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N-Substituted 1,2-Dihydroquinolines as Anticancer Agents: Electronic Control of Redox Stability, Assessment of Antiproliferative Effects, and Mechanistic Insight

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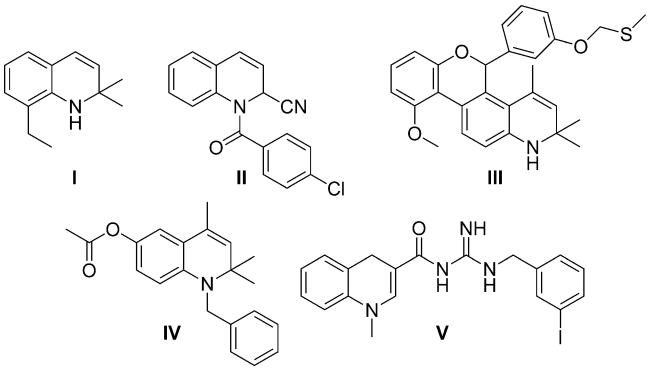
Quinolines and quinolones are privileged core structures in various therapeutic agents, especially those used against infectious diseases. Antimalarial drugs such as chloroquine, mefloquine, and premaquine,[1] and antibacterial agents such as ciprofloxacin, levofloxacin, and norfloxacin,[2] are some of the well-known examples from these groups. Our laboratory recently reported the synthesis of 4- and 2-quinolones from Baylis–Hillman acetates and amines through an $S_N2' \rightarrow S_NAr \rightarrow (\Delta^{3,4}-\Delta^{2,3}$ shift)$\rightarrow$oxidation sequence involving 1,2-dihydroquinolines (DHQs) as key intermediates.[3] Subsequent analyses showed that these DHQs, though sensitive to light and oxygen, can gain stability if the aromatic ring is substituted with electron-withdrawing groups. Because compounds such as menadione, ascorbic acid, and piperlongumine can elicit an antiproliferative response by directly or indirectly perturbing the redox balance in rapidly proliferating cells, we envisaged that DHQs will also be interesting candidates for further study.[4] Results from our investigations in this direction are presented here.

A literature search revealed that DHQs have not received significant attention in drug design, likely due to their decreased stability. Synthetic DHQ derivatives with 1,2- or 2,2-disubstitution pattern have been shown to act as anti-inflammatory agents (e.g., compound **I**)[5] or HIV-1 reverse transcriptase inhibitors (e.g., compound **II**).[6] In addition, derivatives of 2,2,4-trisubstituted DHQs are well known as lipid peroxidation inhibitors,[7] glucocorticoid receptor modulators (e.g., compound **III**),[8] and antioxidants.[9] Interestingly, esters of 2,2,4-trimethyl-1,2-dihydroquinoline-6-ols (e.g., compound **IV**) were found to be redox-active due to the possibility of quinone imine formation through electron transfer after deacetylation by trypanosomal esterases.[10] A number of DHQ derivatives from this group possess antitrypanosomal activity with IC_{50} values in the nanomolar range. Their ability to exert strain on the antioxidant machinery of the parasite by generating reactive oxygen species (ROS) is believed to be responsible for the activity.

Replacement of the C2 and C4 hydrogen atoms by alkyl groups (as in compound **III**) or acylation of the ring nitrogen



(as in compound **II**) is likely to lower the propensity of the compound to undergo oxidative transformations. The tendency of 1,4-DHQs to undergo redox transformation in biological milieu was made use of by Foucout and co-workers in the development of a chemical delivery system (CDS) for central nervous system (CNS)-active agents, such as γ-aminobutyric acid (GABA) and *meta*-iodobenzylguanidine (**V**).[11] These compounds, when conjugated to 1,4-DHQ, were able to cross the blood brain barrier with ease, and the formation of quinolinium salt inside the cells led to accumulation of the agent through an ion trap mechanism. Because cancer cells are more susceptible than normal cells to redox imbalance, DHQs are attractive candidates for selectively targeting malignant cells.[4]

Synthesis of 1,2-DHQ derivatives **5**–**7** typically involves formation of the Baylis–Hillman adduct from the appropriate aromatic aldehyde and α,β-unsaturated carbonyl compound,[12] its acetylation to form intermediate **3**, and subsequent tandem S_N2'–S_NAr reactions using an amine of interest.[3,13] The required 1,2-DHQs were obtained in moderate to good yields (Scheme 1).

