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Original article

Cytotoxicity of new alkylamino- and phenylamino-containing polyfluorinated derivatives of 1,4-naphthoquinone

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ABSTRACT

Fluorinated derivatives of 1,4-naphthoquinone are highly potent inhibitors of Cdc25A and Cdc25 phosphatases and growth of tumor cells. Five new N-substituted polyfluorinated derivatives of 2-amino-1,4-naphthoquinone were synthesized and their mutagenic and antioxidant properties in *Salmonella* cells, as well as cytotoxicity in human myeloma (RPMI 8226), human mammary adenocarcinoma (MCF-7), mouse fibroblasts (LMTK) and primary mouse fibroblast cells (PMF) were studied. 2-*tert*-Butylamino-3,5,6,7,8-pentafluoro-1,4-naphthoquinone (**1**) inhibited the growth of normal control and tumor cells at the same concentration. Three compounds: 2-diethylamino-3,5,6,7,8-pentafluoro-1,4-naphthoquinone (**2**), 2-ethylamino-3,5,6,7,8-pentafluoro-1,4-naphthoquinone (**3**), 2-phenylamino-3,5,6,7,8-pentafluoro-1,4-naphthoquinone (**4**) exhibited a 50% decrease in the growth of cancer cells at low and comparable concentrations (2.4–8.6 μM) while being remarkably less cytotoxic toward normal LMTK and PMF cells. Quinones (**1**)–(**4**), but not 2-phenylamino-3-methyl-5,6,7,8-tetrafluoro-1,4-naphthoquinone (**5**), efficiently suppressed spontaneous mutagenesis in *Salmonella* cells, while all compounds **1**–**5** decreased the mutagenic effect of H_2O_2 on bacterial cells. Their possible perspectives as anticancer drugs are shortly discussed.

1. Introduction

Cyclin-dependent kinases (Cdk) are central regulators of the eukaryotic cell cycle, which phosphorylate proteins responsible for the activation of structural and regulatory genes in transitions between G1, S, G2, and M cell cycle phases. Cdc25A, B and C are members of the family of dual specificity protein phosphatases regulating cyclin-dependent kinases by removing phosphate groups from the cyclin-dependent kinases and thus activating the cyclin–Cdk complexes, which control the cell cycle progression [1,2]. Therefore, abnormalities in kinases and phosphatases, which are the key enzymes of these protein phosphorylation signaling pathways, are closely linked with many human diseases including cancer. Cdc25A and Cdc25B have been shown to be overexpressed in a number of tumors of various origins [3]. The putative involvement of the Cdc25 phosphatases in tumorigenesis makes them potential targets for cancer therapy [4,5].

Several of numerous investigated substances were found to inhibit the enzymes of Cdc25 family [6,7], and NSC 95397 (2,3-bis[2-hydroxyethylthio]-1,4-naphthoquinone) from the National Cancer Institute Library was shown to be the most potent Cdc25 inhibitor [8]. *p*-Naphthoquinones and 7-aminoquinoline-5,8-quinones are core structures of potential inhibitors of Cdc25 phosphatases [8,9], including NSC 663284 [10]. Previous studies suggested that quinoline-5,8-quinones can inactivate the Cdc25 family phosphatases either by Michael addition [11] or oxidation of the catalytic cysteine [12]. Quinoline-5,8-quinone derivatives substituted at the C2 and C4 positions were shown to effectively inhibit Cdc25B and cancer cell growth [13].

Overall, in vivo use of quinones presents a major challenge because of their toxicity [14,15]. Although most quinones have been reported to inhibit Cdc25 by sulfhydryl arylation at the quinone nucleus, the redox properties of quinones can also generate reactive oxygen species (ROS) [14], which may be toxic to normal tissues and thus reduce the therapeutic utility of quinones. Quinone radicals can also damage DNA and mitochondria through the formation of H_2O_2 and other ROS and reactive nitrogen species (RNS) [16–18]. One strategy for overcoming the intrinsic toxicity of quinones might be to use derivatives that are more stable in their reduced

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state and thus are less likely to initiate formation of radicals and indiscriminately damage cells. Interestingly, the fluorinated quinone compound F-Cpd5 (5,6,7,8-tetrafluoro-2-(2-mercaptoethanol)-3-methyl-[1,4]-naphtoquinone) in contrast to its parent compound 2-(2-mercaptoethanol)-3-methyl-[1,4]-naphtoquinone (Cpd5) was predicted not to generate ROS [15]. The calculated reduction potential of F-Cpd5 was suggestive of much higher possible therapeutic index [13]. This was supported by the observation that F-Cpd5 generated significantly less ROS than Cpd5; F-Cpd5 was three times more potent than Cpd5 in inhibiting the hepatoma Hep3B cell growth [18–20]. Also, F-Cpd5 inhibited mitogen-induced DNA synthesis in normal rat hepatocytes 12-fold less than in Hep3B cells [19].

