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Nitrobenzylcarbamate prodrugs of cytotoxic acridines for potential use with nitroreductase gene-directed enzyme prodrug therapy

Christian Asche,[a] Pascal Dumy,[a] Danièle Carrez,[b] Alain Croisy[b] and Martine Demeunynck[a,*]

[a]*CNRS UMR 5616 and ICMG-FR260, Université Joseph Fourier, BP53, 38041 Grenoble Cédex 9, France*
[b]*INSERM Imagerie Intégrative, Institut Curie Recherche, Laboratoire Raymond Latarjet, Centre Universitaire, 91405 Orsay Cédex, France*

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Abstract—The synthesis, solvolytic behaviour and cytotoxicity of novel 4-nitrobenzyl carbamates and carbonates derived from 3-amino-4-hydroxymethylacridine **1** are described. Compounds **2** and **6** are both substrates for *Escherichia coli* nitroreductase and the highly active lead structure **1** is liberated upon incubation of the two compounds in the presence of NTR and its cofactor NADH. Additionally, the cytostatic activity of **2** and **6** against human HT29 colon carcinoma cell lines is decreased 80-fold and 360-fold, respectively, indicating their suitability and potency as prodrugs for either gene-directed enzyme prodrug therapy or antibody-directed enzyme prodrug therapy.

Most of the currently used antitumour agents act by antiproliferative mechanisms and are not active on a particular cell type but more likely on a specific phase of cell cycle. Therefore, their often dose-limiting side effects result from cytotoxic effects on normal cells such as the rapidly dividing cells from bone marrow or gut mucosa. Prodrug design is an important strategy to decrease these unwanted toxic side effects and to improve the tumour selectivity of anticancer agents. Promising strategies comprise tumour-specific localization of exogenous enzymes capable of converting an inactive prodrug into its active cytotoxic form.[1–4] For two of these strategies the relevant enzymes may be site-specifically delivered to tumour cells either by an antibody-enzyme conjugate which binds to tumour-specific antigens (antibody-directed enzyme prodrug therapy, ADEPT)[5] or by incorporating the gene encoding for the enzyme into the DNA of the tumour cells (suicide gene therapy or gene-directed prodrug therapy, GDEPT).[6–8] The aerobic nitroreductase (NTR) isolated and cloned from *Escherichia coli*[9] is such an enzyme currently under investigation for both ADEPT and GDEPT. In conjunction with NADH or NADPH it reduces the aromatic nitro groups of substrates into their corresponding hydroxylamines.[10] The best known example of a prodrug activated by NTR is CB 1954 (5-aziridin-1-yl)-2,4-dinitrobenzamide) converted into a powerful crosslinking agent after reduction of either its 2- or 4-nitro group.[11] This drug exhibits an up to 1000-fold increased activity against NTR-transfected cell lines.[12] Crystal structures of NTR reveal that there are only few specific contacts between the enzyme and the ligand within the active site which probably explain the observed very broad substrate specificity.[13,14] Based on these findings various prodrugs of well-known antiproliferative agents including, for example, mitomycin C,[15] actinomycin D,[15] doxorubicin,[15] some pyrollobenzodiazepines[16] and enediynes[17] have been developed. All of them are attached via their amino nitrogen to a 4-nitrobenzyloxycarbonyl moiety which becomes self-immolative after enzymatic reduction.[15,18] From the electronic point of view, the replacement of a strongly electron-withdrawing group (Hammet $\sigma_p = +0.78$, R-NO_2) by an electron-donating residue (Hammet $\sigma_p = -0.34$, R-NHOH) facilitates the fragmentation by

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* Corresponding author. Tel.: +33 476 514429; fax: +33 476 514946; e-mail: martine.demeunynck@ujf-grenoble.fr