DHQs **5**–**7** differ in the nature of the substituent on the aromatic ring. In order to get an understanding of their cytotoxic effects, the viability of HeLa, SiHa and SW480 cell lines after incubation with these compounds was studied by MTT assay, and the results are presented in Table 1. Among these, the 6,7-difluoro- and 6-nitro-substituted DHQs (**5** and **7.1**, respectively) were more potent than the 5,6,7,8-tetrafluoro-substituted de-

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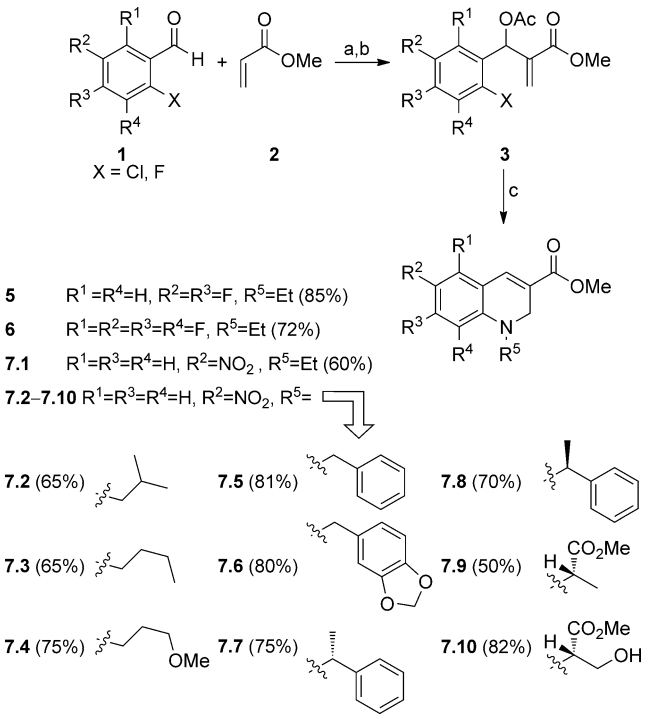

Scheme 1. Synthesis of dihydroquinolines **5**, **6**, and **7.1**–**7.10**. *Reagents and conditions*: a) 1,4-diazabicyclo[2.2.2]octane (DABCO), THF, 27 °C, 24–48 h; b) AcCl, Et_3N, CH_2Cl_2, 0 °C→RT, 1–2 h; c) R^5NH_2 (**4**), NMP (*N*-methyl pyrrolidone), K_2CO_3, 27 °C, 1–12 h. Full protocol details and characterization data are given in the Experimental Section and Supporting Information.

rivative **6**. Considering the inhibitory effects and the ease of synthesis of compound **7.1**, a subset of compounds (**7.2**–**7.10**) was then prepared by varying the nature of the N-substitution and subjected to cytotoxicity evaluation. Interestingly, all of these compounds showed antiproliferative effects in a concentration-dependent manner and had IC_{50} values in the range of 5.2–84 μM (Table 1). 1-Phenylethylamine- and serine-based DHQs **7.7**, **7.8** and **7.10** were superior in terms of their inhibitory activities. As the oxidation of these DHQs after cellular uptake is a possibility, we prepared quinolones **8**–**12**, quinoline **13** and quinolinium salts **14**–**15** to investigate whether such activation in vivo is responsible for the observed cytotoxicity.[3] When assayed against HeLa cells, the IC_{50} values of these compounds were found to be greater than 100 μM suggesting that the biological effects from such species, if at all, would be rather weak; this does not undermine the effect from redox reactions involving DHQs in which quinolinium ions could be a byproduct.