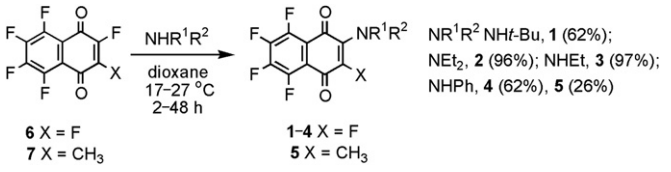
The rationale to study inhibitors of Cdc25 phosphatases is looking for better inhibitors of tumor cells growth having no toxic, mutagenic or carcinogenic properties. Since all naphthoquinones including the polyfluorinated compound F-Cpd5 inhibit Cdc25 phosphatases [8–20], it is reasonable to suggest that some new polyfluorinated naphthoquinones will also inhibit these enzymes to some extent. As only the data concerning toxic, mutagenic or carcinogenic properties could provide a solid judgement regarding possible applications of new compounds as antitumor drugs, a measurement of their affinity for Cdc25 phosphatases seems to be of secondary importance. Taking this into account, we have recently synthesized four new *n*-butylamino and two sulfur-containing derivatives of polyfluoro-1,4-naphthoquinone and analyzed their mutagenic and antioxidant properties using special type of *Salmonella* cells, as well as their cytotoxicity in human and mouse tumor cells, and primary mouse fibroblast cells [21]. Interestingly, all six compounds demonstrated different antioxidant and mutagenic properties in the bacterial system and efficiency to suppress the growth of cancer cells at significantly lower concentrations than normal cells.

In this study, five new polyfluorinated derivatives of 1,4-naphthoquinone containing *tert*-butylamino, diethylamino, ethylamino, and phenylamino groups were synthesized and their cytotoxicity against cancer and normal cells as well as ability to protect bacterial cell from mutagenesis were compared.

2. Chemistry

Syntheses of compounds **1**–**4** were based on amino-defluorination of hexafluoro-1,4-naphthoquinone **6** with *tert*-butyl-, diethyl-, ethylamine and aniline, respectively. Compound **5** was prepared by phenylaminodefluorination of 2-metylpentafluoro-1,4-naphthoquinone (**7**) (Scheme 1).

Quinone **6** reacted easily with aliphatic amines at 17–27 °C to afford corresponding 2-alkylaminopentafluoro-1,4-naphthoquinones **1**–**3**. After 20 h with *tert*-$BuNH_2$, quinone **6** gave a mixture of **1** (~80%) and the starting compound **6** (~20%). Longer reaction times did not change the percentage of the major product **1**, but, besides **6**, products of the further transformations of quinone **1** were also found in the reaction mixture. Quinone **1** was isolated by TLC in 62% yield. Quinones **2** and **3** were obtained for 2 h virtually as individual compounds in practically quantitative isolated yields. The reaction of starting compound **6** with aniline at room temperature for 15 h gave quinone **4** (~90% in the product mixture) alongside with the products of its further transformation (~10%). Quinone **4** was isolated by TLC in 62% yield. After 48 h, quinone **7** with aniline gave compound **5** (~80% in a product mixture) alongside with the products of its further transformation (~20%). Quinone **5** was isolated by TLC in 26% yield.



Scheme 1. Synthesis of compounds **1**–**5**.

Compounds **1**–**5** have been characterized by ^1H and ^{19}F NMR spectra, elemental analysis, or high resolution mass-spectroscopy. Indicative was the position and appearance of the F^3 signals of quinones **1**–**4** compared with that of the starting quinone **6** (δ, ppm vs. hexafluorobenzene as an internal reference: F^2, F^3 22.1; F^5, F^8 25.5; F^6, F^7 18.6). In the spectrum of quinone **3**, this signal was significantly high-field shifted (δ 5.3 ppm) relative to quinone **6** by an electron-donating effect of the ethylamino group. Compared to this, in the spectra of quinones **1**, **2** and **4** the F^3 signals were somewhat shifted to the lower field (**1** 16.5, **2** 18.7, **4** 28.1 ppm), presumably due to the greater or lesser suppression of this effect by the out-of-plane deviation of bulky substituted amino groups by their steric interaction with F^3 and, in the case of **4**, by nitrogen π-conjugation with the phenyl substituent. In all these cases, the F^3 signal looked like a singlet somewhat widened by weak spin couplings with F^5–F^8. The F^5–F^8 signals in the spectra of **1**–**5** were located (δ 23–27 for F^5, F^8 and 14–20 ppm for F^6, F^7) closely to their counterparts in the ^{19}F NMR spectra of **6** and **7**, their multiplicities being typical for *ortho* disubstituted tetrafluorobenzenes ($^3J_{F5,F6}$, $^3J_{F6,F7}$, $^3J_{F7,F8}$ 19–20, $^4J_{F5,F7}$, $^4J_{F5,F8}$, $^4J_{F6,F8}$ 10–16 Hz). The NH resonances in the ^1H spectra of quinones **1**, **3** and **4** appeared as slightly widened singlets at δ 5.39, 5.74 and 7.13 ppm respectively; in case of **5**, between 7.3 and 7.4 ppm.

