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The cytotoxicity and mechanisms of 1,2-naphthoquinone thiosemicarbazone and its metal derivatives against MCF-7 human breast cancer cells

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Abstract

We have investigated the antitumor functions and mechanisms of 1,2-naphthoquinone-2-thiosemicarbazone (NQTS) and its metal complexes (Cu^{2+}, Pd^{2+}, and Ni^{2+}) against MCF-7 human breast cancer cells. The cells were dosed with these complexes at varying concentrations, and cell viability was measured by a sulforhodamine B (SRB) method. To study mechanisms of action, the complexes were incubated with topoisomerase II (topo II) and supercoiled DNA, linear DNA, nicked open DNA, and relaxed DNA were detected by agarose gel electrophoresis. The results revealed that these complexes are effective antitumor chemicals in inhibiting MCF-7 cell growth, with Ni-NQTS being the most effective among the complexes studied. Our data also indicated that Ni-NQTS is more effective than the commercial antitumor drug, etoposide, based on IC_{50} values. The mechanistic study of action showed that metal complexes of NQTS, NQ, and NQTS can only stabilize the single-strand nicked DNA, but not double-strand breakage intermediates. In addition, metal derivatives of these ligands, but not the parent NQ and NQTS, exerted an antagonizing effect on topoisomerase II activity. In summary, chemicals with or without metal derivatives might possess different chemical–topoisomerase II–DNA interactions.

Keywords: Anticancer chemicals; Cytotoxicity; Metal derivatives of 1,2-naphthoquinone-2-thiosemicarbazone; MCF-7 cells

Introduction

Many derivatives of naphthoquinone (NQ) and thiosemicarbazone (TS) are antitumor active and have been used as antitumor drugs. These chemicals include menadione, beta-lapachone (*o*-naphthoquinone) (Dubin et al., 2001; Frydman et al., 1997a; Lai et al., 1998; Pardee et al., 2002; Pink et al., 2000; Planchon et al., 2001; Vanni et al., 1998), and HCTs (α-(*N*)-heterocyclic carboxaldehyde) (Agrawal and Sartorelli, 1978; Finch et al., 1999). One of the mechanisms of antitumor action of these naphthoquinone derivatives is the stabilization of the cleavable complexes formed by topoisomerase II (topo II) and DNA leading to apoptosis (Fujii et al., 1992; Frydman et al., 1997b; Neder et al., 1998). Topoisomerase is a nuclear enzyme that adjusts the topological state of DNA by breaking and resealing DNA strands resulting in alterations in the linking number. There are two major categories of topoisomerases, topoisomerase I (topo I) and topo II. Topo II regulates the topological structure of DNA by transient breaking and rejoining of double-stranded DNA in an ATP-dependent manner (Chung et al., 1992; Muller et al., 1988; Wang et al., 2001).

In vitro studies have shown that some naphthoquinone-derivative drugs such as menadione and etoposide (VP-16) can inhibit topo II by stabilizing the intermediate forms of enzyme–DNA complexes (cleavable complexes) (Matsumoto et al., 2001; Wang et al., 2001). The stabilizing effect is mainly due to the alkylation of thiol residues on the topo II–DNA complex (Neder et al., 1998; Wang et al., 2001).

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Protein denaturant treatment of this cleavable complex results in single-stranded and double-stranded DNA breaks that lead to apoptosis. It is through this cellular process that topo II inhibitors exert their antitumor activity (Barry et al., 1993; Kamesaki et al., 1993).

Most thiosemicarbazone derivatives, such as HCTs, exert their antitumor function through inhibiting ribonucleotide reductase (RR) activity (Booth et al., 1974; Moore and Sartorelli, 1984; Finch et al., 1999). RR catalyzes the synthesis of the deoxyribonucleotides required for DNA synthesis. It contains two identical R1 subunits and two identical R2 subunits, and the catalytical core of the R2 subunits contains a nonheme iron-stabilized tyrosyl free radical. Once the iron center is destabilized, the enzyme is no longer active. It is known that HCTs are effective iron chelators and their existence in cells either destabilize or damage the nonheme iron-stabilized tyrosyl free radical and inhibit the catalytical function of RR (i.e., RR inhibitors) (Li et al., 2001).

1,2-Naphthoquinone-2-thiosemicarbazone (NQTS) was first synthesized in 1979 (Luque de Castro et al., 1979). It can easily chelate with metal ions such as Zn^{2+}, Cd^{2+}, Cu^{2+}, Ag^{+}, and Pb^{2+} in weakly acid medium, and has been used to determine the amount of each metal in water, tobacco, food, etc., by measuring the absorbance of the chelated products (Silva and Valcarcel, 1982). Because NQTS is a derivative of both naphthoquinone and thiosemicarbazone, it is a reasonable prediction that NQTS can be used as an effective antitumor drug. However, to date, there have been no studies to evaluate the antitumor function of NQTS.

The synthesis of 4-hydroxy-3-methyl-1,2-naphthoquinone-1-thiosemicarbazone (HM-NQTS), which has a similar structure to that of NQTS, was recently reported (Saha et al., 2002). It was found that HM-NQTS has in vitro antitumor activity against the MCF-7 cell line, and that this activity is significantly enhanced by metal complexation, among which copper-HM-NQTS is the most effective. However, there are no studies yet of the mechanisms of action of this class of antitumor agents.