There are a number of anticancer agents that exert their effect by inducing apoptosis, a process characterized by plasma membrane blebbing, externalization of phosphatidyl-

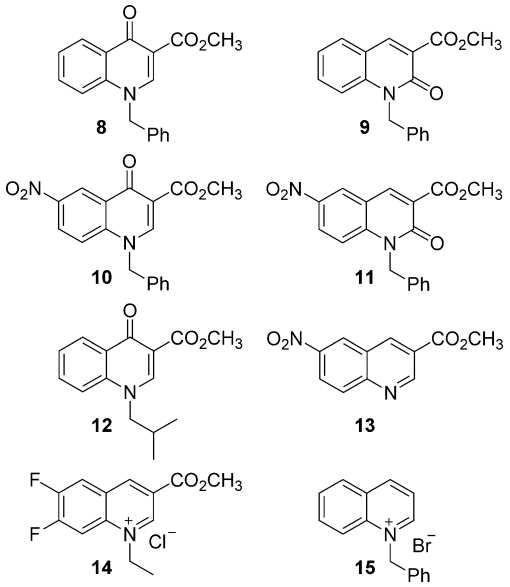

Table 1. Cytotoxic effects of 1,2-dihydroquinoline (DHQ) derivatives against three human cancer cell lines.[a]

Compd	IC_{50} [μM]		
	HeLa	SiHa	SW480
5	47.8 ± 1.0	32.4 ± 6.1	33.2 ± 0.4
6	> 100	> 100	> 100
7.1	42.4 ± 4.0	47.3 ± 1.4	37.3 ± 2.0
7.2	20.3 ± 0.5	24.1 ± 4.6	18.8 ± 0.7
7.3	19.6 ± 1.5	18.0 ± 0.3	12.6 ± 0.4
7.4	29.7 ± 0.8	27.1 ± 1.0	20.4 ± 0.7
7.5	27.4 ± 1.0	23.0 ± 0.3	20.5 ± 0.6
7.6	34.4 ± 2.9	27.3 ± 2.1	19.0 ± 1.0
7.7	17.6 ± 3.5	5.2 ± 0.9	5.7 ± 0.3
7.8	13.9 ± 0.3	7.5 ± 0.2	9.0 ± 0.3
7.9	84.0 ± 2.1	72.5 ± 3.5	60 ± 0.8
7.10	8.5 ± 0.3	6.8 ± 1.8	5.2 ± 0.1

[a] Cells were treated with various concentrations of test compound and incubated for 72 h; DMSO was used as the vehicle-only control; cell viability was determined by MTT assay, and the IC_{50} values were calculated from the results. Data represent the mean ± SD of three independent experiments performed in triplicate.

serine, cell shrinkage, nuclear and cytoplasmic condensation, cytochrome c release, activation of caspases, and DNA fragmentation.[14] In order to assess the cellular effects of exposure to DHQs, the most active compounds (**7.10** and **7.7**) were subjected to further analysis. Because cleavage of DNA into oligonucleosomal-sized fragments is an indication of apoptosis, these compounds were first evaluated in a DNA fragmentation assay according to the reported protocol.[15] Western blot analysis of lysates from HeLa cells treated with these DHQs clearly showed DNA laddering, which point toward their ability to induce apoptosis (Figure 1).

Apoptosis is accompanied by a loss of mitochondrial membrane potential that can be studied using JC-1, a cationic dye capable of undergoing a reversible change in fluorescence emission.[16] High membrane potential promotes aggregation of the dye inside the mitochondria, where it fluoresces orange. At the same time, cells with low potential contain monomeric JC-1 in the cytoplasm, where it fluoresces green. During our studies, the dye was found localized in the mitochondria of DMSO-treated (control) HeLa cells (Figure 2a), where it formed

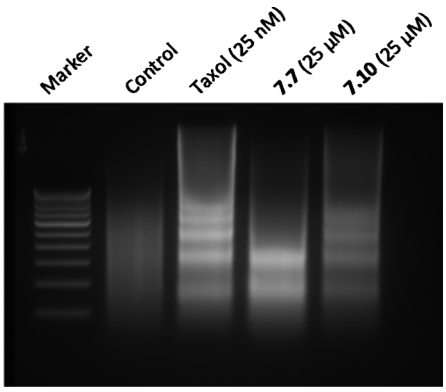

Figure 1. DNA fragmentation induced in HeLa cells by DMSO (control), Taxol (25 nM), **7.7** (25 μM) and **7.10** (25 μM).