3. Biological studies

At the first step, we have analyzed the inhibition of the growth of four cell lines by quinones **1**–**5** using tumor cell lines from human myeloma RPMI 8226, human mammary adenocarcinoma MCF-7, mouse fibroblasts LMTK and primary mouse fibroblast cell line (PMF). Fig. 1 shows typical curves of inhibition by compounds **1**–**4** of the growth of RPMI cells. The results obtained for compounds **1**–**5** with all types of cells are summarized in Table 1. It can be seen that quinone **1** demonstrated nearly the same IC_{50} for cancer cells RPMI and MCF-7 (2.5–3.5 μM) and mouse PMF and LMTK (IC_{50} = 3.1–6.3 μM) cells. Compound **4** was characterized by the same IC_{50} values for two types of cancer cells (2.4 μM), which was comparable

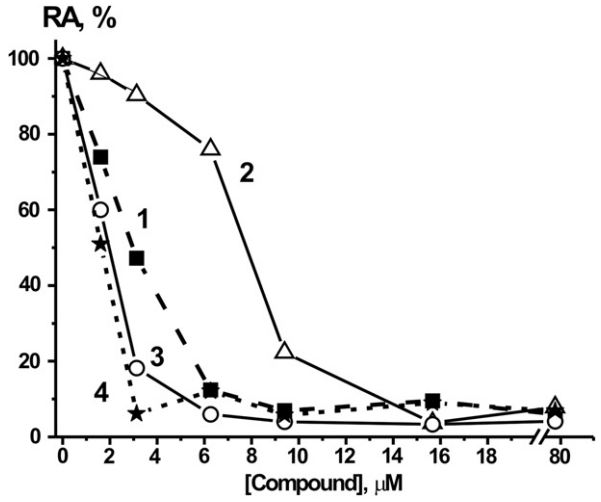


Fig. 1. Effects of four fluorinated compounds on the growth of RPMI cells. The average error in three experiments for any compound concentration did not exceed 5–15%.

Table 1
Cytotoxicity (IC_{50}) of polyfluorinated derivatives of 1,4-naphthoquinone towards different mammalian cell lines.

Compound	IC_{50}, μg/mL (μM)			
	RPMI 8226	MCF-7	LMTK	FMS
1 (2-*tert*-butylamino-3,5,6,7,8-pentafluoro-1,4-naphthoquinone)	1.1 ± 0.2 (3.5 ± 0.6)[a]	0.8 ± 0.1 (2.5 ± 0.4)	2.0 ± 0.2 (6.3 ± 0.7)	1.0 ± 0.1 (3.1 ± 0.3)
2 (2-diethylamino-3,5,6,7,8-pentafluoro-1,4-naphthoquinone)	2.2 ± 0.6 (6.9 ± 1.8)	1.3 ± 0.1 (4.1 ± 0.4)	20.0 ± 3.0 (62.7 ± 9.4)	9.0 ± 1.8 (28.2 ± 5.6)
3 (2-ethylamino-3,5,6,7,8-pentafluoro-1,4-naphthoquinone)	0.7 ± 0.1 (2.4 ± 0.1)	2.5 ± 1.1 (8.6 ± 1.4)	3.2 ± 0.5 (11.0 ± 0.7)	9.0 ± 4.3 (30.9 ± 14.4)
4 (2-phenylamino-3,5,6,7,8-pentafluoro-1,4-naphthoquinone)	0.8 ± 0.03 (2.4 ± 0.1)	0.8 ± 0.07 (2.4 ± 0.2)	4.0 ± 0.6 (11.8 ± 1.8)	2.5 ± 0.3 (7.4 ± 0.7)
5 (2-phenylamino-3-methyl-5,6,7,8-tetrafluoro-1,4-naphthoquinone)	7.5 ± 0.5 (22.4 ± 1.3)	4.5 ± 0.4 (13.4 ± 1.1)	36.0 ± 6.0 (104.4 ± 17.9)	45%[b]
Control compound F-Cpd5: 5,6,7,8-tetrafluoro-2-(2-mercaptoethanol)-3-methyl-[1,4]naphtoquinone [21]	4.7 ± 0.29 (14.8 ± 0.9)	54.9 ± 6.7 (173.0 ± 21.0)	No inhibit. at 173 μM	n.d.[c]

[a] Mean ± S.D. from three independent experiments.
[b] When the cytotoxicity was low, the percent of inhibition of cell growth at the highest used concentration (25 μg/mL) of the compound was determined.
[c] n.d., not determined.