In the past, it has been shown that the metal derivatives are more effective than their parent ligand in anticancer activity (Malon et al., 2001; Saha et al., 2002). One of the many reasons is that the binding affinity of metals to proteins or enzymes will change the interaction process of them with DNA, thereby affecting the DNA replication and cell proliferation (Saha et al., 2002). Here we synthesized a series of metal derivative compounds of NQTS, Cu-NQTS, Ni-NQTS, and Pd-NQTS, and investigated their efficacy of antitumor activity and possible mechanisms of action against the MCF-7 cell line. The antitumor effect was studied in vitro by assessing the inhibitory effect on cell growth. The mechanistic study is greatly complicated by the contributions to cytotoxicity of the antitumor activity of the derivatives of both naphthoquinone and thiosemicarbazone. Thus, in this present study, we only focused on the stability effect of these compounds on topo II and DNA complexes. Because etoposide is an effective, well-known antitumor drug and exerts its antitumor function mainly by stabilizing the cleavable complex formed by DNA and topo II (Hande, 1998), it was used as a positive control.

Experimental procedures

Chemicals and reagents

1,2-Naphthoquinone, thiosemicarbazide, and metal salts were products of highest chemical grade (Aldrich, Saint Louis, MO, USA). Solvents were purified according to standard procedures. Thiosemicarbazide hydrochloride (TS.HCl) was prepared following the method previously reported (Coghi et al., 1976). DMEM-F12 medium, 1% penicillin-streptomycin, and fetal calf serum were purchased from Invitrogen (Carlsbad, CA, USA). Dextran-coated charcoal (DCC) was purchased from Acros Organics (St. Louis, MO, USA). Sulforhodamine B (SRB) was bought from ICN Biomedicals (Irvine, CA, USA). The topo II drug screening kit was purchased from TOPOGEN (Columbus, OH, USA). Proteinase K, dimethyl sulfoxide (DMSO), and acetic acid were obtained from Sigma (Saint Louis, MO, USA). The ApoAlert LM_PCR Ladder Assay Kit was purchased from CLONTECH Laboratories (Palo Alto, CA, USA).

Synthesis of NQTS

Thiosemicarbazide hydrochloride (TS.HCl) (0.13 g, 1.0 mmol) dissolved in hot distilled water (3.0 ml) was added to a suspension of NQ (0.16 g, 1.0 mmol) in hot ethanol (50.0 ml). A clear orange solution formed immediately. The solution was heated to reflux on a water bath for 4 h. The orange precipitate formed was then filtered off, washed several times with warm ethanol, and dried in vacuum. The molecular structure is shown in Fig. 1.

Synthesis of metal complexes of NQTS

The metal salts $CuCl_2{\cdot}2H_2O$ (0.17 g, 1.0 mmol), $NiCl_2{\cdot}6H_2O$ (0.12 g, 0.5 mmol), and K_2PdCl_4 (0.33 g, 1.0 mmol)

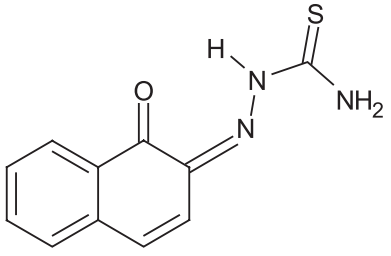

Fig. 1. Structure of 1,2-naphthoquinone thiosemicarbazone (NQTS). The procedures for synthesizing NQTS are given in Experimental procedures section.

were individually dissolved in 3.0 ml hot distilled water and mixed with NQTS (0.24 g, 1.0 mmol) in 50 ml hot ethanol, respectively. The mixtures were refluxed on a water bath for 3–5 h depending on the nature of metal cation used. The complexes, which formed during refluxing, were filtered off, washed with hot ethanol, and then dried in vacuum. The structures of these three metal-NQTS derivatives are shown in Fig. 2. The detailed information on the chemical synthesis and structural properties of these complexes is reported (Afrasiabi et al., 2004).

Stability and solubility of metal-NQTS complexes

The Job's method of continuous variation was used to evaluate the stability constants of the synthesized metal complexes spectrophotometrically (Baran and Erk, 1994; Job, 1928). A series of solutions of varying mole fractions of metal were prepared and their absorbance was plotted against the mole fraction of metal-NQTS at a fixed wavelength. The log K_f values were found to be 5.47, 4.40, and 4.58 for Cu-NQTS, Ni-NQTS, and Pd-NQTS complexes, respectively, which are in the same range as reported for other thiosemicarbazone metal complexes (Balaban et al., 2003). The stability of these metal-NQTS complexes in air was also tested. By exposing these chemicals in the air for 6 months, we found that there were no testable changes in physical appearance and chemical structures.

The solubilities of these three metal-NQTS complexes and NQTS were determined by using *Test Method Protocol for Solubility Determination* developed by the National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicology Methods (NICEATM) (NTP, 2003). The solubilities were found to be 0.2, 0.2, 0.4, and 0.25 mM for NQTS, Cu-NQTS, Ni-NQTS, and Pd-NQTS, respectively.