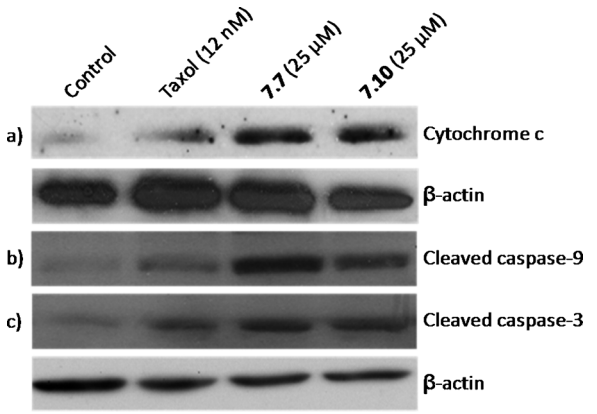

Figure 3. Western blots obtained after exposing HeLa cells to Taxol (12 nM), **7.7** (25 μM), and **7.10** (25 μM). The following effects were observed: a) cytochrome c release into cytosol, b) activation of cleaved caspase-9, and c) activation of cleaved caspase-3.

orange fluorescent aggregates. However, there was a notable loss of mitochondrial membrane potential after 24 h treatment of the cells with Taxol and **7.10**, as indicated by green fluorescence (Figure 2 b,c respectively).

Caspases are a family of cytosolic cysteine proteases involved in the initiation and execution of apoptosis.[17] In mitochondrion-dependent apoptosis, cytochrome c is released into the cytosol where it binds with Apaf1 and procaspase-9 forming an apoptosome complex, which then gives caspase-9; subsequent activation of downstream effectors such as caspase-3 triggers apoptosis.[18] In order to see whether DHQs operate through a mitochondrion-mediated mechanism, HeLa cells were incubated with **7.7** (25 μM) or **7.10** (25 μM) for 48 h and analyzed for cytochrome c, cleaved caspase-9, and cleaved caspase-3 expression by Western blot. Results indicate that these compounds trigger release of cytochrome c, and cleaved caspases 9 and 3, which confirmed that these DHQs induce apoptosis via a mitochondrial pathway (Figure 3).

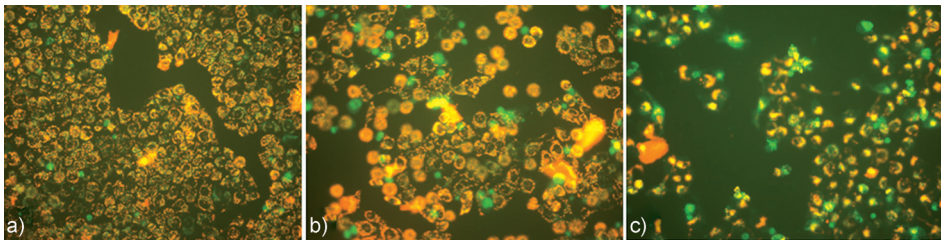

Figure 2. Fluorescence microscopic images of HeLa cells stained with JC-1 after 24 h exposure to a) DMSO, b) Taxol (12 nM), and c) **7.10** (25 μM).

Subsequently, flow cytometry was performed to evaluate the effect of DHQs on the cell cycle.[19] HeLa cells were treated with **7.10** (12.5 μM) for 24 h, fixed, stained by propidium iodide (PI) and analyzed using DMSO and Taxol (12 nM) as negative and positive controls, respectively (Figure 4). Compound **7.10** caused depletion of cells in the G0/G1 phase with a concomitant accumulation of cells in the G_2/M phase indicating a G_2/M arrest (Figure 4 a). An increase was also observed in the apoptotic fraction; the S phase, however, seemed unaffected relative to the DMSO-treated control cells. Treatment of cells for 24 h with **7.10** resulted in 26% apoptotic cells whereas only 8.8% apoptotic cells were seen in vehicle-only control cells (Figure 4 b). Similarly, there were 26.8% of G_2/M-phase cells compared with 13.1% in vehicle-only control cells (Figure 4 c). These data were determined to be statistically significant ($p < 0.01$) suggesting that **7.10** and related compounds induce G_2/M phase cell-cycle arrest.