with those for **1**, while 50% inhibition of LMTK (IC_{50} = 11.8 μM) and FMS (IC_{50} = 7.4 μM) cells was observed at its 3–5-fold higher concentrations. Cytotoxicities of **1** and **3** toward the tumor cells were dependent upon the cell line. While compound **2** suppressed the growth of MCF 1.7-fold better (4.1 μM) than of RPMI cells (6.9 μM), quinone **3** was 3.6-fold more active in the case of RPMI (2.4 μM) than MCF (8.6 μM) cells. While both **2** and **3** exhibited comparable IC_{50} (28.2 and 30.9 μM) in the case of FMS cells, quinone **2** was ~5.7-fold less cytotoxic than **3** towards LMTK cells. Overall, compounds **2**, **3**, and **4** suppressed the growth of LMTK and FMS cells at the concentrations 3.1–15.3-fold higher than was required for suppression of the tumor RPMI and MCF cells, while this difference for quinone **1** was lower (1.1–2.6-fold) (Table 1).

Compound **5** inhibited the growth of cancer MCF (13.4 μM), RPMI (22.4 μM) and LMTK (104.4 μM) cells at different and significantly higher concentrations compared with **1**–**4** (Table 1), but the growth of normal FMS cells was depressed only by 45% at a high concentration (74.4 μM). The data indicate that compounds **2**–**5** can better suppress the growth of tumor cells than of normal mammalian cells in comparison with quinone **1**, while the ratio of relative cytotoxicities of these derivatives towards different types of normal and tumor cells is individual for the every compound.

Recently we have synthesized F-Cpd5 as reported [15] and compared its growth-inhibiting properties with those for several new compounds [21]. Three of them: 2-*n*-butylamino-3,5,6,7,8-pentafluoro-1,4-naphthoquinone (**I**), (2,2'-dithiodi-2)-3,5,6,7,8-pentafluoro-1,4-naphthoquinon-2-ylamino)ethane (**II**), and 2-(2'-methylthioethyl)amino-3,5,6,7,8-pentafluoro-1,4-naphthoquinone (**III**) exhibited IC_{50} 3.7–5.9-fold lower than that for F-Cpd5 (IC_{50} = 14.8 ± 0.9 μM for RPMI and 173.0 ± 21.0 μM for MCF cells) [21].

The structures of **1**–**5** resemble that of F-Cpd5 and above mentioned naphtoquinones (**I**–**III**), therefore one could expect similar effects of all these compounds on the Cdc25 phosphatases and growth of tumor cells. For RPMI cancer cells, F-Cpd5 demonstrated IC_{50} = 14.8 ± 0.9 μM [21] comparable with the IC_{50} for the least active compound **5** but it was 2.1–6.2-fold worse inhibitor than quinones **1**–**4**, while all compounds **1**–**5** inhibited the growth of MCF cells 13–72-fold better than F-Cpd5 (Table 1).

It is known that some compounds interacting with many cell targets at the same time may be polyfunctional and possess antioxidant properties, or, on the contrary, may be mutagenic or carcinogenic [22–24]. Some quinone derivatives can act as topoisomerase inhibitors via DNA intercalation and reduction of quinone moiety by oxidoreductases [25–27] and form ROS/RNS

