing agents have been attractive targets for the development of new anti-cancer drugs. Paclitaxel derivatives, such as vinblastin and vincristine, that act on the tubulin, form the largest group of interest.[1–4] In recent years, 3 major binding sites on tubulin namely, the vinca, the taxus and the colchicine (C-BS)–binding sites, were identified.[5] Nguyen group has established 2 classes of C-BS antagonists: those compounds that are structurally related to colchicines and those that are structurally unrelated to colchicines.[6]

Besides plant oriented drugs, a large number of synthetic small molecules especially phenyl-3-(2-chloroethyl)ureas (CEU, Figure 1; A) and congeners have been synthesized and shown as colchicine–binding site antagonists.[7–20]The pharmacophoric moiety N-(2- chloroethyl)urea plays an anchoring action similar to the trimethoxy phenyl moiety of colchicinoids. The other part of the molecule provides specific steric contacts occurring between the 2-chloroethylamino moiety of CEUs and key amino acids nearby the C-BS.[16,17,21–23]

Nitric oxide (NO), a reactive nitrogen intermediate, is a short-lived free radical. In many animal cells, this free radical is synthesized via the L-arginine to L-citrulin pathway, which is mediated by nitric oxide synthase (NOS). A number of reports indicate that compounds containing organic nitrates have been used for the treatment of several diseases such as blood pressure regulation, inflammation, and infection.[18–20,24–26] Moreover, the NO and NO-related species have delineated anti-proliferative, cytotoxic, and apoptotic effects of NO on several tumour cells including leukaemia, pancreatic, hepatic, and colon cancer.[18,24–28,30]

Inspired by these results, the combination of the pharmacophore of aliphatic nitroureas with the bifunctional moiety of aromatic nitrogen mustards, we synthesized [(2-chloroethyl)ureido] benzoic acid (compounds 3-8) and nitric oxide releasing derivatives of [(2-chloroethyl)ureido] benzoic acid esters (compounds 9-14) as potential anti-cancer agents (Figure 1; B). In this work, we have attempted to understand the potential anti-proliferative effects of these compounds in particular on the non-small cell lung carcinoma model. In our hands, with its significant anti-proliferative effects on A549 cells, and relatively mild and negligible cytotoxic effect on L929 fibroblast control cells, the compound 10 (1-(2-nitrooxyethyl)-3-[(2-chloroethyl)ureido]benzoate) appears to be a better candidate for a potential anti-cancer therapy for non-small cell lung carcinomas. Further studies need to be performed, both in vitro cell culture assays and in vivo animal models in order to safely offer this compound as an anti-cancer reagent for clinical use.

Experimental

Chemical methods

General

All chemicals with appropriate purity were purchased from Sigma-Aldrich, St. Louis, US. Melting points were determined in capillary using a Mettler Toledo FP62 apparatus and given uncorrected. IR spectra were recorded

