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Synthesis, aerobic cytotoxicity, and radiosensitizing activity of novel 2,4-dinitrophenylamine tethered 5-fluorouracil and hydroxyurea

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Abstract—Two novel dual functional agents, 3[3-(2,4-dinitro-phenylamino)-propyl]-5-fluoro-1*H*-pyrimidine-2,4-dione **7** and *N*-[3-(2,4-dinitro-phenylamino)-propoxy]urea **8**, resulting from linkage of 2,4-dinitrophenylamine through three carbon atoms with 5-fluorouracil **5** and hydroxyurea **6**, respectively, were prepared and their in vitro aerobic cytotoxicities in HT-29 cell line with and without radiation were determined. Compounds **7** and **8** unlike their components were not cytotoxic but showed radiosensitizing activity.
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2,4-Dinitrophenylamine derivatives have shown antitumor activity[1] and several mono-[2] and dinitrophenylamine mustards[3] **1** and **2**, and dinitroaziridines[3] **3** have been reported as hypoxia activated alkylating bioreductive agents (Fig. 1). In these compounds, cellular reduction of the nitro group to more electron-donating species activates nitrogen of mustard by electron release through the aromatic ring and generates reactive alkylating species.[4] The cytotoxicity of these compounds in the normal oxygenated cells in which the initially reduced species can be readily re-oxidized by molecular oxygen is by mechanism other than reduction of the nitro group and has been attributed to the parent nitro compounds[2] or bio-activation by NADPH: quinine oxidoreductase (DT diphrase) which can act as an oxygen-insensitive reductase.[3] These results suggest that compounds resulting from replacement of nitrogen mustard of 2,4-dinitrophenylamine mustards **1** and **2** with other antitumor agents that do not require bio-reductive

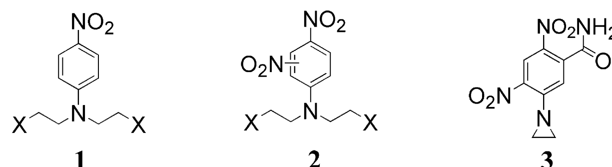

Figure 1.

metabolism activation might have similar or enhanced aerobic cytotoxicity and led us to investigate cytotoxicity of 2,4-dinitrophenylamine-linked 5-fluorouracil **7** and 2,4-dinitrophenylamine-linked hydroxyurea **8**. In addition since a number of clinical trials have shown a benefit for the combined use of ionizing radiations with 5-fluorouracil[5] **5** or hydroxyurea[6] **6** and aromatic nitro compounds are known as electron affinic radiosensitizers[7,8] evaluation of in vitro radiosensitizing activity of dual function agents **7** and **8** in comparison with their component, that is, *N*-(propyl)-2,4-dinitrophenylamine **4**, 5-fluorouracil **5**, and hydroxyurea **6**, was investigated. Since in several bioreductive compounds which have also shown aerobic cytotoxicity, nitroaromatics are linked through 3 methylene groups to other antitumor

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agents with different mechanisms of action,[9] the length of the chain in compounds **7** and **8** of this study was chosen as 3. However, synthesis of analogs of **7**, and **8** with 2, or 4 linking methylene groups is under investigation in order to determine the importance of the length of tether on the activity.

Of the compounds prepared in this study, only formation of *N*-(propyl)-2,4-dinitrophenylamine **4** in 48% yield through staggered addition of sodium hydride and propyl iodide to the solution of 2,4-dinitrophenylamine in THF during the synthesis of 1-alkoxy-2-alkylbenzimidazoles has been reported.[10] In our hands this compound could be prepared in 91% yield by refluxing a solution of 1-chloro-2,4-dinitrobenzene with 1-propylamine in ethanol (Scheme 1) for 4 h followed by evaporation of the solvent and crystallization of the residue from a mixture of CH_3OH–ether (Scheme 1). Compounds **7** and **8** were prepared by the reaction of 5-fluorouracil **5** and hydroxyurea **6** with 1-(3-bromopropyl)-2,4-dinitrophenylamine **9** which in turn was prepared by ultrasound promoted reaction of 2,4-dinitrophenylamine with 1,3-dibromopropane (Scheme 2). All the synthesized compounds were identified by IR, ^{1}HNMR, mass spectroscopic data, and elemental analyses.[11–13]