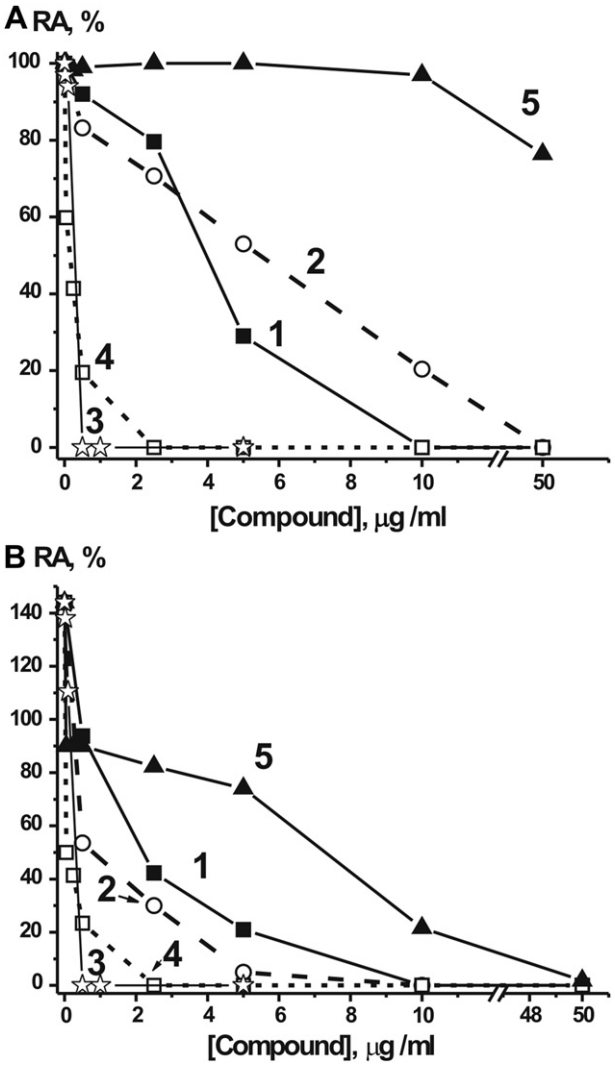


Fig. 2. Analysis of the mutagenic and antioxidant activity of compounds **1**–**5** by a standard Ames test [28] using the *S. typhimurium* strain TA102 in the absence (A) and in the presence of 3 mM H_2O_2 (B). The average error in three experiments for any compound concentration did not exceed 5–15%. The number of revertants in the absence of H_2O_2 was taken for 100%.

Table 2
IC_{50} values characterizing suppression of spontaneous and H_2O_2-induced mutagenesis by polyfluorinated derivatives of 1,4-naphthoquinone.

Compound	IC_{50}, μg/mL (μM)[a]		
	Suppression of spontaneous mutagenesis (from 100 to 50%)	Suppression of H_2O_2-induced and spontaneous mutagenesis	
		From 150 to 100%	From 100 to 50%
1 (2-*tert*-butylamino-3,5,6,7,8-pentafluoro-1,4-naphthoquinone)	3.9 ± 0.4 (12.2 ± 1.2)	0.45 ± 0.4 (1.4 ± 0.14)	2.2 ± 0.2 (6.9 ± 0.7)
2 (2-diethylamino-3,5,6,7,8-pentafluoro-1,4-naphthoquinone)	5.4 ± 0.5 (18.5 ± 1.9)	0.25 ± 0.03 (0.86 ± 0.09)	0.78 ± 0.08 (2.7 ± 0.27)
3 (2-ethylamino-3,5,6,7,8-pentafluoro-1,4-naphthoquinone)	0.2 ± 0.03 (0.69 ± 0.06)	0.14 ± 0.015 (0.094 ± 0.009)	0.3 ± 0.03 (1.0 ± 0.1)
4 (2-phenylamino-3,5,6,7,8-pentafluoro-1,4-naphthoquinone)	0.2 ± 0.03 (0.59 ± 0.06)	0.03 ± 0.003 (0.089 ± 0.009)	0.05 ± 0.005 (0.15 ± 0.015)
5 (2-phenylamino-3-methyl-5,6,7,8-tetrafluoro-1,4-naphthoquinone)	24% at 50 μg/ml (149 μM)[b]	0.25 ± 0.02 (0.75 ± 0.08)	7.3 ± 0.7 (21.8 ± 2.2)
Control compound F-Cpd5: 5,6,7,8-tetrafluoro-2-(2-mercaptoethanol) -3-methyl-[1,4]naphtoquinone [21]	(0.81)	n.d.[c]	(0.44)

[a] Mean ± S.D. from three independent experiments.
[b] When the suppression of revertant formation was low, the percent of suppression at the highest used concentration (50 μg/mL) of the compound was determined.
[c] n.d., not determined.

that damage DNA [16,17]. Thus, drug may be more successful when they are not mutagenic at least at the therapeutic concentrations.

The *Salmonella typhimurium* strain TA102 is often used both for evaluation of mutagenicity of different compounds and for detection of their antioxidant properties as judged from suppression of spontaneous mutagenesis in this strain and from a decrease in the mutagenisity of known oxidants, usually H_2O_2 [28].

The mutagenic and antioxidant activities of compounds **1**–**5** were first estimated in the Ames test [28] using the *S. typhimurium* strain TA102 similar to [29]. The test for mutation induction in the Ames assay is performed by calculating the reversion frequencies from histidine auxotrophy to prototrophy in a response to different mutagenic substances. The compound concentration that causes 50% bacterial revertants growth inhibition (IC_{50}) was determined. Quinone **5** did not markedly affect the reversion frequencies (24% of suppression at 149 μM), while compounds **1** and **2** caused a significant decrease (50% at at 3.9 ± 0.4 and 5.4 ± 0.5 μg/mL or 12.2 ± 1.2 and 18.5 ± 1.9 μM, respectively) in the spontaneous appearance of mutants, while complete suppression of cell growth was observed at concentrations ≥10–50 μg/mL (Fig. 2A, Table 2). At the same time, compounds **3** and **4** displayed the significantly lower and nearly the same IC_{50} values (0.2 ± 0.03 μg/mL or 0.6–0.7 μM) and completely suppressed the appearance of mutants at approximately 0.5 and 2.5 μg/mL (1.6 and 7.4 μM), respectively (Fig. 2A, Table 2).