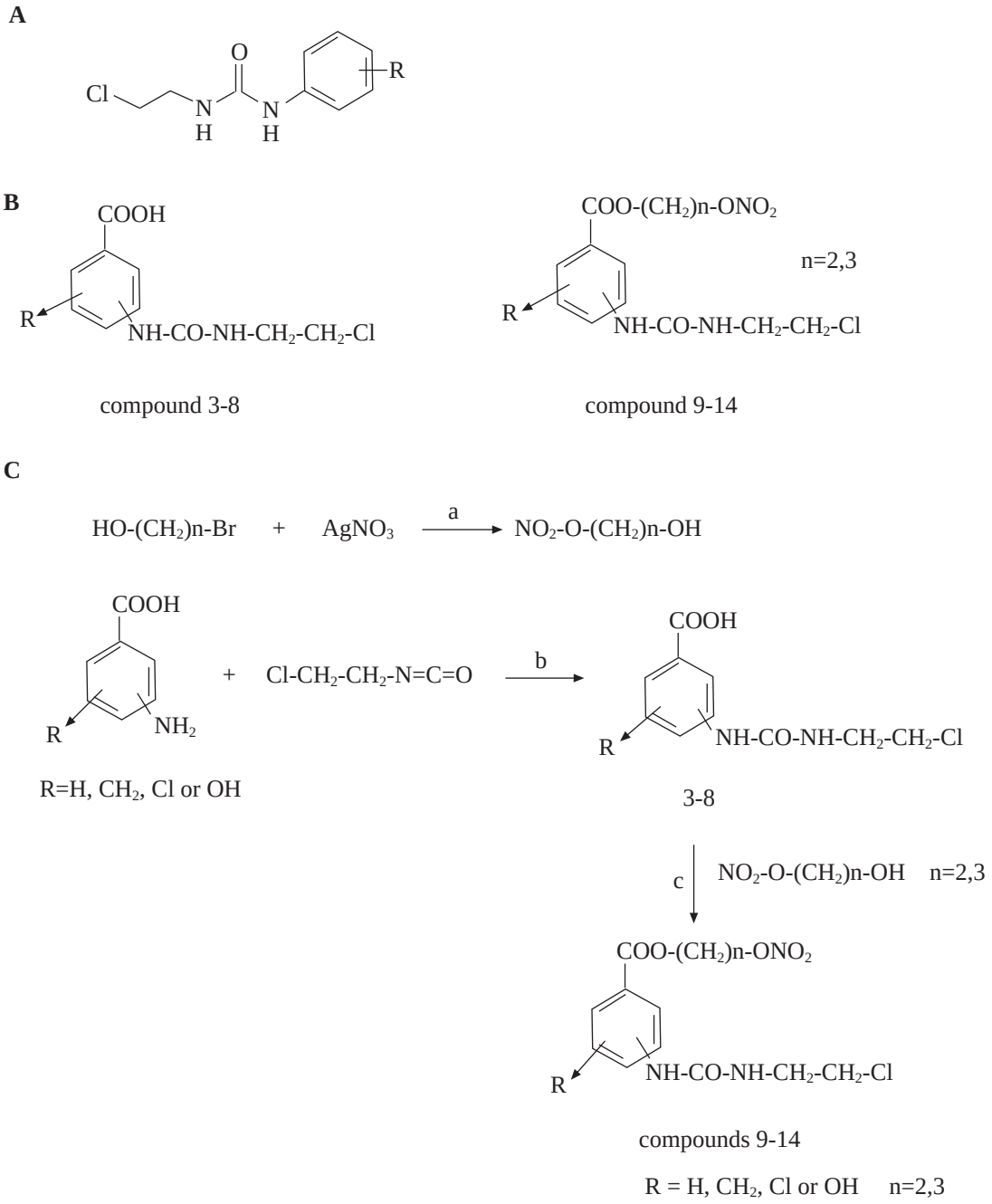


Figure 1. (A) Structure of phenyl chloroethylurea [R=Cyclohexene, t-Butyl, CH_3-$(CH_2)_7$-, OH-$(CH_2)_5$-]; (B) structures of [(2-chloroethyl)ureido]benzoic acid and corresponding esters for compounds 3-8 (left panel) and compounds 9-14 (right panel); (C) Flowchart for synthesis of ureidobenzoic acid derivatives (compounds 3-8) and final nitrooxyalkyl ureidobenzoate structures (compounds 9-14) used in the biological assays. The reagents used are a-acetonitrile, b-ethanolic K_2CO_3, and c-dichloromethane DCC and DMAP.

on a Perkin Elmer Spectra I FT IR spectrometer. The ^{1}H-NMR spectra were taken on a Varian Mercury-400 MHz FT spectrometer using DMSO-d_6. Chemical shifts (δ) were given in ppm, relative to TMS. The mass spectra were recorded on a waters ZQ micro mass LC-MS spectrometer using the ESI (+) method.

General procedure A: The preparation of nitrooxyalcohols (1-2)

A solution of 2-bromoethanol or 3-bromopropanol (23 mmol) in 20 ml of acetonitrile was added dropwise to a solution of silver nitrate (35 mmol) in 20 ml acetonitrile, and stirred over night at room temperature in dark and dry conditions. The formed silver bromide was filtrated and organic layer was evaporated under reduced pressure. The residue was dissolved in diethyl ether, and washed with saturated NaCl solution and distilled water respectively. The ether phase was evaporated. The oily product was dried in vacuum.

General procedure B: The preparation of ureidobenzoic acid (3-8)

A solution of amino benzoic acid derivates (0.012 mmol) in 30 ml of saturated $KHCO_3$ (water or ethanol as solvents) was shaken vigorously with 2-chloroethyl isocyanate derivate (0.012 mmol) for 2 h. The solution was acidified with concentrated HCl and the formed ureido compounds were filtrated. If necessary, the final compounds were crystallized from ethanol. The purity of the compound was detected by TLC (benzene/methanol 6:4).

General procedure C: The preparation nitrooxyalkyl ureidobenzoate (9-14)

A solution of 1.2 mol DCC, 0.2 mol DMAP, and 1.1 mol corresponding ureidobenzoic acid in 20 ml of dichloromethane was stirred in a flask. Then nitrooxyalcohol was added to this mixture, stirred for 5 h at dark and dry conditions in room temperature and left overnight. The mixture was filtered and organic layer was evaporated in vacuum. The residue was crystallized with acetone.

Nitroxy-ethanol (1)

The compound was synthesized from 2-bromoethanol and silver nitrate according to the procedure A. Pale yellow oil, yield 10%.

3-Nitroxy-propan-1-ol (2)

The compound was synthesized from 3-bromopropanol and silver nitrate according to the procedure A. Pale yellow oil, yield 20%.

2-[(2-Chloroethy)ureido] benzoic acid (3)

Compound 3 was synthesized from 2-aminobenzoic acid and 2-chloroethylisocyanate using general method B. Yield 85%; mp 299 °C.
IR (KBr) ν: 3338 (OH), 3306(NH), 1680(CO), 1604 (CO) cm^{-1}
^{1}H- NMR (DMSO-d_6) δ,(ppm) 11.9 (s, 1H, OH), 8.5 (s, 1H, NH), 8.15-8.25 (m, 1H, Ar), 8.05-8.10 (m, 1H,

Ar), 7.55-7.65 (m, 2H, Ar), 7.2 (t, 1H, NH J: 5 Hz), 4.05 (t, 2H, CH_2, J: 7 Hz), 3.85 (t, 2H, CH_2, J: 7 Hz).
MS (ESI) (m/z) $[M+H]^+$ 243.3

3-[(2-Chloroethyl)ureido] benzoic acid (4)