To investigate the possibility of DHQ-induced apoptosis through the formation of ROS, a cell-based assay using 2′-7′-dichlorodihydrofluorescein diacetate (DCFH-DA) was carried out.[20] Intracellular ROS were detected using the redox-sensitive fluorescent dye DCFH-DA, a membrane permeable fluorogenic reagent that measures the ROS activity within a cell. After diffusion into the cell, DCFH-DA is deacetylated by cellular esterases to a non-fluorescent compound, which undergoes oxidation by ROS to the highly fluorescent derivative 2′,7′-dichlorofluorescein (DCF). The production of ROS was determined by measuring the intensity of the fluorescence (Figure 5 a). Relative DCF fluorescence intensities were quantitated from the representative images of cells treated with **7.7** (42.23 ± 0.004) and **7.10** (32.45 ± 0.78) and compared with the control group (6.069 ± 0.03) (Figure 5 b). A significant increase in intracellular ROS was observed in cells subjected to oxidative stress injury caused by DHQs **7.7** and **7.10** but not in the control cells. To determine whether intracellular ROS increment originates directly from the compound or indirectly after its effect on mitochondria, the DCFH-DA assay was carried out with varying incubation times (i.e., 15 min, 30 min, 1 h, 6 h, and 12 h). The results indicate that oxidative stress induced by DHQs is very rapid, and hence the generated ROS is likely due to a direct effect (for details, see the Supporting Information).