It is known that some compounds reacting as antioxidants efficiently decrease the toxic and mutagenic effect of H_2O_2 [28,29]. In the Ames test, H_2O_2 was added to TA102 cells at the optimal concentration, 3 mM [28,29], and the test compound concentrations varied (Fig. 2B). At low concentrations (≤0.05 μg/mL or 0.09–1.4 μM), all five 1,4-naphthoquinone derivatives under study suppressed efficiently the H_2O_2-dependent formation of mutants from 144 to 100% of revertants that was observed for the control containing no H_2O_2. The dependencies observed at higher concentrations of **1** and **3** (Fig. 2B) were comparable with those found for **3** and **4** in the absence of H_2O_2 (Fig. 2A, Table 2). At the same time, quinones **2** and **4** decreased the formation of revertant cells from 100 to 50% in the presence of H_2O_2 at 4–7-fold lower concentrations (0.78 ± 0.08 and 0.05 ± 0.005, μg/mL or 2.7 ± 0.27 and 0.15 ± 0.055 μM, respectively) than in the absence of hydrogen peroxide. Interestingly, while without H_2O_2 compound **1** was a better suppressor of spontaneous revertant formation than **2**, in the presence of H_2O_2 a reverse situation was observed (Fig. 2, Table 2). Compound **5**, demonstrating no detectable effect on the frequency of the spontaneous cell reversion up to high concentration, 50 μg/mL (149 μM) (Fig. 2A), was a very good suppressor of H_2O_2-dependent mutant formation and effectively decreased it from 144 to 100% at 0.25 μg/mL (0.75 μM) (Fig. 2B). At concentrations 0.5–5.0 μg/mL, compound **5** only slightly (~10%) decreased the frequency of mutants, while at higher concentrations it was a more effective suppressor of reversions (Fig. 2B). While the reference F-Cpd5 possessed low activity toward tumor cells (IC_{50} = 14.8–173 μM), it suppressed efficiently spontaneous (IC_{50} = 0.81 μM) and, especially, H_2O_2-indiced mutagenesis (IC_{50} = 0.44 μM). Similar behavior was previously observed for 2-(2′-methylthioethyl)amino-3,5,6,7,8-pentafluoro-1,4-naphthoquinone (**VI**), which became ~20-fold more effective suppressor of H_2O_2-dependent mutagenesis [21]. One cannot exclude that compounds **2**, **4** and previously described F-Cpd5 and **VI** possessing higher activity in the presence than in the absence of H_2O_2 can be metabolized or react with components of mammalian cells or cell peroxides to yield products with a higher protective function or general cytotoxicity.

The relative cytotoxicities of the compounds under study in the suppression of all types of mammalian cell growth (Table 1) overall did not correlate with their ability to decrease the bacterial revertant formation, since quinones **1**, **2** and **5** restrained tumor cell growth better than the formation of bacterial revertants while **3** and **4** showed an opposite tendency (Tables 1 and 2). Taken together, one may suggest these polyfluorinated naphthoquinones to play a dual role, being both antioxidants suppressing spontaneous and H_2O_2-induced formation of revertants and potential inhibitors of cell phosphatases. However, one cannot exclude that these compounds may be to some extent cytotoxic toward mammalian and bacterial cells, without being associated with the inhibition of Cdc25 phosphatases.

According to [18–20], functionalized polyfluoro-1,4-naphthoquinones may be more promising inhibitors of Cdc25 as compared to 1,4-naphthoquinone. It is known that some quinone derivatives can form ROS/RNS and damage DNA [16,17]. A complete absence of a compound-dependent increase in the mutant formation in the presence and in the absence of H_2O_2 indicates that quinones **1**–**5** are not mutagenic themselves and counteract both spontaneous mutagenesis and the toxic and mutagenic effects of H_2O_2. It should be mentioned that compounds **1**–**4** can be considered effective antioxidants, while **5** possesses reduced antioxidant activity.

Since quinone **1** possesses practically the same cytotoxicity towards cancer and control normal mammalian cells, it cannot be considered promising as an inhibitor of Cdc25 and a specific suppressor of cancer cell growth.

4. Conclusion

Polyfluorinated derivatives of 1,4-naphthoquinone are less active in generation of ROS and may be promising inhibitors of Cdc25 as compared to 1,4-naphthoquinone itself [15,18–20]. Our data indicate that compounds **3** (2-ethylamino-3,5,6,7,8-

pentafluoro-1,4-naphthoquinone) and **4** (2-phenylamino-3,5,6,7,8-pentafluoro-1,4-naphthoquinone) show the best potential, exerting a stronger cytotoxic effect against cancer cells in comparison with normal mammalian cells (Table 1) and efficiently protecting bacterial cells from mutagenesis both in the presence and the absence of H_2O_2, all investigated properties of these two compounds being better than for F-Cpd5, but comparable with the analogous properties of previously described naphthoquinones (**I**) and (**II**). Compound **2** is of lower efficiency in both cases (Table 2). Quinone **5** is unlikely a promising candidate for being an anticancer drug owing to its low activity in restraining the growth of cancer cells and suppressing reversions in bacterial cells.