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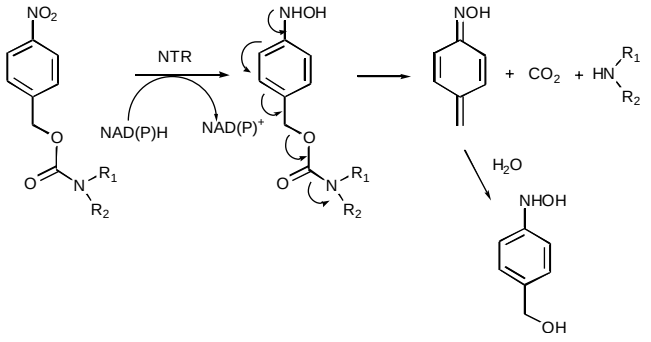
stabilizing the developing positive charge on the benzylic carbon and finally releases the free cytotoxic drug (Scheme 1).

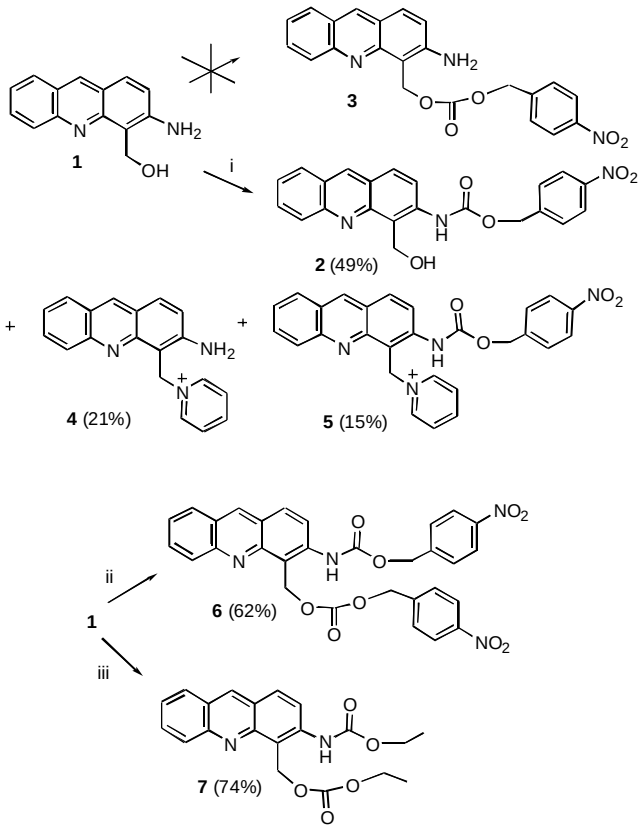
Within our ongoing research on antitumour acridines, a series of compounds derived from 3,6-diaminoacridine have been developed.[19] Some of these molecules belong to the rare class of intercalating-alkylating agents which are able to produce an electrophilic quinone–imine methide species directly at the intercalation site.[20] The lead structure 3-amino-4-hydroxymethylacridine **1** showed encouraging growth inhibitory effects on murine L1210 leukaemia, human A549 lung and human HT29 colon cancer cell lines in the nanomolar range, but appeared to be toxic in vivo. Thus, this acridine seemed to be a good candidate for the development of novel 4-nitrobenzyloxycarbonyl prodrugs activated by NTR with the hope of reducing the systemic toxicity of the active drug.

The development and the synthesis of compound **1** were described elsewhere.[21] Carbamate **2**[22] was prepared starting from **1** in a pyridine–dichloromethane mixture with 1 equiv of 4-nitrobenzyl chloroformate in 49% yield (Scheme 2). This reaction was found to be regioselective. The conceivable formation of carbonate **3** was not observed. Instead we isolated compound **4** as a by-product in 21% yield resulting from an addition of the nucleophilic solvent pyridine to the quinone–imine methide intermediate. This reaction is another evidence for the high alkylating potency of compound **1**. In the same way, the formation of the carbamate **5** was also observed by HPLC and electrospray mass spectrometry up to an extent of 15% during the reactions, this compound could not be isolated. Using 3.4 equiv of the acyl chloride the mixed carbamate/carbonate **6**[23] was obtained in 62% yield. Additionally, we similarly synthesized compound **7**[24] using ethyl chloroformate instead of 4-nitrobenzyl chloroformate. The yield was optimized up to 74% by using 15 equiv of ethyl chloroformate.