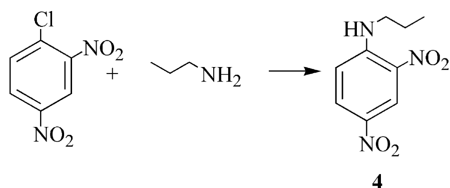
The radiosensitizing activity of compounds **7** and **8** was initially determined in SP2 cell line. Results of the preliminary experiments showed that this cell line was radiosensitive and cell death was caused by combination of radiosensitization and radiation effect which by itself in the absence of compounds reduced the number of cells to 90% of control. Therefore, experiments with this cell line were not continued and HT-29 cell line which has been reported[14,15] and was found in this study to be resistant to the effects of radiation doses more than 1 GY was used to determine the radiosensitizing activity of the tested compounds.

The experiments for determination of cytotoxicity[16] of compounds were carried out in 96-well culture dishes and cells were seeded at the density of 10^4 cells/well. The stock solutions of compounds **4**–**8** in DMSO were diluted with media and added into each well of dish. Culture mediums containing DMSO in concentration equal to those incubations treated with the tested compound served as control. Cell incubation was then kept at 37 °C under an atmosphere of 95% air:5% CO_2. The response of cells to compounds **4**–**8** with and without radiation was evaluated by determining the cell survival by MTT[17] assay after 20 h. Survival was scored by comparing treated cells to untreated cells with the tested compounds (control) and is expressed as percentage of cell survival. For radiation experiments, incubations were irradiated with different doses of the radiation at the dose rate of 1.75 GY/min by a ^{60}Co source. Control cells underwent the same procedure but without irradiation.

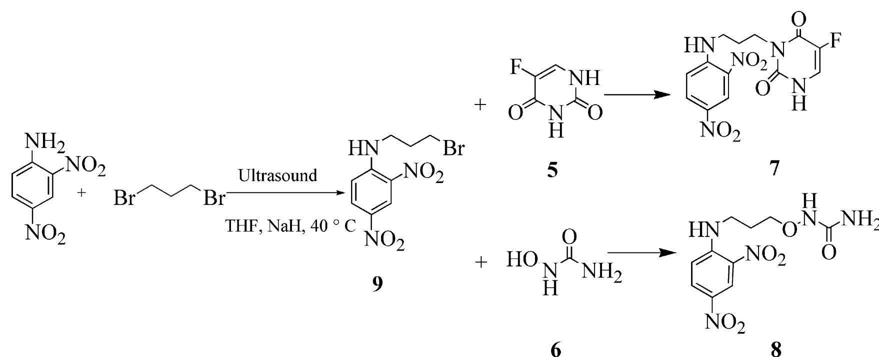
In order to determine the cell viabilities by clonogenic assay,[18] cells after treatment with the tested compounds and/or radiation were plated into culture dishes at very low concentration to yield 10^2–10^3 cells/dish, returned to 37 °C incubation, and maintained for 10 days. Cells were then fixed with formalin, stained with methylene blue, and colonies containing more than 50 cells were counted. The number of colonies of the irradiated and unirradiated dishes was compared and expressed as percentage of the survival by clonogenic assay.

All experiments were carried out with three wells three times in duplicate resulting in a minimum of 18 single data. Statistical analysis was performed using SPSS 11.0 for window (SPSS Inc, Chicago, IL, USA) and descriptive statistics are shown as arithmetic mean ± standard deviation. Independent samples' *t*-test was used to investigate the differences between irradiated and unirradiated cells treated with the tested compounds and *p* value smaller than 0.05 was considered statistically significant.

The response of HT-29 cell line to compounds **4**–**8** at concentrations of 2.5, 5, 10, and 20 μM with and with-



Scheme 1.



