Anticancer Activity of 3-Demethylubiquinone Q2. In Vivo Experiments and Probable Mechanism of Action

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Abstract. Background: 3-Demethylubiquinone Q2 (3DMUbQ2), isolated from the ascidian Aplidium glabrum and later synthesized, is known as a natural product inhibiting EGF-induced malignant JB6 P+ CI 41 cell transformation. However, its in vivo anticancer properties and probable mechanism of this action have not been studied. Materials and Methods: Preventive and curable effects of 3DMUbQ2 on mice with inoculated Ehrlich carcinoma tumors were examined by magnetic resonance tomography. Capability to inhibit human tumor cell colony growth and induce their apoptosis was investigated using the anchorage-independent phenotype expression assay in soft agar and flow cytometry. Results: 3DMUbQ2 inhibits the growth of the solid Ehrlich carcinoma in mice, especially using the prophylactic scheme of administration (50% inhibition). It inhibits the phenotype expression of HT-460, HCT-116 and SK-MEL-28 human tumor cells and induces apoptosis of these cell lines, as well as that of HL-60 and THP-1 tumor cells. Conclusion: 3DMUbQ2 and other related marine polyprenylquinones have potential for development of a new antitumor agent in cancer prophylactics and treatment and should be further investigated.

Structurally similar to ubiquinones, linear polyprenylquinones and hydroquinones are commonly found in a variety of organisms, especially in marine invertebrates and algae (1, 2). Some natural products of this structural series demonstrate various biological effects such as toxic activity against predatory fish (3), and also inhibitory actions on leukotriene formation (4), the enzyme system TOPO II (5) and ATPases (6). Recently, we reported the isolation and synthesis of a novel unusual linear diprenylquinone (Figure 1) from the marine ascidian Aplidium glabrum (7). This compound, 3-demethylubiquinone Q2 (3DMUbQ2), is a much more closed analog of classical ubiquinones and differs from the latter only in a short polypropenyl chain and the absence of a methyl group. 3DMUbQ2 is not a potent cytotoxin, but it demonstrated good cancer preventive activity on JB6 CI 41 cell transformation activated by EGF (8). However, in vivo anticancer properties of 3DMUbQ2 and analogs as well as the molecular mechanism of its action against tumor cells have not been examined.

The purpose of the present work is to study the in vivo anticancer activities of 3DMUbQ2 and to carry out a series of in vitro experiments on different human tumor cells to elucidate mechanism of this action.

Materials and Methods

General procedures. The analysis of the onset of apoptosis and the cell cycle were performed by flow cytometry using the Becton Dickinson FACSCalibur (BD Biosciences, San Jose, CA, USA). Cell colonies in the anchorage-independent phenotype expression assay were scored using the LEICA DM IRB inverted research microscope (Leica Mikroskopie und Systeme GmbH, Germany) and Image-Pro Plus software, version 3.0 for Windows (Media Cybernetics, Silver Spring, MD, USA). The size of solid Ehrlich carcinomas in mice for experimental studies was measured using a PharmaScan US 70/16 tomograph (Bruker, Germany) with a BGA 09P coil, at a magnetic field intensity of 7.0 T and frequency of 300 MHz.

Reagents, cell culture and animals. Minimum essential medium (MEM), RPMI medium, and McCoy’s medium were from Gibco Invitrogen Corporation (Carlsbad, CA, USA). Fetal bovine serum (FBS) was from Gemini Bio-Products (Calabasas, CA, USA). Penicillin/streptomycin and gentamycin were from Bio-Whittaker (Walkersville, MD, USA). L-glutamine was from Mediatech, Inc. (Herndon, VA, USA). The Annexin V-FITC Apoptosis Detection Kit was from BD Biosciences (San Jose, CA, USA). The protein concentration of solutions was determined using the BCA assay (Pierce, Rockford, IL, USA) and Bradford reagent (Bio-Rad, Hercules, CA, USA)).