Compound 4 was synthesized from 3-aminobenzoic acid and 2-chloroethylisocyanate using general method B. Yield 80%; mp 215-216 °C
IR (KBr) ν: 3357 (OH), 3283 (NH), 1690 (CO), 1611 (CO) cm^{-1}.
^{1}H- NMR (DMSO-d_6) δ (ppm): 9.0, (s, OH, 1H), 8.0 (s, NH, 1H), 7.55-7.65, (m, Ar, 1H), 7.40-7.50 (m, Ar, 1H), 7.25-7.35 (m, Ar, 2H), 6.50 (t, NH,1H J: 5 Hz), 3.70 (t, CH2, 2H J: 7 Hz), 3.50 (t, CH_2, 2H J: 7 Hz).
MS (ESI) (m/z) $[M+H]^+$ 243.12

3-[(2-Chloroethyl)ureido]-2-methylbenzoic acid (5)

Compound 5 was synthesized from 3-Amino-2-methyl benzoic acid and 2-chloroethylisocyanate using general method B. Yield 88%; mp 277-278 °C
IR (KBr) ν: 3301(OH and NH), 1691 (C=O), 1600, (C=O) cm^{-1}.
^{1}H- NMR (DMSO-d_6) δ (ppm): 12.1 (s OH, 1H), 8.1 (s, NH, 1H), 7.75-7.85, (m 1H), 7.35-7.45 (m, Ar, 1H), 7.7.15-7.25 (m, Ar,1H), 6.70 (t NH, 1H J: 5 Hz), 3.70 (t, CH_2, 2H J: 7Hz), 3.40 (t, CH_2, 2H,J:7 Hz), 2.30 (s, CH_3, 3H).
MS (ESI) (m/z) $[M+H]^+$ 257.23

4-[(Chloroethyl)ureido] benzoic acid (6)

Compound 6 was synthesized from 4-aminobenzoic acid and 2-chloroethylisocyanate using general method B. Yield 83%; mp 215-216 °C
IR (KBr) ν: 3329 (OH), 3176 (NH), 1761(C=O), 1703 (C=O) cm^{-1}.
^{1}H- NMR (DMSO-d_6) δ (ppm): 12.9 (s, OH, 1H) 9.0 (s, NH, 1H), 8.1 (s, Ar, 1H), 7.65, (m Ar, 1H), 7.50 (m, Ar, 1H), 7.20 (m, Ar, 1H), 6.50 (t, NH, 1H, J: 5 Hz), 3.70 (t, CH_2, 2H, J: 7Hz), 3.40 (t, CH_2, 2H J: 7 Hz).
MS (ESI) (m/z) $[M+H]^+$ 243.20

3-[(2-Chloroethyl]ureido]-6-chlorobenzoic acid (7)

Compound 7 was synthesized from 3-amino-6-chlorobenzoic acid and 2-chloroethylisocyanate using general method B. Yield 95%; mp 238-239 °C
IR (KBr) ν: 3332 (OH, NH), 1709 (C=O), 1690 (C=O) cm^{-1}.
^{1}H- NMR (DMSO-d_6) δ (ppm): 13.3 (s, OH, 1H) 9.0 (s, NH, 1H), 8.0 (s, Ar, 1H), 7.5, (m, Ar, 1H), 7.4 (m, Ar, 1H), 6.55 (t, NH, 1H, J: 5 Hz), 3.60 (t, CH_2, 2H, J: 7Hz), 3.35 (t, CH_2, 2H, J: 7 Hz).
MS (ESI)(m/z) $[M+H]^+$ 278.09

3-[(2-Chloroethyl]ureido]-4-hydroxybenzoic acid (8)

Compound 8 was synthesized from 3-amino-4-hydroxybenzoic acid and 2-chloroethylisocyanate using general method B. Yield 95%; mp 300 °C
IR (KBr) ν 3379 (OH), 3340 (NH), 1677 (C=O), 1632 (C=O) cm^{-1}
^{1}H- NMR (DMSO-d_6) δ (ppm): 12.8 (s, OH, 1H, 10.7 (s, OH, 1H), 8.6 (s, NH, 1H), 8.2 (s, Ar, 1H), 7.4, (m, Ar, 1H), 7.2 (m, Ar, 1H), 6.80 (t, NH, 1H, J: 5 Hz), 3.60 (t, CH_2, 2H, J: 7Hz), 3.40 (t, CH_2, 2H, J: 7 H).
MS (ESI)(m/z) $[M+H]^+$ 259.45.

1-(2-Nitrooxyethyl)-2-[(2-Chloroethyl)ureido]benzoate (9)

Compound 9 was synthesized from [2-(2-chloroethyl)ureido]benzoic acid and 2-nitroxy-ethanol according to the general procedure C. Yield 43%; mp 143-144 °C
IR (KBr) ν :3329 (NH), 1735 (C=O), 1602 (C=O) cm^{-1}.
^{1}H- NMR (DMSO-d_6) δ (ppm): 8.8 (s, NH, 1H), 8.2 (s, Ar, 1H), 7.65 (m, Ar, 1H), 7.45 (m, Ar, 1H), 7.35 (m, Ar, 1H), 6.4 (t, NH, 1H, J: 5 Hz), 4.95 (t, CH_2, 2H, J:7Hz), 4.55 (t, CH_2, 2H, J: 7Hz), 3.85 (t, CH_2, 2H, J: 7 Hz), 3.4(t, CH_2, 2H, J: 7 Hz).
MS (ESI)$[M+H]^+$ 332.66