We have previously[20] shown that compounds such as **1** react as potential electrophiles by formation of highly reactive quinone–imine methide intermediates during an acid catalyzed reaction. The chemical reactivity of the new compounds **2**, **6** and **7** was investigated in a



Scheme 1. Bioactivation of 4-nitrobenzyl carbamates by NTR.



Scheme 2. Synthesis of the prodrugs. Reagents and conditions: (i) *para*-nitrobenzyl chloroformate (1 equiv), pyridine, 0 °C to rt, 8 h; (ii) *para*-nitrobenzyl chloroformate (3.4 equiv), pyridine, 0 °C to rt, 8 h; (iii) ethyl chloroformate (15 equiv), pyridine/CH_2Cl_2, 0 °C, 1 h.

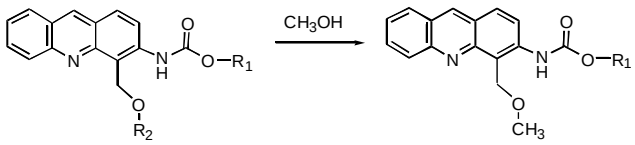
similar way. Their solvolytic behaviour was studied in methanol at 40 °C in the dark. We also studied the influence of increasing amounts of acid on both the nature of the reaction products and the kinetics of their formation. The disappearance of the starting compound was followed by HPLC and reaction products were subsequently isolated and identified by NMR and mass spectrometry.

For all reactions pseudo-first-order kinetics were observed. The calculated rates as well as half-lives are summarized in Table 1. As already reported for the *N*-methyl analogue of 3-amino-4-hydroxymethylacridine **1**[20] the reaction products were almost quantitatively the corresponding ethers,[25] which is another evidence for the high reactivity of this series of acridines (Scheme 3). The uncatalyzed reaction appeared to be much faster for all three products than for the *N*-methyl analogue of **1** ($t_{1/2}$: 66 h, k: $1.7\ 10^{-4}$ min^{-1}). Surprisingly and in contrast to our former studies,[20] the rates decrease with increasing amounts of acid indicating that the reaction is not acid catalyzed. We propose that the presence of the electron-withdrawing *para*-nitrobenzylcarbamate instead of the electron-rich amino group disfavours the formation of the quinone–imine–methide intermediate. This effect is strengthened in acid conditions by the protonation of the carbamoyl group.

Table 1. Solvolytic data

Solvent:	MeOH					
Compound:	**2**		**6**		**7**	
No. of eq. *p*-TsOH	$t_{1/2}$ (min)	$k \times 10^4$ (min^{-1})	$t_{1/2}$ (min)	$k \times 10^4$ (min^{-1})	$t_{1/2}$ (min)	$k \times 10^4$ (min^{-1})
0	156	44.4	347	20.0	72	96.4
2.5	298	23.3	485	14.3	108	64.5
5	1310	5.3	1645	4.2	163	42.5
7.5	2454	2.8	2537	2.7	468	14.8
10	3008	2.3	3275	2.1	603	11.5

Reactions were carried out in the dark at 40 °C. The final concentration was 1.67×10^{-4} M for each compound. Reactions were followed by HPLC.



2 R_1 = 4-NO_2-Bzl, R_2 = H
6 R_1 = 4-NO_2-Bzl, R_2 = 4-NO_2-Bzl-OCO
7 R_1 = C_2H_5, R_2 = C_2H_5OCO
8 R_1 = 4-NO_2-Bzl
9 R_1 = C_2H_5

Scheme 3. Methanolysis.