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Kit was from Medical & Biological Laboratories (Watertown, MA, USA). The human tumor cell lines, HT-460, HL-60, THP-1, HCT-116, and SK-MEL-28 were obtained from the American Type Culture Collection (Rockville, MD, USA) and were cultured at 37°C and 5% CO₂ in RPMI (for HT-460, HL-60, and THP-1 cells), McCoy’s (for HCT-116), or MEM (for SK-MEL-28), containing 10% FBS, 2 mM L-glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin. Information regarding the genetic background of these cell lines is available online. This study adhered to the “Principles of Laboratory Animal Care” (9). White non-linear mice weighing about 20 g each and inoculated with Ehrlich carcinoma were used for study of the in vivo anti-neoplastic and cancer preventive activities of the 3DMUbQ2.

Study of toxicity in mice. The toxicity of 3DMUbQ2 in mice was determined by Karber’s method (10). The LD₅₀ of 3DMUbQ2 for non-linear mice was found to equal 35 mg/kg, and LD₅₀=60 mg/kg.

Magnetic resonance tomography. Ascites tumors from Ehrlich carcinoma were inoculated into animals under the right shoulder blade at the concentration of 5 million/ml. Treatment was started one day before (for the study of cancer preventive effect) or one day after the transplantation of the tumor. 3DMUbQ2 was dissolved in a mixture of DMSO and H₂O (1:1-v/v) or in EtOH and H₂O (1:1-v/v) and injected into the mouse intraperitoneally, once or twice at a specific time, at a final 3DMUbQ2 concentration of 30 mg/kg in a volume of 0.1 ml. Antineoplastic preventive and therapeutic effects of 3DMUbQ2 were estimated by measuring the size of the solid Ehrlich carcinoma on the 6th, 9th, 12th, 15th and 18th day correspondingly. It was found that 50% DMSO or 50% EtOH solutions of 3DMUbQ2 showed almost the same activity. When 3DMUbQ2 was used as a therapeutic preparation and injected into mice at the same dose in 50% DMSO one day after tumor inoculation, the inhibition of tumor growth was about 20-25% (Figure 2C).

3DMUbQ2 inhibited the growth of solid Ehrlich carcinoma in mice. The inhibition of solid Ehrlich carcinoma growth after treatment with 3DMUbQ2 was studied in vivo using white non-linear mice. Further investigation of 3DMUbQ2 as an anticancer agent requires to study its activity in different solvents, so we studied the action of 3DMUbQ2 using DMSO-H₂O, 1:1 or EtOH-H₂O, 1:1 solutions. Treatment was started one day before (cancer preventive effect) or one day after the transplantation of the tumor (anticancer effect). The growth of the solid Ehrlich carcinoma in these mice was significantly (by about 50%) inhibited with the preventive injection of 3DMUbQ2 at a total dose of 30 mg/kg (LD₅₀=35 mg/kg) dissolved in 50% DMSO or 50% EtOH (Figure 2A or 2B, correspondingly). It was found that 50% DMSO and 50% EtOH solutions of 3DMUbQ2 showed almost the same activity. When 3DMUbQ2 was used as a therapeutic preparation and injected into mice at the same dose in 50% DMSO one day after tumor inoculation, the inhibition of tumor growth was about 20-25% (Figure 2C).

3DMUbQ2 inhibited phenotype expression of human tumor cell lines. When the natural quinone 3DMUbQ2 was assayed for inhibition of colony formation using the anchorage-independent phenotype expression assay in soft agar, it was established that it inhibited phenotype expression of HT-460, HCT-116 and SK-MEL-28 cells (Figure 3A, 3B, and 3C) in a dose-dependent manner. Specifically, 50% inhibition of the colony formation of these cells by 3DMUbQ2 in soft agar against HT-460, HCT-116 and SK-MEL-28 cells was indicated at 50.5 µM, Germany). Statistical analysis of the data was conducted using the Mann-Whitney U-test non-parametric method. Data are represented as the mean±SD from 8 mice in two independent experiments.

Anchorag-independent phenotype expression assay. The ability of 3DMUbQ2 to inhibit phenotype expression of human tumor cells was evaluated using the anchorage-independent soft agar assay as described elsewhere (11).

Apoptosis assay using flow cytometry. The induction of early and late apoptosis by 3DMUbQ2 was analyzed by flow cytometry as described elsewhere (11).