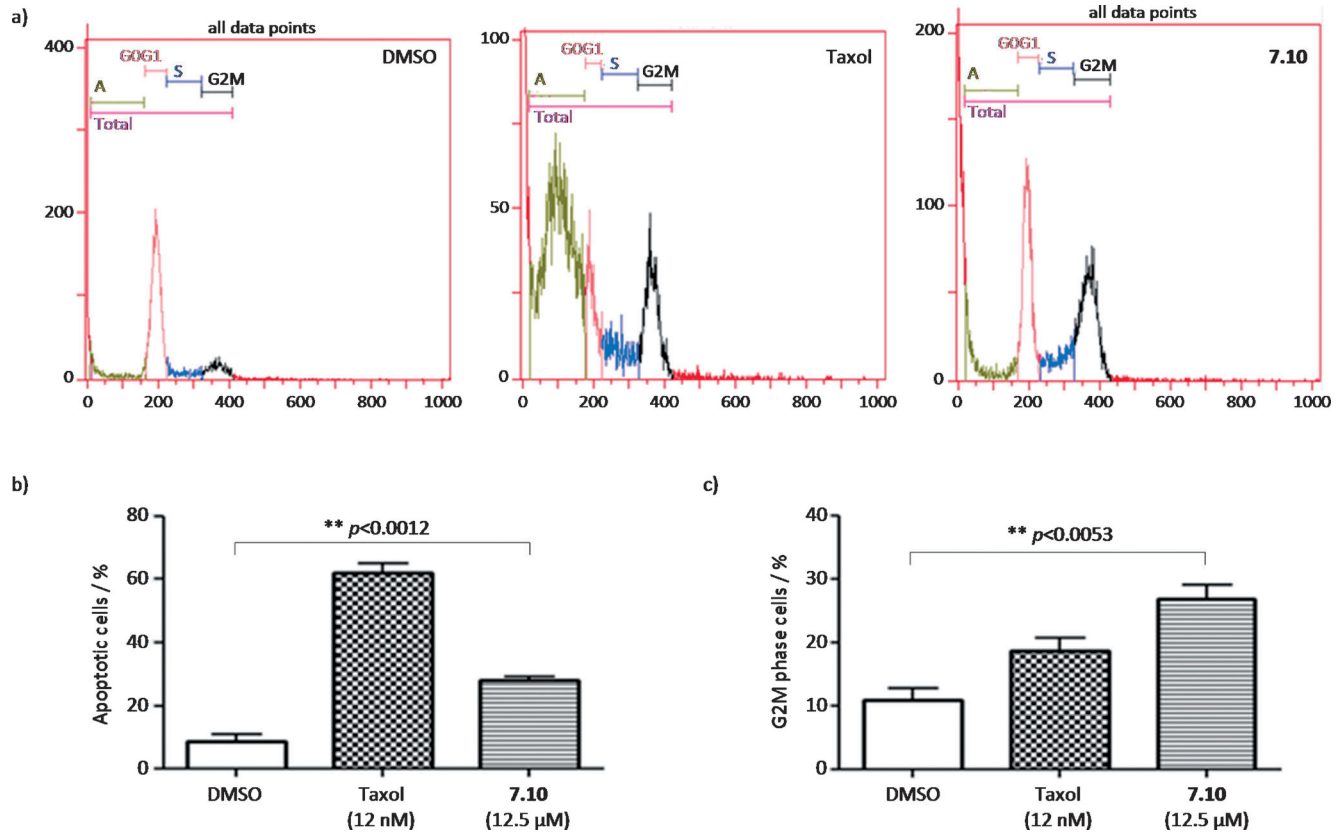

Figure 4. Flow cytometric analysis of HeLa cells. a) Cell-cycle histograms of HeLa cells treated with DMSO, Taxol (12 nM), and **7.10** (12.5 μM). Statistical analysis of b) percentage of apoptotic cells, and c) percentage of G_2/M phase cells. Data represent the mean ± SEM of three independent experiments performed in triplicate.

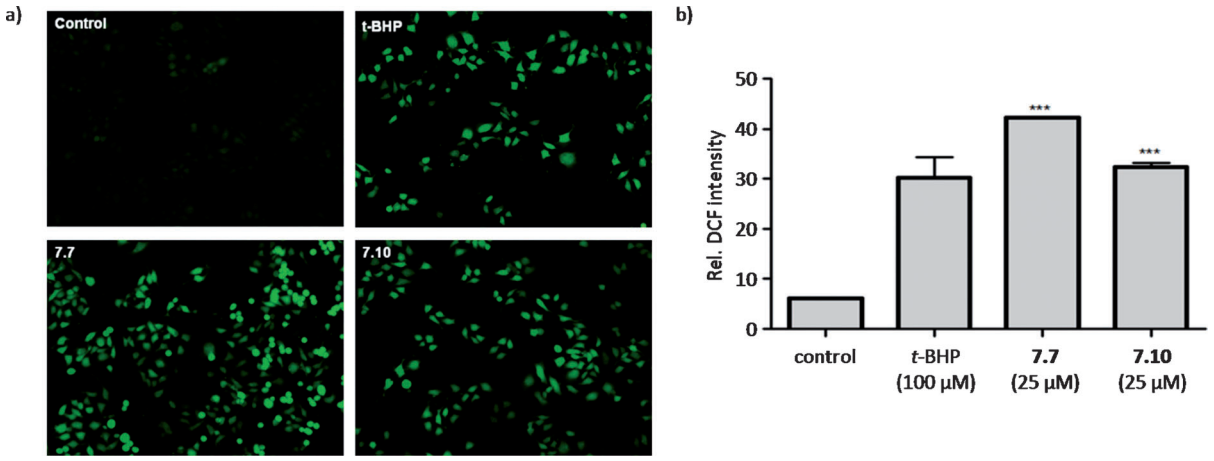

Figure 5. Dihydroquinolines (DHQs) induce ROS accumulation in HeLa Cells. a) Representative fluorescent photomicrographs showing relative (rel.) 2′,7′-dichlorofluorescein (DCF) fluorescence in cells treated with DMSO (control), **7.7** (25 μM), **7.10** (25 μM), and *tert*-butyl hydroperoxide (*t*-BHP) (100 μM). b) Quantification of reactive oxygen species (ROS) production as determined by DCF fluorescence. b) Bar graphs presenting the ROS accumulation data. Values represent the mean ± SEM of $n = 2$ independent experiments performed in triplicate; *** $p < 0.001$ vs. control.