Scheme 2.

out irradiation is represented in Figure 1. These concentrations were chosen on the basis of pre-test measurements that showed at concentration lower than 2.5 μM, compound **5** did not show cytotoxicity and concentrations higher than 20 μM did not further potentiate the cytotoxicity and radiosensitizing activity of the tested compounds. Results showed that while 5-fluorouracil **5**, hydroxyurea **6**, and *N*-(propyl)-2,4dinitrophenylamine **4** were cytotoxic on this cell line and reduced the viability of cells to 65%, 25%, and 20% of controls, respectively, the dual functional agents **7** and **8** were not cytotoxic. These findings are in agreement with the published studies that alkylation of 5-fluorouracil **5**[19] and hydroxyurea **6**[20] with groups that are not labile results in the formation of inactive compounds.

To investigate the radiosensitizing properties of compounds **7** and **8**, incubations were irradiated with the dose 4 GY of radiation since at this dose compounds **7** and **8** at minimum concentrations of 2.5 μM showed radiosensitizing activity. In order to determine the optimum time for exposure of cells to the tested compounds before irradiation, incubations were irradiated at 0.5, 1, 2, and 4 h after treatment with 10 μM of the tested compounds **7** and **8**. Results of the clonogenic forming tests presented in Figure 3 showed that the number of surviving cells was minimum by irradiation at 1 h after exposure and the viability of cells did not change with time over the duration of the growth studies. As a result, the radiosensitizing properties of the compounds **4–8** were determined at 1 h after addition to the incubation mediums.

A comparison of results shown in Figure 1 reveals that while dual functional agents **7** and **8** were radiosensitizer and reduced viability of cells to 60% of controls, compounds **4** and **5** in contrast to reports[5,6] under experimental conditions of this study had no radiosensitizing activity.

On the basis of the results of these experiments it may also be concluded that the aerobic cytotoxicity of phenylamine mustards **3** is related to their alkylating moieties since analogous compounds **7** and **8** resulting from replacement of this moiety with 5-fluorouracil **5** or hydroxyurea **6** were not cytotoxic in HT-29 cell line. As it can be found from Figure 2, the change in concentration of compounds had no appreciable effects on their cytotoxicities.