The ability of NTR to reduce the two 4-nitrobenzyloxycarbonyl compounds **2** and **6** was evaluated with an enzyme assay[26] where phosphate-buffered solutions (pH 7.0) of **2** and **6** (10 μM) were incubated with NADH (50 μM) and NTR (2 μg/ml) at 37 °C. Time course of the reactions was followed by HPLC. Both compounds were found to be substrates for NTR. Formation of the highly cytotoxic 3-amino-4-hydroxymethylacridine was observed for the monosubstituted carbamate **2** as well as for the bisubstituted carbamate/carbonate **6** upon bioreduction by NTR. We have checked that in the absence of NTR, the starting compounds **2** or **6** are stable. As an example the release of lead acridine **1** from carbamate **2** as a function of time is shown in Figure 1. Interestingly, both compounds reached only end points of about 45%. These findings could perhaps be explained by an inhibition of the enzyme by one or more of the

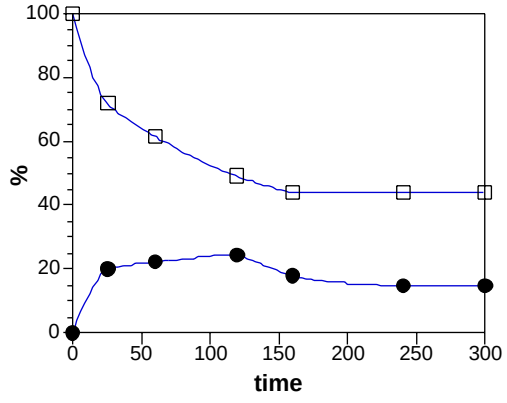


Figure 1. Release of lead acridine **1** (circles) from prodrug **2** (squares; 10^{-5} M) upon incubation with *Escherichia coli* nitroreductase (2 μg/ml) and NADH (5×10^{-5} M) in phosphate-buffered solution, pH 7. Reactions were followed by HPLC.

Table 2. In vitro cytostatic activity against various cell lines

Compound:	**1**	**2**	**6**	**7**
HT29 IC_{50}[a]	0.025	2.5	9	4.5
A549 IC_{50}	0.005	3.5	>10	n.t.[b]
V79 IC_{50}	n.t.	3.5	>10	n.t.

[a] Concentration (μM) necessary for 50% of cell growth inhibition.
[b] n.t., not tested.

reaction products, particularly the main product 3-amino-4-hydroxymethylacridine **1**, which was already found to possess alkylating properties. This hypothesis may be supported by the apparent disappearance of **1** as a function of time. It should be noted however that the same effect is observed in the presence of larger amount of enzyme.

The in vitro antiproliferative properties of the new compounds were evaluated using human HT29 colon carcinoma cell lines, A 549 (human pulmonary adenocarcinoma) and V 79 (Chinese hamster lung fibroblast).[27] The results are summarized in Table 2. Compounds **2**, **6** and **7** exhibited cytostatic activities at micromolecular concentrations. As expected, carbamoylation of the 3-amino group and additionally of the 4-hydroxymethyl group led to a significant decrease in activity. Carbamate **2** is the most active molecule, with an IC_{50} value of 2–3 μM, that is to say 80-fold less active than the unsubstituted lead structure **1** on HT29 cells. The mixed carbamate/carbonate **6** was even found to be 360-fold less active (IC_{50} value of 9 μM) on the same cell line. These differences are even more pronounce on A549 cell lines.

The activities of *para*-nitrobenzyloxy and ethoxy carbonyl compounds (**6** and **7**, respectively) are in the same range indicating that the presence of bulky substituents is not a limitation for cytotoxicity.