In summary, the metal complexes of NQTS are highly stable and moderately soluble in the cell culture medium, according to the above tests.

Cell culture

The MCF-7 cell line was obtained from the Karmanos Cancer Institute (Detroit, MI, USA). Cells were maintained in DMEM F12 medium supplemented with 10% calf serum and 100 IU/ml penicillin, and grown at 37 °C in a 4% CO_2 humidified environment.

Assessment of cytotoxicity

Cells were inoculated into 24-well plates at a density of 15 000 cells per well and allowed to attach for 72 h in 2.00 ml experimental medium of 5% calf serum treated with dextran-coated charcoal to remove all steroids. Single chemicals were prepared in DMSO and tested in a series dilution containing six to eight concentrations, with each dilution tested in quadruplicate per assay. Four wells containing appropriate solvent but without test chemicals were used as negative controls. Each single chemical was tested

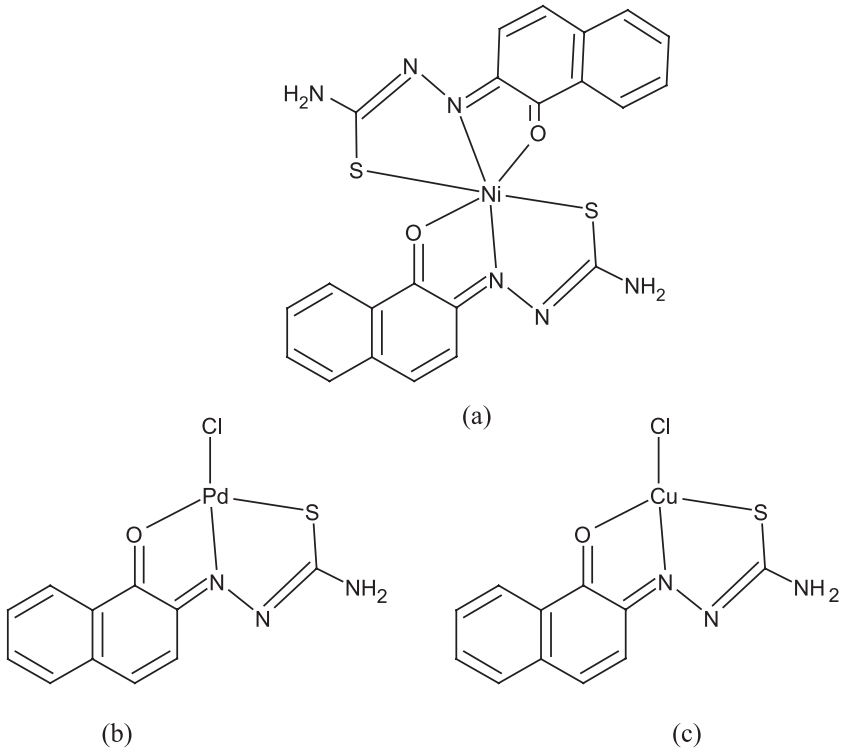

Fig. 2. The molecular structures of metal complexes of NQTS. (a) Ni-NQTS; (b) Pd-NQTS; (c) Cu-NQTS. The detailed procedures for synthesizing these metal complexes of NQTS were described in the Experimental procedures section.

at least three times. Three days after exposure, the experiment was terminated during the late exponential phase of proliferation. The cell numbers in each well was determined using the sulforhodamine B (SRB) assay (Skehan et al., 1990). The experimental medium was discarded, and the 24-well plates were washed with 500 μl cold phosphate-buffered saline per well. The cells were then fixed for at least 40 min with 200 μl cold 10% (w/v) trichloroacetic acid (TCA). After the TCA was discarded, the cells were washed three times with tap water and dried completely under a hood at the room temperature. Staining of the cells was performed by adding 250 μl solution of 0.2% sulforhodamine B in a 1% acetic acid to each well. After 20 min, the staining solution was discarded and the cells were washed several times with 1% acetic acid until the washing solution became colorless. After complete drying, the dye was dissolved in 300 μl cold 10 mM Tris buffer (pH 10.5) per well, and extinction at 550 nm (reference 630 nm) was measured in duplicate per well with a microplate reader (FLOURstar, BMG Labtechnologies, Durham, NC, USA) by transferring aliquots of 100 μl into wells of 96-well plates. The IC_{50} values were determined using a nonlinear regression in GraphPad Prism software (GraphPad software Inc., San Diego, CA, USA). One-tailed unpaired Student's *t* test was used for significance testing. The level of *P* value was set at 0.05.

Time course of cytotoxicity

The cells were seeded into six 24-well plates as described in *Assessment of Cytotoxicity*. At the fourth day after plating, Cu-NQTS, NQTS, Ni-NQTS, and Pd-NQTS dissolved in DMSO were diluted to 3, 3, 3, and 10 μM, respectively, using experimental medium. The cell media in each lane of six plates were replaced by experimental medium containing only one drug. Thus, four lanes were used for the time course studies of Cu-NQTS, NQTS, Ni-NQTS, and Pd-NQTS, respectively. The medium in the fifth lane in each plate was replaced with experimental medium without drug to serve as control. At designated time periods of 4, 8, 14, 26, 48, and 72 h after drug dosage, the cell growth was assessed with the SRB method. The time course of cell viability was plotted using the GraphPad Prism software. The results were analyzed by one-tailed unpaired Student's *t* test. The level of *P* value was set at 0.05.