Although cancer cells depend on ROS-based signaling for hyperproliferation, they are very sensitive to fluctuations in redox status. For this reason, compounds capable of inducing redox imbalance can potentially target such cells. While reactivity and interaction with multiple targets might be considered a drawback for drug development, if good selectivity can be achieved, these same features could help overcome resistance, which is a major concern for many anticancer drugs in clinical use. Active interest in the area of redox chemotherapy is evident from drug candidates such as 2-methoxyestradiol

(phase II), disulfiram (phase II), motexafin gadolinium (phase III), and β-lapachone (phase II), which have all reached advanced stages of clinical development.[4a,21] The results presented here clearly show that 1,2-DHQs induce oxidative stress in HeLa cells. As normal cells are known to be less susceptible to small fluctuations in ROS levels due to the presence of antioxidant defense mechanisms, the compounds discussed here can be considered as leads for the development of new drug candidates. The ready availability of starting materials, ease of synthesis, and possibility of structural diversification for structure–activity relationship studies are all advantages of this class of compounds. Efforts to discover highly potent compounds in this class, preferably with IC_{50} values in the low nanomolar range, and investigations to identify the specific targets, if any, of these compounds are currently underway.

In conclusion, we have discovered a novel series of 1,2-dihydroquinoline (DHQ) derivatives with promising anticancer activities. Detailed biochemical studies showed that they are capable of inducing apoptosis as evidenced by DNA fragmentation, disruption of mitochondrial membrane potential, cytochrome c release, and activation of caspase-9 and caspase-3. Their ability to induce oxidative stress in cancer cells was unequivocally confirmed by DCFH-DA fluorescence assay. Flow cytometric analysis of HeLa cells treated with a representative compound (**7.10**) revealed that these DHQs cause cell-cycle arrest in the G_2/M phase. Taken together, these observations point toward the operation of an intrinsic pathway of apoptosis in the presence of these compounds. Detailed structure–activity relationship studies and efforts to gain an understanding of their biological effects at a molecular level are currently in progress.

Experimental Section

General procedure for the preparation of dihydroquinolines: A stirred solution of the Baylis–Hillman acetate (1 equiv) in *N*-methyl pyrrolidone (6 mL mmol^{-1}) under a nitrogen atmosphere was treated with K_2CO_3 (1.5 equiv). The appropriate amine (2 equiv) was then added to the reaction mixture, and stirring was continued at room temperature for 1–12 h. After completion (determined by TLC), water was added until the solution became turbid, and the mixture was stirred for an additional 30 min. The precipitated product was isolated by filtration, washed with water and dried in vacuo. This near-pure material was then purified further to remove trace impurities by column chromatography (EtOAc/hexanes; gradient elution). Isolated yields of dihydroquinolines ranged from 50–85%. Spectral data of representative examples (**7.7** and **7.10**) are given below, and that of other compounds are given in the Supporting Information.

(*R*)-Methyl 6-nitro-1-(1-phenylethyl)-1,2-dihydroquinoline-3-carboxylate (7.7): Reaction of the appropriate Baylis–Hillman acetate **3** ($R^1=R^3=R^4=H$, $R^2=NO_2$, X=Cl) (200 mg, 0.64 mmol) for 5 h according to the procedure described above yielded **7.7** as a yellow gummy solid (162 mg, 75%): $R_f=0.43$ (EtOAc/hexanes, 1:4); ^{1}H NMR (400 MHz, $CDCl_3$): $\delta=8.00$ (dd, $J=9.2$, 2.4 Hz, 1H, Ar*H*), 7.90 (d, $J=2.8$ Hz, 1H, Ar*H*), 7.41–7.29 (m, 6H, Ar*H*, –C_4H), 6.64 (d, $J=9.2$ Hz, 1H, Ar*H*), 5.20 (q, $J=6.8$ Hz, 1H, N–C*H*), 4.35 (d, $J=16.0$ Hz, 1H, N–CH_aH_b), 4.14 (d, $J=16.0$ Hz, 1H, N–CH_aH_b), 3.76 (s, 3H, OCH_3), 1.69 ppm (d, $J=6.8$ Hz, 3H, CH_3–CH); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta=165.2$, 151.2, 139.0, 137.6, 134.1, 129.1 (2C), 128.4, 128.1, 127.0 (2C), 126.0, 122.8, 119.6, 110.0, 55.8, 52.1, 43.3, 15.5 ppm; IR (neat): $\tilde{\nu}=1708$, 1601, 1574, 1499, 1436, 1324, 1289, 1233, 1165, 1106 cm^{-1}; HRMS-ESI: *m/z* $[M+K]^+$ calcd for $C_{19}H_{18}N_2O_4K$: 377.0904, found: 377.0897.