We have tested the influence of NTR pre-incubation on the cytotoxicity of **2** and **6** (Fig. 2). At 5 μM, in the presence of NTR, no significant difference is observed for compound **2**, which appears highly toxic for the three cell lines. At lower doses, especially with A 549 cells, this molecule becomes toxic when incubated with NTR. This observation is also true for **6**, which has no intrinsic toxicity at the tested doses and displays some cytostatic activity in the presence of NTR. In that case, HT 29 appears more sensitive than A 549 cells. Under our experimental conditions, incubation with NTR and NADH is highly cytotoxic for V79 cells, thus rendering difficult the assessment of NTR effect.

These results provide evidence that masking the 3-amino group and additionally the 4-hydromethyl group of lead acridine **1** by carbamoylation leads to a significant decrease in cytostatic activity. However, the molecules keep their good electrophilic properties even if the mechanism of reaction differs from what was found for the unsubstituted compound **1**. The two 4-nitrobenzyloxycarbonyl protected compounds **2** and **6** found to be substrates for NTR might be useful as prodrugs for GDEPT or ADEPT strategies.

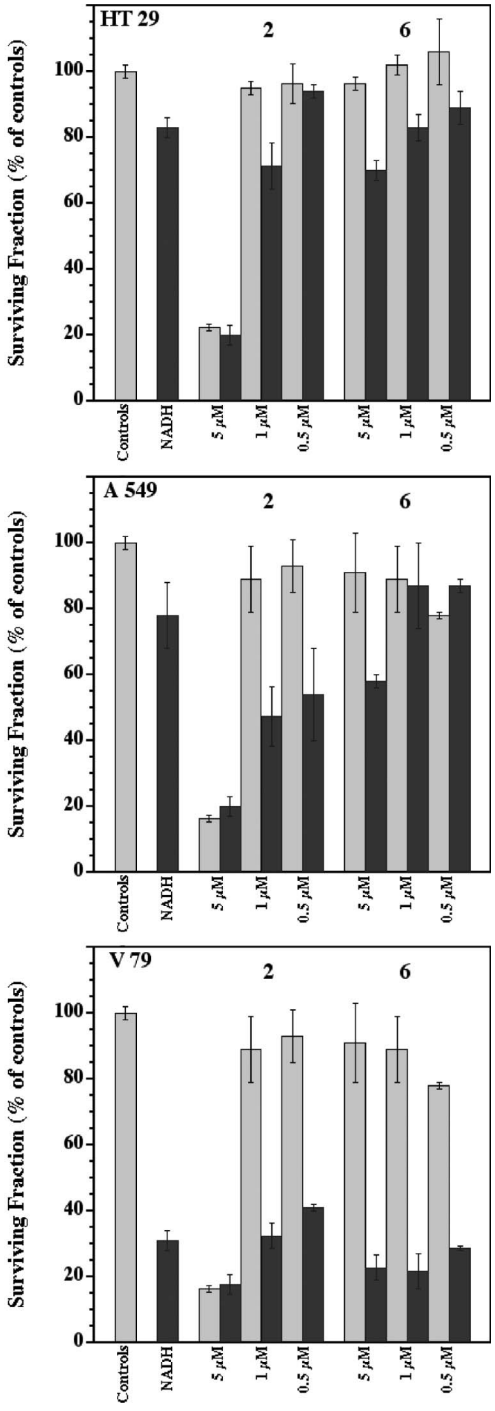


Figure 2. Cytotoxic effect on the three tested cell lines of compounds **2** and **6** at three standard doses (5, 1 and 0.5 μM) alone (▒) or in the presence of nitroreductase (NTR) and NADH (■). Survival of untreated and NTR/NADH treated controls is given for reference.