1-(2-Nitrooxyethyl)- 3-[(2-chloroethyl)ureido]benzoate (10)

Compound 10 was synthesized from 3-(2-chloroethyl)benzoic acid and 2-nitroxyethanol according to the general procedure C. Yield 40%; mp 105.-106 °C
IR (KBr) ν :3341 cm^{-1} (NH), 1724(C=O), 1620 (C=O) cm^{-1}.
^{1}H- NMR (DMSO-d_6) δ (ppm): 9.0 (s, NH, 1H), 8.15 (s, Ar, 1H), 7.70, (m, Ar, 1H), 7.55 (m, Ar, 1H), 7.4 (m, Ar, 1H), 6.40 (t, NH, 1H, J: 5 Hz), 4.85 (t, CH_2, 2H, J: 7 Hz), 4.60 (t, CH_2, 2H, J: 7 Hz), 3.85 (t, CH_2, 2H), 3.40 (t, CH_2, 2H, J:7 Hz).
MS (ESI)$[M+H]^+$ 332.49

1-(Nitrooxypropyl)- 3-[(2-chloroethyl)ureido]-2-methylbenzoate (11)

Compound 11 was synthesized from 3-[(2-chloroethyl)ureido]-2-methylbenzoic acid and 2-nitroxpropanol according to the general procedure C. Yield 45%; mp: 97 °C
IR (KBr) ν :3335 cm^{-1} (NH), 1730 (C=O), 1605 cm^{-1} (C=O) cm^{-1}.
^{1}H- NMR (DMSO-d_6) δ (ppm): 8.2 (s, NH, 1H), 8.0 (m, Ar, 1H), 7.80 (m, Ar, 1H), 7.55 (m, Ar, 1H), 6.90 (t, NH, 1H), 4.85 (t, CH_2, 2H, J: 5 Hz), 4.60 (t, CH2, 2H, J: 7Hz), 3.85 (t, CH_2, 2H, J:7 Hz), 3.40 (t, CH_2, 2H, J: 7Hz), 2.15 (s, CH_3, 3H), 2.05 (m, CH_2, 2H).
MS (ESI)$[M+H]^+$ 360.70

1-(Nitrooxypropyl)-4-[(2-chloroethyl)ureido]benzoate (12)

Compound 12 was synthesized from 4-(2-chloroethylureido)benzoic acid and 2-nitroxpropanol according to the general procedure C. Yield 45%; mp 180-181 °C

IR (KBr) ν :3105 (NH), 1735 (C=O), 1638 (C=O) cm^{-1}.
^{1}H- NMR (DMSO-d_6) δ (ppm): 8.20 (s, NH, 1H), 8.0 (m, Ar, 1H), 7.80, (m, Ar, 1H), 7.40 (m, Ar, 2H), 5.80 (t, NH, 1H, J: 5Hz), 4.75 (t, CH_2, 2H, J: 7Hz), 4.50 (t, CH_2, 2H, J: 7Hz), 3.65 (t, CH_2, 2H, J: 7Hz), 3.40 (t, CH_2, 2H, J: 7Hz), 1.80(m CH_2, 2H).
MS (ESI)$[M+H]^+$ 346.12.

1-(2-Nitrooxyethyl)-6-chloro-3-[(2-chloroethyl)ureido]benzoate (13)

Compound 13 was synthesized from 3-[(2-chloroethyl]ureido]-6-chlorobenzoic acid and 2-nitroxethanol according to the general procedure C. Yield 40%; mp < 30 °C
IR (KBr) ν : 3115 (NH), 1725 (C=O), 1628 (C=O) cm^{-1}.
^{1}H- NMR (DMSO-d_6) δ (ppm): 9.20 (s, NH, 1H), 7.80 (d, Ar, 1H), 7.55 (m Ar, 1H), 7.35, (d, Ar, 1H), 6.65 (t, NH, 1H, J: 5Hz), 4.70 (t, CH_2, 2H, J: 7Hz), 4.55 (t, CH_2, 2H, J: 7Hz), 3.85 (t, CH_2, 2H, J: 7Hz), 3.40 (t, CH_2, 2H, J: 7Hz).
MS (ESI)$[M+H]^+$ 367.65

1-(2-Nitrooxyethyl)-4-hydroxy-3-[(2-chloroethyl)ureido]benzoate (14)

Compound 14 was synthesized from [2-(chloroethyl)ureido]-4-hydroxybenzoic acid and 2-nitroxyethanol according to the general procedure C. Yield 38%; mp: 150 °C
IR (KBr) ν: 3198 (OH), 3298(NH), 1753(C=O), 1708 (C=O) cm^{-1}.
^{1}H- NMR (DMSO-d_6) δ (ppm): 10.2 (s, OH, 1H), 8.80 (s, NH, 1H), 8.20 (s, Ar, 1H), 7.70 (m, Ar, 1H), 7.45 (m, Ar, 1H), 6.35(t, NH, 1H, J: 5Hz), 4.80 (t, CH_2, 2H, J: 7Hz), 4.30 (t, CH_2, 2H J: 7Hz), 3.55 (t, CH_2, 2H, J: 7Hz) 3.40 (t, CH_2, 2H, J: 7Hz).
MS (ESI)$[M+H]^+$ 314.22

Biological assays and reagents

Cell Lines

Cell lines used were from American Type Culture Collection (Rockville, MD): A549, human non-small cell lung carcinoma; L929 mouse fibro sarcoma. A549 was maintained in F-12K Ham's medium supplemented with L-glutamine, sodium bicarbonate, and fetal bovine serum. L929 was grown in a minimum essential medium (MEM) supplemented with fetal bovine serum. Cells were cultured in a humidified atmosphere of %5 of CO_2 and 95% air at 37 °C and used during their exponential growth phase.