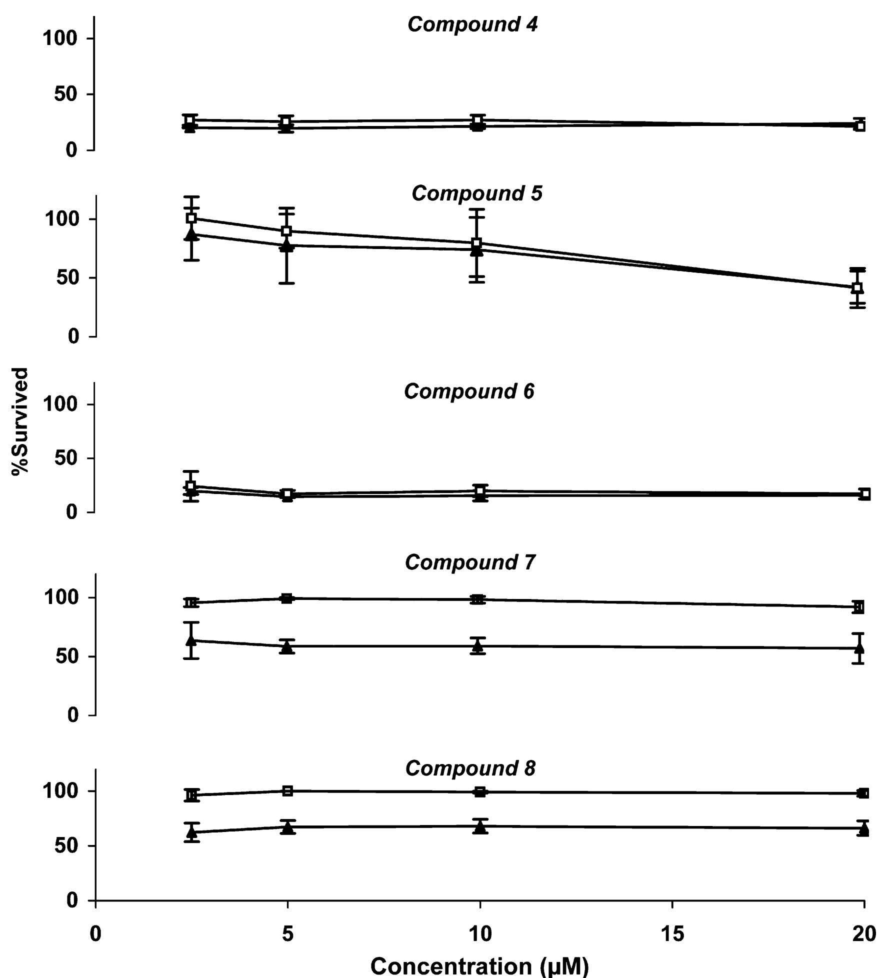


Figure 2. Survival of HT29 cells without (□) and upon (▲) Gamma-irradiation (4 Gy) after 1 h. incubation with different concentrations of the compounds **4–8** assessed by MTT assay. Data points were obtained from three independent experiments, and the standard deviations are given for each point.

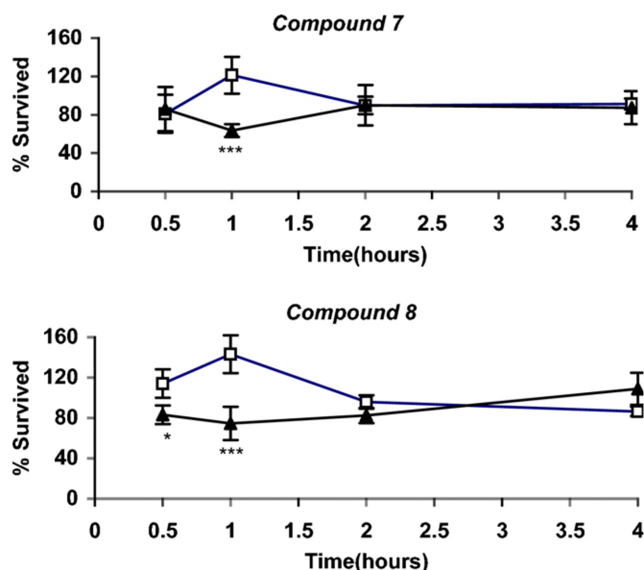


Figure 3. Clonogenic survival of HT29 cells without (□) and upon (▲) Gamma-irradiation (4 Gy) at different times after incubation with 10 μM of the compounds **7** and **8**. Data points were obtained from three independent experiments, and the standard deviations are given for each point ($^{*}p < 0.05$, $^{***}p < 0.001$).

The radiosensitizing activity of both compounds **7** and **8** was high and did not increase significantly with increase in the dose of radiation as it was found from the values which were obtained by dividing the radiation dose without over the radiation dose with the tested compounds for the same survival levels.[21] These values for compound **7** at doses of 4, 8, and 12 G were equal to 1.64, 1.66, and 1.67, where it reduced the viability of cells to 60.8%, 59.22%, and 59.56% of controls, respectively. For compound **8** the corresponding values were 1.77, 1.68, and 1.95 for reduction of cell survival to 56.63%, 59.41%, and 51.07% of controls, respectively, which statistically were not significantly different.

From results of this investigation it seems that further exploration of compounds **7** and **8** may be appropriate since these compounds were not cytotoxic but increased the sensitivity of the normal oxygenated cells to radiation. The use of these compounds in conjunction with tumor radiotherapy might result in much greater toxicity to tumor than normal tissues.