In vitro topoisomerase II inhibitor screening

The topo II drug screening kit (TOPOGEN) was used in this study. The reaction system contained 2 μl of 10 × cleavage buffer, 1 μl RYG DNA substrate (supercoiled), 2 μl of 1 mM test compound or a control inhibitor (etoposide), 4–6 units of purified topo II, and H_2O (volume variable, make up to 20 μl). The reaction was incubated at 37 °C for 30 min, terminated with 2 μl SDS, and then subjected to proteinase K (50 μg/ml) digestion at 37 °C for 15 min. After extraction with a mixture of chloroform and isoamyl alcohol (24:1), the sample was loaded onto 1% agarose gel containing 0.5 μg/ml ethidium bromide along with the marker DNA. The gel electrophoresis was run at 2 V/cm until the dye front traveled about 80% of total gel length. The linear DNA and nicked open DNA were visualized under a UV light at 254 nm.

To detect the antagonizing effect of each drug on topo II, the reaction conditions and the reagents were the same as described above except that 2 μl of 10× assay buffer was used instead of cleavage buffer. The relaxed DNA band formed in each reaction was then visualized under a UV light.

Apoptosis

Approximately 1×10^6 MCF-7 cells were treated for 7 h by Ni-NQTS at varying concentrations, 10, 20, 40, 60, 80, 100, and 120 μM, or at a concentration of 10 μM for 4.5, 7, 8, 10, 12, 24.5, and 48 h. The control group was without drug treatment. The cells were harvested by trypsin. After 3000 rpm centrifugation for 5 min, 1 ml of phosphate-buffered saline (PBS) buffer was added to wash the cells. The cells were precipitated again by centrifugation followed by resuspension in 500 μl PBS, and then the DNA was extracted with the method described by Eymin et al. (1997).

After DNA extraction, two methods were applied to detect the nucleosomal DNA ladders to determine whether any nicked-open DNAs or linear DNAs were produced during the drug treatment. The first method used 1% agarose gel electrophoresis to separate the DNA fragments in the samples under a voltage of 2 V/cm. Ethidium bromide of 0.5 μg/ml was maintained in Tris–Boric acid–EDTA (TBE) buffer to facilitate UV absorbance detection of the separated DNA fragments. The second method was to amplify the DNA fragments in the samples using the ApoAlert LM_PCR Ladder Assay Kit. The analysis followed the protocol in the kit. Briefly, DNA was mixed with adaptor oligonucleotides and ligation buffer followed by incubation at 55 °C for 10 min. The adaptor oligonucleotides were allowed to anneal with DNA by cooling to 10 °C for 1 h. After adding T4 DNA ligase into the mixture, it was incubated overnight at 16 °C. Finally the adaptor-ligated DNA was subjected to PCR amplification. The PCR setting was as follows: 72 °C for 8 min followed by 20–35 cycles with each cycle at 94 °C for 1 min and 72 °C for 3 min.

Results

Determination of IC_{50} of each chemical against MCF-7 cells

After 72 h exposure in the concentration range of 1–20 μM, the IC_{50} values of each individual metal derivative of NQTS were determined. To compare the cytotoxicity with

those of other compounds, the cytotoxicity of NQTS, NQ, and etoposide were studied in parallel. The dose–response curves of each chemical were shown in Fig. 3 whereas the IC_{50} values were summarized in Table 1. The IC_{50} value of etoposide was 3.82 ± 0.04 μM (mean ± SD). Except for Pd-NQTS, the IC_{50} values of NQTS, Cu-NQTS, and Ni-NQTS were significantly smaller than that of NQ (Ps < 0.05, n = 3). Except for NQTS (P = 0.086, n = 3), the IC_{50} value of Ni-NQTS is significantly smaller than those of etoposide (P < 0.0001, n = 3), Cu-NQTS (P = 0.02), Pd-NQTS (P = 0.001), and NQ (P = 0.007).

Time course of cytotoxicity

In order to assess the time response of MCF-7 cells to each chemical, a time course study of cytotoxicity was conducted. A dosage of 3 μM was used for Ni-NQTS, NQTS, and Cu-NQTS, whereas a 10 μM dosage was applied for Pd-NQTS. These two chosen concentrations were close to their IC_{50} values. Moreover, the same concentration of Ni-NQTS, NQTS and, Cu-NQTS was used to compare their relative inhibitory effect on MCF cell growth. Overall, the cell viability of these four chemicals decreases steeply within a period of 48 h, with Cu-NQTS decreased more significantly at 14 h of exposure (P = 0.01, n = 5) than the other three (Fig. 4).