Cell cycle assay using flow cytometry. The influence of 3DMUbQ2 on the cell cycle in HL-60 and THP-1 cells was analyzed by flow cytometry as described elsewhere (11).

Statistics. The statistical computer program, Statistica 6.0 for Windows (StatSoft, Inc., Tulsa, OK, USA, 2001) was used for analysis of the obtained data.

Results

3DMUbQ2 inhibited the growth of solid Ehrlich carcinoma in mice. The inhibition of solid Ehrlich carcinoma growth after treatment with 3DMUbQ2 was studied in vivo using white non-linear mice. Further investigation of 3DMUbQ2 as an anticancer agent requires to study its activity in different solvents, so we studied the action of 3DMUbQ2 using DMSO-H₂O, 1:1 or EtOH-H₂O, 1:1 solutions. Treatment was started one day before (cancer preventive effect) or one day after the transplantation of the tumor (anticancer effect). The growth of the solid Ehrlich carcinoma in these mice was significantly (by about 50%) inhibited with the preventive injection of 3DMUbQ2 at a total dose of 30 mg/kg (LD₅₀=35 mg/kg) dissolved in 50% DMSO or 50% EtOH (Figure 2A or 2B, correspondingly). It was found that 50% DMSO and 50% EtOH solutions of 3DMUbQ2 showed almost the same activity. When 3DMUbQ2 was used as a therapeutic preparation and injected into mice at the same dose in 50% DMSO one day after tumor inoculation, the inhibition of tumor growth was about 20-25% (Figure 2C).

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Figure 2. Inhibition of Ehrlich carcinoma growth by 3DMUbQ2 using the prophylactic scheme of administration (A and B), or one day after the inoculation of the tumor (C). Ascites tumors from Ehrlich carcinoma were inoculated into animals under the right shoulder blade at a concentration of 5 ml/mL. Treatment was started one day before (for the study of prophylactic effect) or one day after the transplantation of the tumor. 3DMUbQ2 was dissolved in a mixture of dimethylsulfoxide (DMSO) and H2O (1:1-v/v) (A and C) or in EtOH and H2O (1:1-v/v) (B) and injected into mice i.p., once or twice at a specific time, at a final 3DMUbQ2 concentration of 30 mg/kg in a volume of 0.1 mL. Antineoplastic preventive and therapeutic effects of 3-demethylubiquinone Q2 were estimated by measuring the size of the solid Ehrlich carcinoma on the 6th, 9th, 12th, 15th, and 18th day after the tumor inoculation. Data are shown as the percent inhibition of tumor growth in mice treated with 3DMUbQ2 compared with that in untreated control mice. An asterisk (*) indicates a significant decrease (p<0.05) in tumor size in treated mice compared to that of untreated mice.

Figure 3. Inhibition of phenotype expression of HT-460 (3A), HCT-116 (3B), and SK-MEL-28 (3C) human tumor cells in soft agar by 3DMUbQ2. The ability of 3DMUbQ2 to inhibit phenotype expression of the human tumor cells was evaluated using the anchorage-independent soft agar assay as detailed in Material and Methods. Cell colonies were scored using a LEICA DM IRB inverted research microscope. Results are expressed as the number of 3DMUbQ2-treated cell colonies compared to the number of untreated control cell colonies expressed as a percentage. Data are represented as means±S.D. of six samples from two independent experiments. An asterisk (*) indicates a significant inhibition by 3DMUbQ2 (p<0.05) compared to untreated control cells.
12.7 μM and 17 μM concentrations, respectively. Concentrations as low as 6.3 μM induced statistically reliable inhibition of colony formation of SK-MEL-28 cells, which along with HCT-116 cells were the most sensitive to the action of 3DMUbQ2.