5. Experimental protocols

5.1. Chemistry

Commercially supplied diethylamine, *tert*-butylamine, and aniline were purified by vacuum distillation (0.03 mm Hg); ethylamine hydrobromide was used without purification. Quinone **6** was prepared according to [30], and compound **7** according to [31]. Dioxan was vacuum distilled (0.1 mm Hg) and dried by molecular sieves. ^{19}F and ^{1}H spectra were recorded on a Bruker AV-300 NMR spectrometer at 282 MHz (^{19}F) or 300.13 MHz (^{1}H) and calibrated according to the chemical shifts of hexafluorobenzene and acetone or chloroform, respectively. Molecular masses were determined on a Finnigan MAT-8200 HRMS instrument.

5.1.1. Typical procedure for the synthesis of quinones *1–5*

A mixture of ethylamine hydrobromide (0.070 g, 0.556 mmol), KOH (0.094 g, 1.675 mmol) and dioxan (2 mL) was stirred for 30 min at room temperature. The precipitate was centrifuged, quinone **6** (0.101 g, 0.379 mmol) was added, and the mixture was stirred at room temperature for 2 h. The red precipitate was collected by centrifugation, washed with water, dried and purified by sublimation (150 °C, 0.03 mm Hg) to give 2-ethylamino-3,5,6,7,8-pentafluoro-1,4-naphthoquinone **3** (0.095 g, 88%) as wine-red crystals, mp 184–185 °C. ^{19}F NMR: δ/ppm 25.8 (dt, F^8), 24.2 (ddd, F^5), 19.8 (ddt, F^6, $J_{6,3} = 4.1$), 15.1 (dt, F^7), 5.3 (br s, F^3), $J_{5,6}$, $J_{6,7}$, $J_{7,8}$ 19.7–19.8 Hz, $J_{5,7}$, $J_{5,8}$, $J_{6,8}$ 10.1–12.1 Hz; ^{1}H NMR: δ/ppm 5.39 (br s, 1H, NH), 3.58 (m, 2H, CH_2), 1.29 (dt, 3H, $J = 7.2$, 0.9 Hz, CH_3). Elemental anal.: calcd for $C_{10}H_6F_5NO_2$: C, 49.50; H, 2.08; N, 4.81. Found: C, 49.23; H, 1.85; N, 4.86.

A mixture of quinone **6** (0.174 g, 0.653 mmol), corresponding amine and dioxan (1.5 mL) was stirred for 20 h (for **1**) or 2 h (for **2**) or 15 h (for **4**) and poured into water (6 mL). The precipitate was collected by centrifugation, dried in vacuum (0.03 mm Hg) at room temperature for 2 h, and purified as described below.

From quinone **6** and *tert*-butylamine (0.086 g, 1.170 mmol), 2-*tert*-butylamino-3,5,6,7,8-pentafluoro-1,4-naphthoquinone **1** (0.128 g, 62%) was obtained after purification by TLC (Silufol, $CHCl_3$–hexane, 3:1) as orange crystals, mp 186–190 °C. ^{19}F NMR: δ/ppm 26.0 (dt, F^8), 24.2 (ddt, F^5, $J_{5,3} = 2.0$), 19.8 (ddt, F^6, $J_{6,3} = 4.5$), 16.5 (br s, F^3), 15.1 (dt, F^7), $J_{5,6}$, $J_{6,7}$, $J_{7,8}$ 19.4–19.8 Hz, $J_{5,7}$, $J_{5,8}$, $J_{6,8}$ 10.1–12.3 Hz; ^{1}H NMR: δ/ppm 5.74 (br s, 1H, NH), 1.43 (s, 9H, $3CH_3$). HRMS ($M^{+\bullet}$, $C_{14}H_{10}F_5NO_2$) calcd 319.06316, found 319.06247.

From quinone **6** and diethylamine (0.071 g, 0.971 mmol), 2-diethylamino-3,5,6,7,8-pentafluoro-1,4-naphthoquinone **2** (0.200 g, 96%) was obtained as dark-red crystals, mp 90–92 °C. ^{19}F NMR: δ/ppm 22.9 (m, F^8), 22.7 (m, F^5), 18.7 (br s, F^3), 17.4 (ddt, F^6, $J_{6,3} = 3.8$), 15.1 (dt, F^7), $J_{5,6}$, $J_{6,7}$, $J_{7,8}$ 18.7–19.4 Hz, $J_{5,7}$, $J_{6,8}$ 8.8–9.1 Hz; ^{1}H NMR: δ/ppm 3.45 (dq, 4H, $J = 7.0$, 1.9 Hz, $2CH_2$), 1.26 (dt, 6H, $J = 0.6$, 7.0 Hz, $2CH_3$). HRMS ($M^{+\bullet}$, $C_{14}H_{10}F_5NO_2$) calcd 319.06316, found 319.06220.