In vitro topoisomerase II inhibitor screening

To assess the mechanism of the cytotoxicity of NQTS and its metal complexes, an in vitro topo II inhibitor screening system was used to study the inhibitory effects of each metal-NQTS complex to topo II. Nicked open DNA was detected as the results of topo II screening with Ni-NQTS (Fig. 5, lane 2). The screening results of other metal derivatives of NQTS were shown in Fig. 6. No linear DNA band was detected in any of the chemicals except for the etoposide positive control. Nicked-open DNA was detected in all the reactions that contained NQ, NQTS, Cu-NQTS, and Pd-NQTS, respectively. Taking these together, NQ, NQTS, Ni-NQTS, Cu-NQTS, and Pd-NQTS were able to stabilize the cleavable complexes formed by topo II and single-strand nicked DNA, but not double-strand cleavage intermediates.

The results in Fig. 7 showed the antagonizing effect of each chemical on the action of topo II on supercoiled DNA.

Table 1
IC_{50} value (μM) of each chemical

Drug	IC_{50} (μM) (mean ± SD)
Ni-NQTS	2.25 ± 0.14
NQTS	3.14 ± 0.92
Cu-NQTS	3.98 ± 1.01
Pd-NQTS	12.94 ± 2.77
NQ	13.37 ± 4.64
Etoposide	3.82 ± 0.04

Each single chemical was tested in a series dilution containing six to eight different concentrations, with each dilution tested in quadruplicate per assay. Three days (72 h) after exposure, the experiment was terminated during the late exponential phase of proliferation and the cell numbers were determined by using sulforhodamine B (SRB) method. The IC_{50} values (mean ± SD) were determined by a nonlinear regression. Each single chemical was tested at least three times.

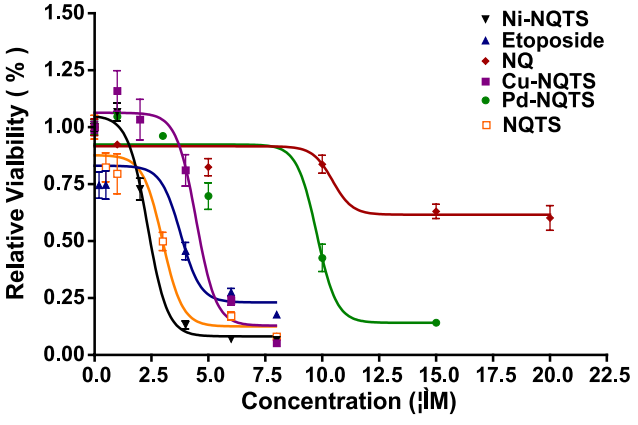

Fig. 3. Dose–response curves of MCF-7 cells to the chemicals tested. The cell density was plated at 15,000 cells per well, with the concentrations of the chemicals ranging between 1 and 20 μM. The cell viability was determined by a sulforhodamine B (SRB) method after 72 h of chemical exposure. Other experimental conditions were described in the Experimental procedures section. The curves are representative ones from different independent experiments. The deviations of the means for NQ and Pd-NQTS in three independent experiments were larger than those of other chemicals (see Table 1); thus, the IC_{50} values of these two chemicals derived from this graph were not close to the calculated IC_{50} values in Table 1 compared to other chemicals.

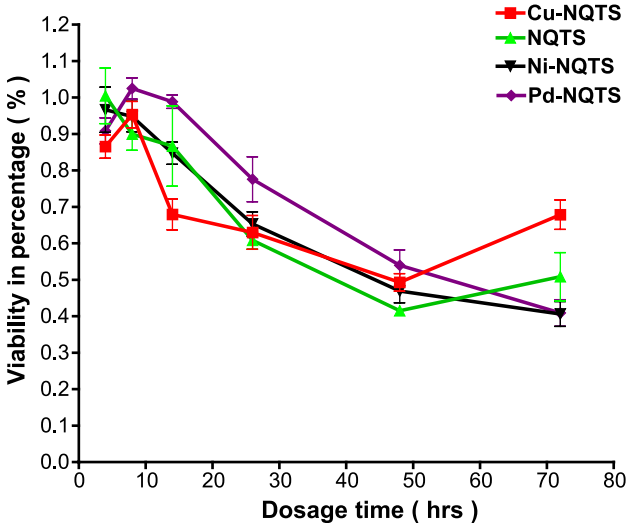

Fig. 4. Time course of cytotoxicity of each drug on MCF-7 cells. The cells were dosed with 3 μM of Cu-NQTS, NQTS, Ni-NQTS, respectively, and 10 μM of Pd-NQTS for 4, 8, 14, 26, 48, and 72 h. The cell viability was determined by sulforhodamine B (SRB) method. Other experimental conditions were described in the experimental section. The curves are representative ones from different independent experiments.