Cell Growth Inhibition Assay

Synthesized samples to be tested were dissolved in less than 0.1% DMSO. Cell growth inhibition assay was performed in A549 cell line with MTS (Promega cat no: G5421) assay. This technique is based on the determination of number of viable cells in culture via quantitation of ATP which signals the presence of metabolically active cells.

Cytotoxic effects of samples were evaluated in L929 cell line with MTS assay as explained before. Cell line was sub cultured at a concentration of 3×10^3 cells/well, in 96 well-plates. The 96 wells were read with Elisa plate reader at 490 nm. Each experiment was carried out 3 times. Cells were plated in 96-well plates at 3×10^3 cells per well in 100 μl of media per well. Control wells were prepared by adding culture medium without cells. Wells were treated with either tenfold dilutions of synthesized samples or vehicle for 3 days. MTT assay was used to quantify viable cells. Absorbance at 490 nm was recorded with an Elisa plate reader. Cell viability was expressed as the percent absorbance relative to that obtained for cells not exposed to synthesized compounds and plotted against log (sample concentration) for the determination of the GI_{50} value.

In vitro Nitric Oxide Release

Due to their poor water solubility, compounds 9-14 were dissolved in DMSO/water mixture (70/30 w/w) at various concentrations. Aliquots were taken from each sample and added to an equal volume of N-naphtylaminoethylamine[0.2%] and sulfanilamide[2%] solution in 3 N hydrochloric acid (Griess reagent) . Appropriate sodium nitrite solutions in the DMSO/water mixture were used for the construction of the standard curve and values were identical to those obtained with aqueous solutions. Nitric oxide release was estimated spectrophometrically at 540 nm.[27]

Results and Discussion

Synthesis of the compounds

[(2-chloroethyl)ureido] benzoic acid and its nitric oxide releasing ester derivatives are small molecules, which are similar to CEU that can be prepared easily by the following procedures as microtubule disrupting agents. The synthesis of the variable nitrooxyalcohol (1-3) was essentially performed by the reaction of appropriate alkyl haloalcohol with silver nitrate yielding nitric ester and silver chloride.[26] The synthesis of ureidobenzoic acid derivatives (compounds 3-8) were achieved by the nucleophilic addition reaction of 2-, 3-, and 4- amino benzoic acid with 2-chloroethyl in ethanolic K_2CO_3 to yield 75-90%.[30] The preparation of final nitrooxyalkyl ureidobenzoate structures (compound 9-14) was performed following a standard esterification procedure involving the reaction of ureido carboxylic acids and nitrooxyalcohols in the presence of dicyclohexylcarbodiimide (DCC) and DMAP in dichloromethane by the method described before with a yield of 38%-45% (Figure 1; C).[31]

Anti-proliferative activities of the compounds

The growth inhibition assays were conducted on A549 (human lung carcinoma) cells and expressed in terms of GI_{50} value (nM), which is the drug concentration required to inhibit the tumour cell growth by 50%. Compounds 4-8, which bear carboxylic acid functional groups on their aromatic rings, show very mild, anti-proliferative activities (Figure 2). Indeed, compound 7, for example, was even seen to exhibit higher proliferative capacity at different concentrations of the compounds for various durations (Figure 2C, cell viability increased from 100% to around 106 %). Viability of A549 cells after exposure to paclitaxel, which is a microtubule-stabilizing agent, for 72 h decreased linearly with the ln (paclitaxel concentration) ($R = 0.8186$) (Figure 3). The GI_{50} value,

calculated at Y = 50 using the linear regression equation of Y = -9,3842x + 92,499, was 4.52. Since similar values were reported by Isbrucker et al,[32] our results appear to be consistent with literature in the sense that paclitaxel used in this study had comparable anti-proliferative activity and that the A549 cells had acceptable paclitaxel sensitivity (Figure 3).