3DMUbQ2 induced apoptosis in the SK-MEL-28, HT-460, HCT-116, HL-60 and THP-1 human tumor cell lines. The ability of 3DMUbQ2 to induce apoptosis in human tumor cell lines is shown in Figures 4 and 5. Apoptosis was induced in human tumor HT-460 (Figure 4A), HCT-116 (Figure 4B), SK-MEL-28 (Figure 4C), HL-60 (Figure 5A) and THP-1 (Figure 5B) cells in a dose-dependent manner. The effect of 3DMUbQ2 at a concentration of 30 μM on the cell cycle distribution of the treated cells was determined and results confirmed that the treatment of HL-60 or THP-1 cells with this quinone led to significant increases in the proportion of apoptotic sub-G1 cells. Among all the studied human tumor cell lines, HCT-116 and SK-MEL-28 cells were the most sensitive to 3DMUbQ2. Statistically reliable induction of apoptosis in these cells was observed at 5 μM of 3DMUbQ2.

Interrelation of early and late apoptosis (Figure 5) was shown in experiments against HL-60 and THP-1 human tumor cells. It was established that in THP-1 cells, at a concentration of 20 μM 3DMUbQ2 induced more early (40%) than late apoptosis (36.6%); in contrast, the same concentration against HL-60 cells gave 66.6% late and only 19.5% early apoptosis.

Discussion

To the best of our knowledge, besides 3DMUbQ2, only one related marine metabolite, 2-(3-methylbuten-2-yl)-[1,4]-hydroquinone (12) was earlier studied for its anticancer and cancer preventive properties using a series of in vivo and in vitro methods. This compound was tested in vivo in mice for its effectiveness against P388 lymphocytic leukemia and showed an inhibitory effect at a dose of 3.12 mg/kg. Its cancer preventive properties were confirmed by the modified Ames assay for mutagenicity and UV against Salmonella typhimurium (13).

In our animal studies, we used MRT as a powerful and versatile imaging method. Monitoring of tumor growth or the response to drug therapy in vivo is one of the most useful applications of MRT. The advantages of the method include the possibility of obtaining a holistic image of a tumor in any projection on a living model and in the absence of radiation. In our experiments, 3DMUbQ2 inhibited the growth of the solid Ehrlich carcinoma in mice at a dose of 30 mg/kg. Besides tumor measurements, the state of the liver, spleen and thymus over the lifetime of the mice was also observed visually and no significant differences in size, appearance or general condition of these immunocompetent organs in the 3DMUbQ2-treated mice were indicated compared to untreated control mice. Similar data were obtained after removing and measuring these organs. This finding

Figure 4. The induction of apoptosis by 3DMUbQ2 in the human tumor HT-460 (A), HCT-116 (B), or SK-MEL-28 (C) cells as measured by flow cytometry as detailed in Material and Methods. Data are represented as the mean ± S.D. from four samples of two independent experiments. An asterisk (*) indicates a significant increase in apoptosis (p<0.05) induced by 3DMUbQ2 compared to untreated control cells.
suggests that even relatively high doses of 3DMUb Q2 have no significant effect on the immune system of mice. To the best of our knowledge, the inhibition of tumor growth using the prophylactic scheme of administration was established for 3DMUb Q2 for the first time.

Our further experiments allowed us to suggest some peculiarities of the molecular mechanism of this action. The data obtained from apoptosis and cell cycle distribution experiments using flow cytometry suggested that 3DMUb Q2 exerts at least some of its cancer preventive properties by...
inducing apoptosis of tumor cells and inhibiting their phenotypic expression. In fact, our results indicated that 3DMUbQ2 effectively induced apoptosis in various human tumor cell lines (Figures 4 and 5). These results also seem to show 3DMUbQ2 might be more effective against melanomas than against colon or lung cancer.

Low molecular weight natural products having no potent cytotoxicity but showing cancer preventive and anticancer properties are attracting more and more attention as good candidates in chemopreventive or chemotherapeutic anticancer strategies. Several of these natural products, for example, resveratrol, a phytoalexin produced in grapewine skin (14), caffeine and (-)-epigallocatechin gallate from tea (15), dammarane tetrool and dammarane diols from Panax ginseng (16) and some others inhibit apoptosis of tumor cells similarly to 3DMUbQ2. However, only a few natural marine products with similar properties have been found to date, as an example, our recent paper on dactylone from Aplysia dactylomela (11) may be given. Based on the obtained data, 3DMUbQ2 and its analogs may be used for the development of a new promising anticancer agent.

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