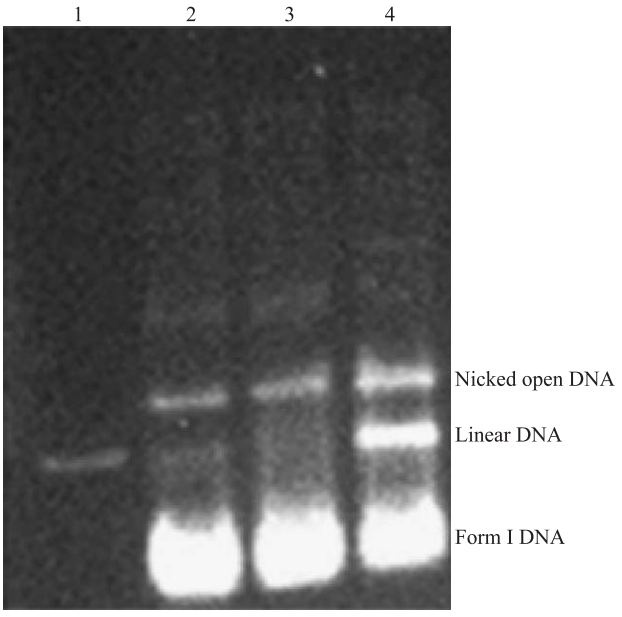

Fig. 5. The stabilizing effect of Ni-NQTS on cleavable complex formed by topoisomerase II and DNA. Ni-NQTS was incubated with topo II and DNA in cleavage buffer for 30 min. After terminated by SDS, the reaction mixtures were either digested by proteinase K (lane 2) or without proteinase K digestion (lane 3). The linear DNA marker was shown in lane 1. Lane 4 was the reaction containing etoposide and digested by proteinase K, which was used as a positive control. Linear DNA band appeared only in lane 4.

Topo II incubated with DNA in assay buffer was used as the positive control (lane 6) whereas the reaction containing only DNA substrate was used as the negative control (lane 7). The relaxed DNA band catalyzed by topo II was formed in the reactions containing NQTS (lane 2) and NQ (lane 5). On the other hand, no relaxed DNA was detected in the reactions containing Cu-NQTS (lane 1), Ni-NQTS (lane 3), and Pd-NQTS (lane 4) indicating the catalyzing function of topoisomerase II on DNA was totally inhibited by those chemicals.

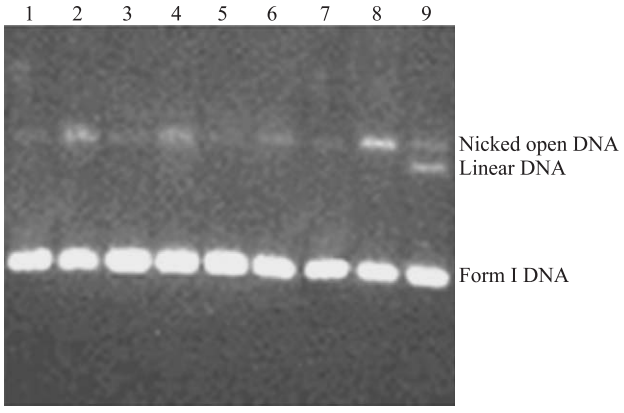

Fig. 6. The stabilizing effect of NQ, NQTS, Cu-NQTS, and Pd-NQTS on cleavable complex formed by topoisomerase II and DNA, respectively. Each chemical was incubated with DNA with or without Topo II in cleavage buffer for 30 min. Lanes 1, 3, 5, and 7 were NQ, NQTS, Cu-NQTS, and Pd-NQTS incubated DNA without Topo II, respectively, and lanes 2, 4, 6, and 8 were NQ, NQTS, Cu-NQTS, and Pd-NQTS incubated DNA with Topo II, respectively. Lane 9 was etoposide positive control that was incubated with topoisomerase II at the same condition. The reactions were terminated by SDS followed by digestion with proteinase K. For all the new chemicals, there was more nicked open DNA formed in the reactions containing topoisomerase II (lanes 2, 4, 6, 8) compared to the reactions without adding topoisomerase II (lanes 1, 3, 5, 7).

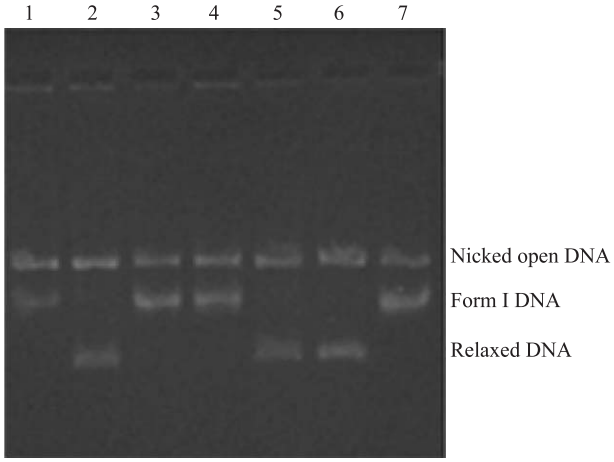

Fig. 7. The antagonizing effect of each drug on topoisomerase II's action on supercoiled DNA. Each drug was incubated with topo II and DNA in assay buffer. Topo II incubated with DNA in assay buffer without using any drug was used as the positive control (lane 6). The reaction containing only DNA substrate was used as the negative control (lane 7). Relaxed DNA band was formed in the reactions containing NQTS (lane 2) and NQ (lane 5). On the other hand, no relaxed DNA was detected in the reactions containing Cu-NQTS (lane 1), Ni-NQTS (lane 3), and Pd-NQTS (lane 4).