Acknowledgment

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11. For the preparation of the compound **7**, a solution of hydroxyurea **6** (76 mg, 1 mmol) and sodium ethoxide (78 mg, 1 mmol) in 20 ml of EtOH was treated with 1 mmol of 1-(3-bromopropyl)-2,4-dinitrophenylamine **9** (305 mg, 1 mmol) and refluxed for 12 h. After evaporation of the solvent under reduced pressure, compound **7** was isolated from the residue by preparative TLC (EtOAc–$CHCl_3$ 1:1) and purified by crystallization from a $CHCl_3$–Hexan mixture to give 135 mg (36%) of **7** as a yellow solid, mp 183–185 °C. IR (KBr) (νmax, cm^{-1}): 3421, 1700, 1600, 1410. ^{1}H NMR (DMSO-d_6 400 MHz) : 9.16 (d, 2H, $J = 2.8$ Hz), 8.88 (d, 1H, $J = 2.8$ Hz), 8.23 (dd, 1H, $J = 2.8$, 9.6 Hz), 7.46 (d, 1H, $J = 6.4$ Hz), 7.22 (d, 1H, $J = 9.6$ Hz), 3.60 (t, 2H, $J = 6.4$ Hz), 3.44–3.46 (m, 2H), 1.84 (m, 2H). MS (Finingan, TSQ-70): *m/z* 353 (M+). Anal. Calcd for $C_{13}H_{12}FN_5O_6$: C, 44.2; H, 3.42; N, 19.82. Found: C, 44.12; H, 3.41; N, 19.85.
12. A solution of 5-fluorouracil **5** (1 g, 7.5 mmol) in 40 ml of DMSO, was treated with K_2CO_3 (1.04 g, 2.5 mmol), KI (5.4 g, 2.5 mmol), and (780 mg, 2.5 mmol) of the compound **9** and the mixture was heated at 70–80 °C for 4 h. The mixture was then treated with water, acidified with HCl, and extracted with $CHCl_3$. The residue after evaporation of the solvent was subjected to preparative TLC ($CHCl_3$–EtOH, 9:1) to isolate compound **8** which was crystallized from a CH_3OH–ether mixture to give 210 mg (24%) of the pure product as yellow solid, mp 107–110 °C. IR (KBr) (νmax, cm^{-1}): 3356, 2919, 1613, 1511, 1332. ^{1}H NMR (DMSO-d_6, 400 MHz) 9.02 (br, 1H, NH), 8.92 (br, 1H, NH), 8.86 (d, 1H, $J = 2.8$ Hz), 8.26 (dd, 1H, $J = 2.8$, 9.6 Hz), 7.26 (d, 1H, $J = 9.6$ Hz), 6.42 (br, 2H, NH), 3.77 (t, 2H, $J = 5.6$ Hz), 3.56–360 (m,2H), 1.90–1.95 (m, 2 H). MS (Finingan, TSQ-70) *m/z* 299.1 (M+). Anal. Calcd for $C_{10}H_{13}N_5O_6$: C, 44.30; H, 4.73; N, 18.79. Found: C, 44.41; H, 4.72; N, 18.81.
13. The key intermediate **9** was prepared by ultrasound promoted reaction of 2,4-dinitrophenylamine (1.86 g, 0.01 mol) with 1,3-dibromopropane (606 mg, 0.01 mol) in the presence of sodium hydride (240 mg, 0.01 mol) in 10 ml of CH_3CN at 40 °C for 10 min and isolated by the flash chromatography ($CHCl_3$) of the residue after evaporation of the solvent and recrystallized from a $CHCl_3$–Hexan mixture to give 2.088 g (68.7%) of the pure **9** as a yellow solid; mp 63–67 °C. IR (KBr) (νmax, cm^{-1}): 3365, 2919, 1613, 1511, 133. ^{1}H NMR ($CDCl_3$ 400 MHz): 9.17 (d, 1H, $J = 2.8$ Hz), 8.62 (br, 1H, NH), 8.31 (dd, 1H, $J = 2.8$, 9.6 Hz), 7.01 (d, 1H, $J = 9.6$ Hz), 3.64–3.66 (m, 2H), 3.54 (t, 2H, $J = 6$ Hz), 2.3 (m, 2H); MS (Finingan TSQ-70) *m/z* 305 (M+). Anal. Calcd for $C_9H_{10}BrN_3O_4$: C, 35.55; H, 3.31; N, 13.82. Found: C, 35.61; H, 3.30; N, 13.77.
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