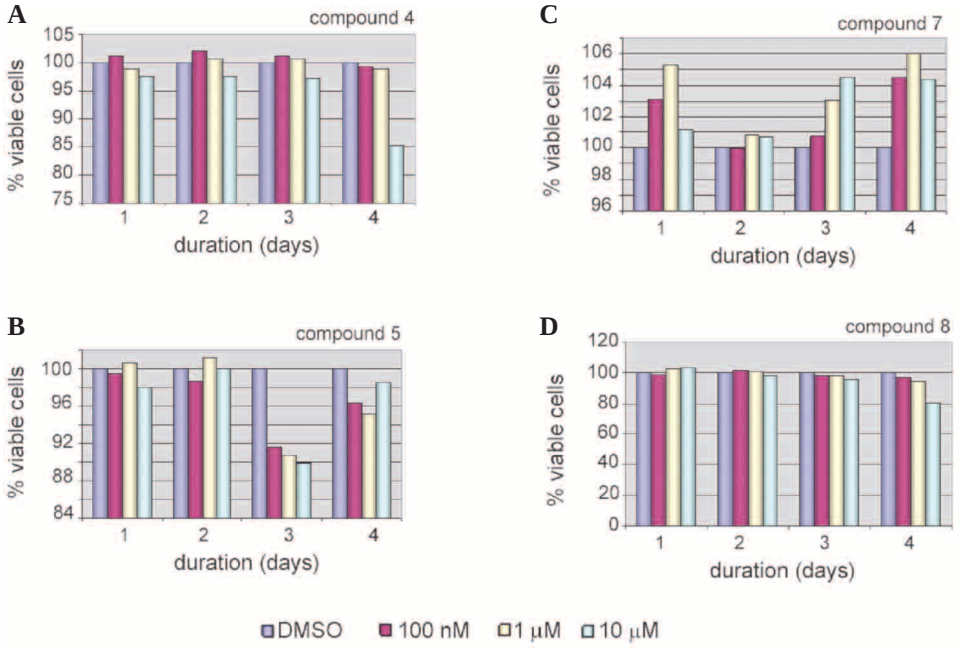


Figure 2. Anti-proliferative activities of compounds 4-8 on A549 non-small lung carcinoma cells. Essentially, compounds 4-8 were dissolved in DMSO and applied on to normally growing A549 cells at the concentrations indicated, using DMSO only as control. The compounds were administered to the cells in the range of 1 μM, 10 μM. and 100 μM, and viability was measured using the MTT assay at various days (1-4) to monitor cell growth over a period of time. (A) compound 4, (B) compound 5, (C) compound 7, and (D) compound 8.

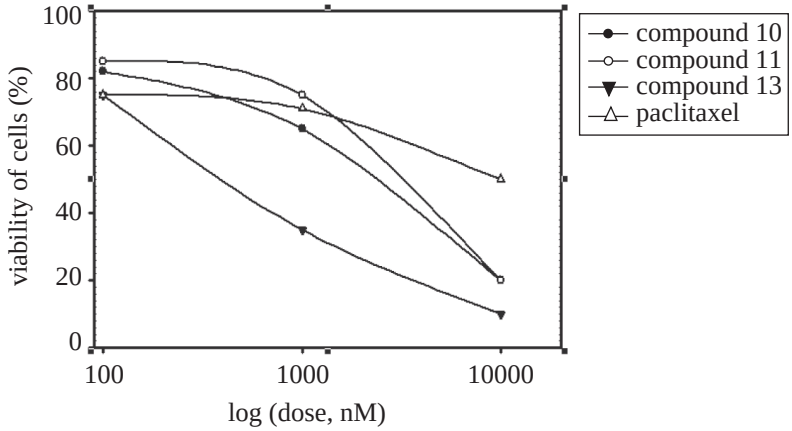


Figure 3. Anti-proliferative activities of compounds 10-13 on A549 non-small lung carcinoma cells. The chemicals were administered to the cells at concentrations 0, 1, and 10 μM and cell viability was established using the MTS assay after 3 days.

We have then tested the growth inhibition or anti-proliferative capacities of compounds 9, 12, and 14 to find only that the anti-proliferative activities at the tested concentrations were very low, similar to compounds 4-8 (data not shown). On the contrary, compounds 10, 11, and 13 showed a growth inhibition potency on A549 cells (GI_{50} values were: 3.0, 3.19, and 2.60 nM, respectively) (Figure 3).

Cytotoxicity of the compounds

In any anti-cancer drug screening approach, it is always necessary that the potential drugs that may have been identified to be effective on tumour cells do not also compromise the integrity or survival of the healthy tissue. To that end, we have used L929 fibroblast cell line as our control cells to test the cytotoxicity of our compounds (following ISO guidelines, Caglayan, Dedeagac and Kurnaz). The cytotoxic activities of the compounds (as well as paclitaxel as control) were assayed in L929 cells during a 4-day period (Figures 4 and 5).