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22. Compound **2**. δ_H ($CDCl_3$) 9.11 (s, br, 1H, NH), 8.72 (s, 1H, H-9), 8.29–8.18 (m, 4H, H-1 or H-2, H-5, H-3′, H-5′), 8.05 (d, 1H, H-1 or H-2, *J* 9.1 Hz), 7.97 (d, 1H, H-8, J 8.5 Hz), 7.81-7.75 (m, 1H, H-6 or H-7), 7.67–7.60 (m, 2H, H-2′, H-6′), 7.57–7.50 (m, 1H, H-6 or H-7), 6.08 (s, 2H, $ArCH_2O$), 5.42 (s, 2H, $NHCOCH_2Bzl$), MS (ESI^+) *m/z* 404 $(M+H)^+$ (calcd for $C_{22}H_{17}N_3O_5$: C, 65.50; H, 4.25; N, 10.42. Found: C, 65.61; H, 4.30; N, 10.30).
23. Compound **6**. δ_H ($CDCl_3$) 9.19 (s, br, 1H, NH), 8.71 (s, 1H, H-9), 8.29–7.99 (m, 5H, aromatic H's), 7.85–7.13 (m, 9H, aromatic H's, solvent overlies signals), 6.14 (s, 2H, $ArCH_2O$), 5.37 + 5.24 (2 × s, 4H, $NHCOOCH_2Bzl$ + $OCOOCH_2Bzl$), MS (ESI^+) *m/z* 583 $(M+H)^+$ (calcd for $C_{30}H_{22}N_4O_9$: C, 61.86; H, 3.81; N, 9.62. Found: C, 61.59; H, 3.92; N, 9.58).
24. Compound **7**. δ_H ($CDCl_3$) 9.04 (s, br, 1H, NH), 8.68 (s, 1H, H-9), 8.25 (d, 1H, *J* 8.8 Hz, H-5), 8.21 (d, 1H, *J* 9.2 Hz, H-1 or H-2), 8.00 (d, 1H, *J* 9.2 Hz, H-1 or H-2), 7.97 (d, 1H, *J* 8.5 Hz, H-8), 7.79-7.73 (m, 1H, H-6 or H-7), 7.53-7.48 (m, 1H, H-6 or H-7), 6.12 (s, 2H, $ArCH_2O$), 4.32 (q, 2H, *J* 7.1 Hz, $OCOCH_2CH_3$), 4.23 (q, 2H, *J* 7.1 Hz, $NHCOCH_2CH_3$) 1.40 and 1.28 (2× t, 2× 3H, *J* 7.1 Hz, $2 \times OCH_2CH_3$), MS (DCI^+, NH_3, isobutane) *m/z* 369 $(M+H)^+$ (calcd for $C_{20}H_{20}N_2O_5$: C, 65.21; H, 5.47; N, 7.60. Found: C, 65.34; H, 5.41; N, 7.52).
25. *General procedure for ether formation*. The appropriate acridine was dissolved in methanol and the reaction mixture was stirred at 40 °C in the dark. The disappearance of the starting compound was followed by HPLC. After the reaction was completed, the mixture was concentrated in vacuo and stored at 4 °C. The solid obtained after 1 h was filtered, subsequently triturated in isopropanol and the solid thus formed was filtered again. *4-Methoxymethyl-3-[(4-nitrobenzyloxycarbonyl) amino]*

*acridine (**8**)*. Compound **8** was obtained as an orange solid in 71% yield. δ_H ($CDCl_3$) 9.01 (s, br, 1H, NH), 8.68 (s, 1H, H-9), 8.35 (d, 1H, *J* 8.8 Hz, H-5), 8.27–8.23 (m, 2H, H-3′, H-5′), 8.16 (d, 1H, *J* 8.6 Hz, H-2), 7.95 (d, 1H, *J* 9.2 Hz, H-8), 7.80–7.67 (m, 1H, H-6 or H-7), 7.63-7.58 (m, 2H, H-2′, H-6′), 7.52–7.41 (m, 1H, H-6 or H-7), 5.63 (s, 2H, AcrCH_2O), 5.37 (s, 2H, NHCOOCH_2Bzl), 3.53 (s, 3H, OCH_3); MS (ESI^+) *m/z* 418 $(M+H)^+$ (calcd for $C_{23}H_{19}N_3O_5$: C, 66.18; H, 4.59; N, 10.07. Found: C, 65.94; H, 4.89; N, 9.99). *3-(4-Ethoxycarbonyl)amino-4-(methoxymethyl)acridine (**9**)*. Compound **9** was obtained as an orange solid in 68% yield. MS (DCI^+, NH_3, isobutane) *m/z* 311 $(M+H)^+$ (calcd for $C_{18}H_{18}N_2O_3$: C, 69.66; H, 5.85; N, 9.03. Found: C, 69.74; H, 6.02; N, 8.90).