(*S*)-Methyl 1-(3-hydroxy-1-methoxy-1-oxopropan-2-yl)-6-nitro-1,2-dihydroquinoline-3-carboxylate (7.10): Reaction of the appropriate Baylis–Hillman acetate **3** ($R^1=R^3=R^4=H$, $R^2=NO_2$, X=Cl) (200 mg, 0.64 mmol) for 12 h according to the procedure described above yielded **7.10** as a yellow solid (176 mg, 82%): $R_f=0.26$ (EtOAc/hexanes, 1:1); mp: 134–137 °C; ^{1}H NMR (400 MHz, $CDCl_3$): $\delta=8.02$ (dd, $J=9.2$, 2.8 Hz, 1H, Ar*H*), 7.92 (d, $J=2.4$ Hz, 1H, Ar*H*), 7.35 (s, 1H, –C_4H), 6.52 (d, $J=9.2$ Hz, 1H, Ar*H*), 4.55–4.48 (m, 2H, N–CH_aH_b/N-C*H*), 4.34 (d, $J=15.2$ Hz, 1H, N–CH_aH_b), 4.25 (dd, $J=11.6$, 5.6 Hz, 1H, CH_aH_bOH), 4.09 (dd, $J=11.6$, 7.2 Hz, 1H, CH_aH_bOH), 3.83 (s, 3H, –OCH_3), 3.81 (s, 3H, –OCH_3), 2.22 ppm (br s, 1H, –O*H*); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta=169.9$, 165.1, 151.0, 139.0, 133.8, 127.9, 125.8, 123.5, 120.7, 110.3, 62.6, 59.8, 53.0, 52.3, 45.6 ppm; IR (KBr): $\tilde{\nu}=3457$, 2954, 1740, 1721, 1640, 1573, 1495, 1434, 1324, 1298, 1221, 1174, 1088, 1010, 929, 817, 742 cm^{-1}; HRMS-ESI: *m/z* $[M+Na]^+$ calcd for $C_{15}H_{16}N_2O_7Na$: 359.0855, found 359.0866.

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Keywords: apoptosis · cytotoxic agents · dihydroquinolines · G_2/M cell-cycle arrest · reactive oxygen species

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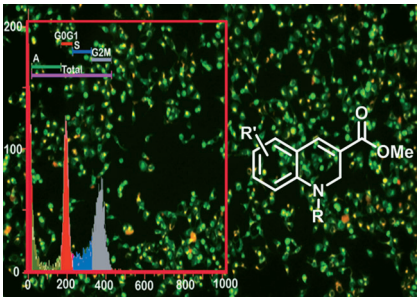
Redox chemotherapy: Antiproliferative activities of a series of N-substituted 1,2-dihydroquinolines capable of causing redox imbalance in cancer cells are presented. Detailed studies showed that these derivatives arrest the cell cycle in the G_2/M phase and induce apoptosis through an intrinsic pathway characterized by loss of mitochondrial membrane potential, DNA fragmentation, cytochrome c release, and activation of caspases 9 and 3.



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N-Substituted 1,2-Dihydroquinolines as Anticancer Agents: Electronic Control of Redox Stability, Assessment of Antiproliferative Effects, and Mechanistic Insight