Apoptosis study

To study apoptosis induced by Ni-NQTS, the MCF-7 cells were treated with Ni-NQTS and DNA was extracted followed by examination of DNA ladders. Two independent methods described in the Experimental procedures section were used to determine DNA ladders. Neither of these two methods detected DNA ladders in the gels (data not shown).

Discussion

We have studied the antitumor functions of 1,2-naphthoquinone-2-thiosemicarbazone (NQTS) and its metal complexes (Cu^{2+}, Pd^{2+}, and Ni^{2+}) against the MCF-7 human breast cancer cells and the possible mechanisms of action of toxicity. Etoposide, which is a NQ derivative and a clinical antitumor drug, was used as a marker to evaluate the relative cytotoxic efficiency of NQTS and its metal derivatives. Statistical analysis of the results showed that except for Pd-NQTS, NQTS and its metal derivatives, Cu-NQTS and Ni-NQTS, had significantly stronger effect than NQ on MCF-7 cell growth inhibition. The IC_{50} value of Ni-NQTS on MCF-7 cytotoxicity was also statistically significantly smaller than that of etoposide, which means that Ni-NQTS is more effective than etoposide (Fig. 3). All of the test

chemicals show steep cytotoxic effects 26 h after dosage, followed by a slower phase of effect (Fig. 4). This finding may provide a new potential antitumor drug that deserves more attention.

To assess the mechanism of the cytotoxicity of NQTS and its metal complexes, an in vitro topo II inhibitor screening system was used to study the inhibitory effects of each metal-NQTS complex to topo II. The assay system was based on the formation of cleavable complexes among supper coil DNA, topo II, and each metal-NQTS compound. A stable cleavable complex would result in a formation of nicked-open circular DNA or linear DNA. The DNA products were then separated by ethidium bromide gel electrophoresis. To verify whether the formation of the linear DNA or nicked-open circular DNA was caused by stabilized cleavable complexes, the entire assay system was divided into two aliquots after the reaction was terminated. One aliquot was digested with proteinase K whereas the other aliquot was not. If stabilized cleavable complexes were formed, proteinase K-treated aliquot would show bands of linear DNA and possibly nicked-open circular DNA. No bands would appear in the aliquot without proteinase K digestion, because the DNA and topo II intermediates would be lost in the interphase during the CIA extraction. The results of topo II screening with Ni-NQTS show that this complex stabilized the cleavable complexes formed by topo II and DNA substrate, which led to single-strand DNA breakage (Fig. 5). Although Ni-NQTS shows a higher level of cytotoxicity (in terms of IC_{50}) than etoposide (Table 1), much less linear DNA was observed in the presence of Ni-NQTS than that observed for etoposide (an NQ derivative). We suspect that this difference may be in part due to ribonucleotide reductase (RR) activity from thiosemicarbazone (TS) moiety leading to less cell growth in Ni-NQTS treatment. Further investigations need to determine the degree of RR activity from TS moiety involving in growth inhibition by Ni-NQTS.

Fig. 6 showed that the etoposide-treated group yielded both nicked-open DNA and linear DNA, whereas other chemical-treated groups only yielded nicked-open DNA. This stabilizing effect experiment was determined twice and the results were similar. Thus, Ni-NQTS, NQ, NQTS, Cu-NQTS, and Pd-NQTS might stabilize the cleavable complexes formed by topo II and single-strand nicked DNA, but not double-strand cleavage intermediates. A similar observation was also reported by Muller et al. (1988). Their study showed that when topo II was trapped as DNA-topo II covalent complex containing single-stranded breaks by SDS, DNA single-strand cleavages would proceed asymmetrically. With the existence of 4′ -(9-acridinylamino)methanesulfon-*m*-anisidide (*m*-AMSA), single-stranded DNA cleavages increased significantly because of the stabilization effect of *m*-AMSA on DNA-topo II covalent complex. A discrepancy was however observed in Pd-NQTS that showed the lowest cytotoxicity but had the highest amount of single-strand break. The discrepancy between cytotoxicity and topo II-mediated DNA cleavage levels was also observed in other studies (Binaschi et al., 1997a, 1997b, 1998, 2000). In those studies, the authors found that dh-EPI was about 2 times more effective than idarubicin in cleavage assay, but idarubicin was 20 times more potent than dh-EPI in cytotoxicity test. They proposed that the discrepancy might be due to both differential cellular pharmacokinetics of two completely different drugs and different genomic sites of DNA cleavages. To understand the difference between the cytotoxicity in the cells and the stabilizing effect in the in vitro system in our study, the behaviors of individual metal-NQTS complexes and their genomic sites of DNA cleavage needs to be studied. In addition, contributions by both metabolism and possible metal dissociation are discussed in detail as below. Nevertheless, NQ, NQTS, Ni-NQTS, Cu-NQTS, and Pd-NQTS may be considered as a different class of chemicals that only stabilize the topo II and nicked-open DNA complexes leading to cytotoxicity.