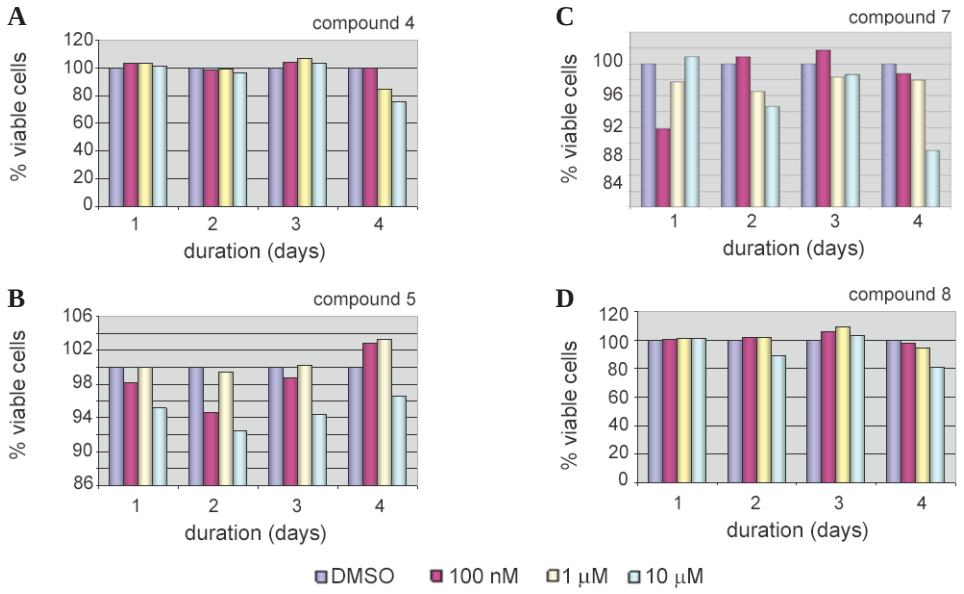


Figure 4. Cytotoxiciy of compounds 4-8 on L929 fibroblast control cells. Essentially, compounds 4-8 were dissolved in DMSO and applied onto normally growing L929 cells at the concentrations indicated, using DMSO only as control. The compounds were administered to the cells in the range of 1 μM, 10 μM. and 100 μM, and viability was measured using the MTT assay at various days (1-4) to monitor cell growth over a period of time. (A) compound 4, (B) compound 5, (C) compound 7, and (D) compound 8.

Compounds 4-8 did not show a very high cytotoxicity in these cells, only a very mild effect, if any, (Figure 4), similar to their mild anti-proliferative activities on A549 cells. Compound 4 at the highest concentration showed a slight decrease in the viability of L929 cells (from 100 % to less than 80 %, Figure 4A), whereas compound 5 showed varying degrees of negligible decrease in viability of cells (around 92% – 100%, Figure 4B). Interestingly, compound 7, which resulted in a slight increase in proliferation of A549 cells, yielded a small decrease in L929 viability (Figure 4C). However none of these changes are considered cytotoxic to our control cell lines.

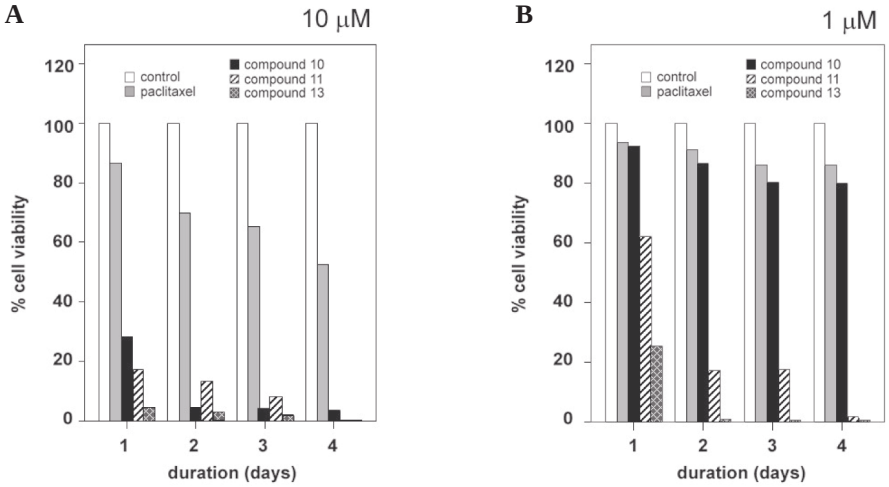


Figure 5. The cytotoxic effects of the compounds 10, 11, 13, and control drug paclitaxel on the viability of L929 cells at doses of (A) 10 μM and (B) 1 μM over a period of 4 days.

Compounds 10-13, however, mostly resulted in a significant decrease in survival and proliferation in L929 cells, indicating that these compounds are more effective in disrupting proliferation in general (Figure 5). Especially at the lower concentration of 1 μM, compounds 11 and 13 were observed to be cytotoxic even to L929 cells (Figure 5B), whereas at higher concentrations compound 13 killed almost all of the cells at the end of day 4, whereas with compound 11 a minor fraction of cells (albeit around 5% only) remained viable (Figure 5A). Paclitaxel was only mildly cytotoxic at higher concentration on these cells (Figure 5A), and 1μM of paclitaxel induced only a small decrease of around 14% during the 4-day period (Figure 5B), parallel to the results previously reported for paclitaxel.[33]

Compound 10, on the other hand, appeared to have a less cytotoxic effect on L929 cells, which was promising (Figure 5). At the lower concentration of 1 μM, compound 10 behaved in a manner similar to paclitaxel, resulting in only a mild death (around 20% death) of cells by the end of day 4 (Figure 5B). At the higher concentration, however, compound 10 was cytotoxic to cells, yielding over 70% cell death even at day 1 (Figure 5A), indicating that further assays need to be performed in order to establish the exact range of drug cytotoxicity to healthy cells in animal models.

NO Release

Our next task was to try and identify the NO-release of compounds 9-14, the group which was observed to have a stronger anti-proliferative effect in the biological assays. The ability of compounds 9-14 to release NO at different concentrations [from 31.25 to 500 μM] was determined and is summarized in Table 1. Essentially, S-nitroso-N-acetylpenicillamine (SNAP) was used as an NO donor reference. Compound 10 and 12 showed maximum NO releasing activity at the 125 and 31.25 μM concentrations. Compound 10 appears to be the most potent NO donor, which could explain its less cytotoxic effect on L929 (see Discussion). Any substituent on the aromatic ring, in this case 6-chloro- (for compound 13) and 4-hydroxy- (for compound 14), or a methyl substituent (as in compound 11) was seen to reduce the NO releasing ability (which could explain the increased

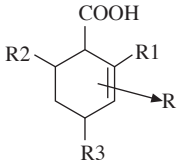
cytotoxicity of these compounds, see Discussion). A nonlinear increase in the amount of released NO is observed with increasing compound concentration, comparable to previous reports in literature.[26]

Table 1. NO releasing effects of synthesized compounds (NO release %).