From quinone **6** and aniline (0.067 g, 0.719 mmol), 2-phenylamino-3,5,6,7,8-pentafluoro-1,4-naphthoquinone **4** (0.136 g, 62%) was obtained after purification by TLC (Silufol, $CHCl_3$–CCl_4, 1:1) as wine-red crystals, mp 211–212 °C. ^{19}F NMR: δ/ppm 28.1 (br s, F^3), 26.6 (dt, F^8), 24.9 (dt, F^5), 20.2 (ddt, F^6, $J_{6,3} = 4.5$ Hz), 16.3 (dt, F^7), $J_{5,6}$, $J_{6,7}$, $J_{7,8}$ 19.2–20.1 Hz, $J_{5,7}$, $J_{5,8}$, $J_{6,8}$ 10.8–12.7 Hz; ^{1}H NMR: δ/ppm 7.34–7.42 (m, 2H, 2CH), 7.20–7.27 (m, 1H, CH), 7.09–7.17 (m, 2H, 2CH), 7.13 (br s, 1H, NH). HRMS ($M^{+\cdot}$, $C_{14}H_{10}F_5NO_2$) calcd 339.03186, found 339.03150.

A mixture of quinone **7** (0.200 g, 0.763 mmol), aniline (0.142 g, 1.526 mmol) and dioxan (2 mL) was stirred for 48 h and worked up as mentioned above. 2-Phenylamino-3-methyl-5,6,7,8-tetrafluoro-1,4-naphthoquinone **5** (0.067 g, 26%) was obtained after purification by TLC (Silufol, $CHCl_3$–CCl_4, 1:2) as red crystals, mp 155–156 °C. ^{19}F NMR: δ/ppm 25.2 (dt, F^5), 23.5 (ddd, F^8), 18.8 (dt, F^7), 14.6 (dt, F^6), $J_{5,6}$, $J_{6,7}$, $J_{7,8}$ 19.5–19.9 Hz, $J_{5,7}$, $J_{6,8}$ 15.5–12.8 Hz, $J_{5,8}$, 10.0 Hz; ^{1}H NMR: δ/ppm 7.32–7.42 (m, 3H, 2CH, NH), 7.13–7.23 (m, 1H, CH), 6.95–7.05 (m, 2H, 2CH), 1.72 (s, 3H, CH_3). HRMS ($M^{+\cdot}$, $C_{17}H_9F_4NO_2$) calcd 335.0569, found 335.0569.

5.2. Biological experiments

5.2.1. Determination of mutagenisity of compounds

In the Ames test, the histidine-dependent strain of *S. typhimurium* TA102 was used, which carries a mutation at the histidine operon [28]. The mutagenic activity of the samples was analyzed by the standard method without metabolic activation [28]. A liquid culture of TA102 was obtained by 16-h growth of cells from a frozen stock at 37 °C in LB medium with penicillin. Then cells were plated on minimal glucose agar, antibiotics and histidine at the density sufficient to obtain isolated colonies. A separate bacterial colony was inoculated into LB medium (5 mL) containing ampicillin (50 μg/mL) and tetracycline (2 μg/mL), and grown with shaking (130 rpm) for 15 h at 37 °C.

The Ames test was carried out using the double-layer method as described in [28]. The overnight culture of bacteria (100 μl) containing one of the tested compounds in different concentrations and, if required, 3 mM H_2O_2, was mixed at 42 °C with 2 mL of liquid 0.6% top agar. The mixture was poured onto plates with a minimal medium containing 0.2% glucose and 3% agar, taking care to distribute the mixture uniformly on the surface of the solid agar. The plates were incubated for 48 h at 37 °C, and the revertants were counted. The cells incubated with H_2O_2 in the absence of compounds analyzed were used as positive controls, and the cells grown in the absence of H_2O_2 and antioxidants served as negative controls for mutation induction. The results are expressed as mean ± standard deviation of at least 3 independent experiments.

5.2.2. Cytotoxicity assays

Tumor cell lines from human myeloma RPMI 8226, human mammary adenocarcinoma MCF-7, mouse fibroblasts LMTK and primary mouse fibroblast cell line (PMF) (~2000 cells per well) were incubated for 24 h at 37 °C in IMDM or RPMI 1640 medium (5% CO_2) and then were treated with compounds **1–5**. After 72 h of cell incubation, the relative amount of live cells was determined using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (a standard colorimetric MTT-test [32]) and the drug concentration that caused 50% cell growth inhibition (IC_{50}) was determined. The results are expressed as mean ± standard deviation of at least 3 independent experiments.

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