To determine whether the cytotoxicity might in part result from the dissociation of the metal-NQTS complexes in cell culture media, the stability and solubility were conducted for each of these metal-NQTS complexes. Based on the data, these metal-NQTS complexes were stable at our study conditions and it was unlikely that the cytotoxicity was caused by NQTS and metals dissociated from metal-NQTS complexes. However, more studies need to be conducted to investigate how these chemicals are metabolized by detoxifying enzymes such as cytochrome P450 enzymes and how this metabolism affect the overall toxicity. In addition to possible hydroxylation or conjugation by phases I and II metabolizing enzymes, it is also unclear whether metals might be cleaved off the compounds by detoxifying enzymes, and metals alone act upon DNA to contribute in part to the observed toxicity. Understanding pathways of metabolism of these newly synthesized chemicals and identification of metabolites and their roles require extensive studies.

It has been shown that Cu(II) derivative of 4-hydroxy-3-methyl-1,2-naphthoquinone-1-thiosemicarbazone has the highest cytotoxicity compared to those of Fe(III), Ni(II), Pd(II), and Pt(II) metal derivatives; this was explained by the generation of Cu(I) species during intracellular enzymatic reduction or greater binding affinity of Cu(I) to the estrogen receptor protein complex (Saha et al., 2002). Therefore, this binding would prevent the protein complex from functioning properly during its interaction with DNA. However, because of the lack of hydroxyl and methyl groups on NQTS, the mechanism of the cytotoxicity of our metal derivatives was expected to be different from those of previous studies. Indeed, our data in Table 1 demonstrated that Ni-NQTS derivative showed a greater cytotoxicity than that of Cu-NQTS complex. In the end, it is worth noting that the cytotoxicity of Pd (II) NQTS complex is weaker than that of the parent ligand NQTS. Both of the

dose–response and time course experiments indicate that among the test chemicals, Ni-NQTS is the most effective one on MCF-7 cytotoxicity.

Studies have shown that most, but not all, of the topo II inhibitors induce apoptosis, and the resulting random-cut DNA ladders can be detected using gel electrophoresis (Li et al., 2001; Onishi et al., 1993; Saura et al., 1997; Tomkins et al., 1994). Even though DNA ladders were not observed in the Ni-NQTS treated group, we could not conclude that the MCF-7 cells treated with Ni-NQTS did not undergo an apoptotic process. Indeed it has been reported that except for traditional DNA ladders, all of the morphological characteristics of apoptosis can be observed in apoptotic MCF-7 cells (Wuerzberger et al., 1998). Thus, examination of morphological characteristics of apoptosis is needed in the future investigation. Initially, we expected that these compounds tested would have similar biochemical behaviors acting through the same mechanism of action; therefore, only Ni-NQTS was tested for apoptosis. However, the negative outcome of Ni-NQTS does not exclude the possibility that the other chemicals will result in random-cut DNA ladders in MCF-7 cells. Thus, all of the chemicals are subject to apoptosis testing, particularly changes in morphological characteristics in our next step of study of these chemicals.

Fig. 7 indicates that Cu-NQTS, Pd-NQTS, and Ni-NQTS have the ability to antagonize the topo II action on DNA because the relaxed DNA formation was totally inhibited after incubating the chemicals with topo II and DNA. Our results with etoposide also showed that it possesses the same function (data not shown). On the other hand, NQTS and NQ do not show such effects. It is worth noting that relaxed DNA was only observed in the chemicals without metals. Even though the role of metals in these chemicals deserves more study to explain the major differences, we believe that there are several possibilities that can cause this antagonizing effect. One possible mechanism is that the metal complexes can interact with DNA and form DNA aggregates by DNA interhelical cross-links and prevent formation of relaxed DNA (Quiroga et al., 1998). Another possibility is that these metal complexes are coordinated with topo II and antagonize metal-NQTS induced formation of topo II–DNA complexes, which will prevent conversion of supercoiled DNA to relaxed DNA (Miller et al., 1998). It is important to know that the metals in metal-NQTS are much more catalytically active compared to metal ions themselves (Padhye et al., 1992; Saha et al., 2002), which enhances the interaction of metal-NQTS complexes with the thiol residues on topo II–DNA complexes. This effect will stabilize the topo II–DNA complexes and prevent formation of relaxed DNA. Though NQ and NQTS did not antagonize topo II on DNA, it did not imply that these two chemicals are less toxic to cells compared to metal-NQTS complexes due to other mechanisms of action stated above.

In summary, we discovered that NQTS and its metal derivatives are effective antitumor candidate drugs in the aspect of MCF-7 cytotoxicity, and Ni-NQTS is statistically the most effective. Moreover, our data indicate that Ni-NQTS is more effective than the commercial antitumor drug, etoposide, based on the IC_{50} values of cell growth inhibition. The studies on mechanism of action showed that metal complexes of NQTS could stabilize the cleavable complex formed by DNA and topo II. Furthermore, our data suggest that metal derivatives of NQTS have an antagonizing effect on topo II activity although the NQ and NQTS themselves do not have. All these evidence indicate that these metal-NQTS complexes can coordinate with topo II and stabilizing the topo II–DNA complexes and also possibly interact with DNA by forming interhelical cross-links. These multi-inhibition mechanisms would greatly enhance cell cytotoxicity compared to that of NQTS.

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