		Compounds					
		C9	C10	C11	C12	C13	C14
Concentration	500 μM	1.56	0.82	1.63	0.29	3.17	2.45
	250 μM	2.32	1.11	2.05	0.44	4.60	3.25
	125 μM	3.83	12.76	3.03	0.61	5.44	8.56
	62.5 μM	3.91	18.94	6.59	10.89	6.59	10.59
	31.25 μM	7.82	58.29	9.97	35.74	8.89	12.28

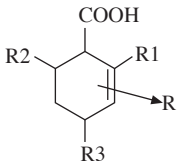
All determinations are performed in triplicates. NO release from the reference NO donor S-nitroso-N-acetylpenicillamine (SNAP) (100 μM in DMSO/water) was found to be 50.0%.

Table 2. Chemical Formulas of ureidobenzoic acid derivatives (compounds 3-8).



Comp.no	position	R	R1	R2	R3
3	2	-NH-CO-NH- CH_2-CH_2-Cl	-	H	H
4	3	-NH-CO-NH- CH_2-CH_2-Cl	H	H	H
5	3	-NH-CO-NH- CH_2-CH_2-Cl	CH_3	H	H
6	4	-NH-CO-NH- CH_2-CH_2-Cl	H	H	H
7	3	-NH-CO-NH- CH_2-CH_2-Cl	H	Cl	H
8	3	-NH-CO-NH- CH_2-CH_2-Cl	H	H	OH

Table 3. Chemical Formulas of nitrooxyalkyl ureidobenzoate derivatives (compounds 9-14).



Comp. no	n	position	R	R1	R2	R3
9	2	2	-NH-CO-NH- CH_2-CH_2-Cl	-	H	H
10	2	3	-NH-CO-NH- CH_2-CH_2-Cl	H	H	H
11	3	3	-NH-CO-NH- CH_2-CH_2-Cl	CH_3	H	H
12	3	4	-NH-CO-NH- CH_2-CH_2-Cl	H	H	-
13	2	3	-NH-CO-NH- CH_2-CH_2-Cl	H	Cl	H
14	2	3	-NH-CO-NH- CH_2-CH_2-Cl	H	H	OH

Conclusion

In this study, we have attempted to screen [(2-chloroethyl)ureido]benzoic acid (compounds 3-8) and nitric oxide releasing derivatives of [(2-chloroethyl)ureido] benzoic acid esters (compounds 9-14) as potential anti-cancer agents specifically against non-small cell lung carcinomas, and tried to determine potential drug candidates that do not cause significant cytotoxicity of the L929 fibroblast cell line as control. Compounds 4-8 unfortunately yielded neither any significant anti-proliferative nor cytotoxic effects. Since these compounds are highly hydrophilic, our conclusion at this moment is that most probably these compounds did not effectively penetrate the cell membrane, and thus did not exert any significant impact on either cell line. Further work needs to be done in the future to determine whether that is indeed the case, and if so, effective delivery methods could be developed to further analyze the potential anti-tumourigenic effects of these compounds. Additionally, other tumour types could be screened for these compounds to better understand whether the mild effects seen in this work are peculiar to the tumour type studied.

Compounds 10-14, however, were observed to be much stronger in both A549 and L929 cell lines. In previous research, pharmacological activities of the several CEU derivatives with hydroxyalkyl chains at the different position of the aromatic ring have been compared, and 3-alkyl derivatives were reported to show much better growth inhibition activity.[17] Since compounds 10-14 tested in this study were 3-substituted derivatives, the results are encouragingly parallel to similar findings in literature. Although the mechanism of cytotoxicity of CEUs have, however, not yet been completely elucidated, it has been suggested that CEUs act through similar nucleophylic alkylation or acylation mechanisms that have been already reported with other alkylating drugs such as chlorambucil and carmustine.[12,23] The pronounced activities of these compounds in our assay system could be due to the high lipophilicity of these compounds, as well as their NO-releasing activities.

It is well known that macrophages use L-Arginine to synthesize nitric oxide (NO) through the inducible NO synthase (iNOS) and the released NO contributes to the tumourigenic activity of macrophages.[25] Fast et al. has previously reported that exogenous NO is cytotoxic to L929 cells.[27] When NO releasing activities of synthesized compounds are evaluated together with growth inhibition activity results, the mild cytotoxicity of compound 10 on L929 cells may be attributed to its NO releasing effects. Although all 3 compounds show anti-proliferative activities due to their NO releasing properties, since compounds 11 and 13 are much more cytotoxic towards L929, they could not be offered as potential anti-tumour drugs. We propose that compound 10 appears to be a better candidate towards drug therapy of non-small cell lung carcinomas, with potentially little harm to the surrounding healthy cells. Further animal models need to be developed in order to confidently establish this compound as a potential clinical candidate.

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