26. Recombinant *E. coli* NTR and β-NADH (β-nicotinamide adenine dinucleotide, reduced form, dipotassium salt) were purchased from Sigma. Enzyme assays were carried out in Eppendorf cuvettes using a thermoblock at 37 °C. 10^{-3} M stock solutions of **2** and **6** in DMSO as well as a 10^{-3} M stock solution of NADH in buffer and a solution of enzyme in buffer containing 2 μg enzyme/ml were prepared. Final compound concentrations of 10^{-5} M were subsequently obtained by pipetting 15 μl into an Eppendorf cuvette and diluting this volume with the appropriate amount of buffer, NADH solution and—at $t = 0$ min—enzyme solution up to a volume of 1.5 ml. For a normal testing the volumes used were: 15 μl of 10^{-3} M compound solution in dimethylsulfoxide; 75 μl of 10^{-3} M NADH stock solution (final concentration: 5×10^{-5} M, 5 equiv); 410 μl of buffer; 1.0 ml of enzyme solution 2 μg/ml. For every single test a 'blank' was also performed which contained: 15 μl of 10^{-3} M compound solution in dimethylsulfoxide; 75 μl of 10^{-3} M NADH stock solution (final concentration: 5×10^{-5} M, 5 equiv); 1410 μl of buffer. This was done in order to test the stability of the compounds under these conditions. Reactions were followed by HPLC as a function of time by removing aliquots (250 μl) and injecting them onto the HPLC column. Peak areas of UV chromatograms were used to determine both the disappearance of the starting compounds as well as the appearance of the reaction products.
27. Cells were cultivated in Dulbecco's MEM supplemented with 10% FCS. Cells from log-phase culture were seeded in 24-microwell plates [1 ml—5×10^4 cells/well for HT 29 (human colon adenocarcinoma, ATCC HTB 38), 10^4 cells/well for A 549 (human pulmonary adenocarcinoma, ATCC CCL 185) and 10^5 cells/well for V 79 (Chinese hamster lung fibroblast, ATCC CCL-93)] and incubated for 2 days. Tested compounds, in dimethylsulfoxide solution, were added under the minimum volume (5 μl) in increasing concentration. For incubation with nitroreductase, 50 μL of a 0.5 mM solution of NADH and 8 μL of nitroreductase (1 μg/mL) were added to each well. Control cells received 5 μl of dimethylsulfoxide alone, and, when appropriate, of NADH, NTR or NADH + NTR at the same concentration described above. Plates were incubated for 24 h, then medium was removed and cells were washed twice with phosphate-buffered saline before addition of fresh medium free of drug. Plates were reincubated for 3 days before evaluation of the cell survival using the MTT assay using 30 min incubation with 100 μg/well of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT, Sigma).[28] After removal of the medium, formazan crystals were taken up with 100 μl of dimethylsulfoxide and absorbance at 540 nm was measured with a microplate reader (Model 450 Bio-Rad), survival was expressed as % of dimethylsulfoxide treated controls. All incubations were carried out at 37 °C in a water-jacketed CO_2 incubator (5% CO_2, 100% relative humidity). Cytostatic activity was expressed as IC_{50}, the concentration that reduced by 50% the number of treated cells relative to controls. IC_{50} values were extracted from regression curves obtained with experimental points.
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