Abstract. We have previously shown that a 2-chloro-1,4-naphthoquinone derivative (TW-92) induces cell death in leukemia cells. TW-92 exhibited relatively high selectivity towards primary Acute Myeloid Leukemia (AML) cells, as compared to normal mononuclear cells. In view of the selectivity of this family of naphthoquinones, novel chloroaminophenylnaphthoquinone isomers with different methyl substitutions on the phenyl ring were synthesized, and their effect on leukemia cells was tested. These compounds induced cell death in U937 human myeloid leukemia cells, which was prominent following 48 h of culture. Structure-activity relationship studies revealed that TW-74, a novel chloronaphthoquinone with a methyl group at the meta (m) position, was the most active derivative in inducing apoptosis. The mechanism underlying cell death induction by TW-74 was further investigated in U937 cells, a monocytic cell line which serves as a sensitive model of apoptosis induction. TW-74 induced rapid activation of Mitogen Activated Protein Kinases (MAPKs). It caused swelling of isolated rat liver mitochondria and an early reduction of mitochondrial membrane potential in intact cells, indicative of a direct effect on mitochondria. Apoptosis induced by TW-74 was accompanied by cytochrome C release and caspase activation. TW-74 induced down-regulation of (BCL2), an anti-apoptotic protein. Furthermore, TW-74 induced selective dose-dependent cell death in primary B-Chronic Lymphocytic Leukemia (CLL) cells. These findings demonstrate that chloronaphthoquinones use common as well as diverse mechanisms for the induction of cell death. The data reported here warrant further studies of the utility of TW-74 in the treatment of CLL.

Naphthoquinones have been studied with regard to their pharmacological properties and such compounds with anticancer activity have been identified (1), alongside with other activities. Quinones such as mitomycin-C (benzoquinone), daunorubicin (anthraquinone) and beta-lapachone (naphthoquinone) are the most clinically used anticancer drugs and are frequently used for the treatment of leukemia. The modes of action of these compounds include induction of apoptosis, inhibition of proliferation and induction of differentiation (2). However, the propensity of leukemia cells to acquire drug resistance, and the cytotoxic effects of these drugs towards normal cells, has lead to a persistent search for alternative therapies.

Naphthoquinones, such as menadione (2-methyl-1,4-naphthoquinone), also known as vitamin K3, have attracted considerable attention, given their broad range of antitumor activities in human cells and a toxicity lower than that exhibited by doxorubicin, daunorubicin and mitomycin-C.

In the search for new antileukemia agents, a large number of naphthoquinones has been synthesized and screened for antileukemic activity (Figure 1). In our previous study, we showed that TW-92, a novel 2-chloro-1,4-naphthoquinone, acts by induction of cell death in leukemia cells via the activation of multiple signaling pathways (3). Considerable attention has been focused on the role of Mitogen Activated Protein Kinases (MAPKs) in signal transduction pathways involved in the regulation of cell survival and death, particularly those related to MAPK modules. Out of these, the Stress Activated Protein Kinase/c-Jun Kinase...
(SAPK/JNK) and p38 MAPKs are primarily induced by local environmental insults (e.g., DNA damage or osmotic stress) and are generally associated with pro-apoptotic actions (4, 5), in contrast, the p42/44 Extracellular Regulated Kinase (ERK) MAPK signaling cascade is induced by mitogenic, and differentiation-related stimuli and is most frequently, although not invariably, associated with pro-survival activity (6, 7).

The BCL2 family of proteins plays a central role in the regulation of apoptosis. Proapoptotic members of this family, such as (BAX), translocate from the cytosol to mitochondria, leading to the release of cytochrome C into the cytosol (8).

Antiapoptotic proteins of the BCL2 family members inhibit apoptosis and their overexpression has been shown to contribute to chemoresistance in Chronic Lymphocytic leukemia (CLL) (9) and in Acute Myelogenous Leukemia (AML) as well (10).

In view of the antileukemic activity of the 2-chloro-1,4-naphthoquinone TW-92 and its selective activity towards leukemia cells, here we studied the antileukemic activity of TW-74, a new member of the 2-chloro-amino-phenyl-1,4-naphthoquinone family.

Materials and Methods

Synthesis of TW-74 [2-chloro-3-(3-methyl-phenylamo)-1,4-naphthoquinone].

2,3-Dichloro-1,4-naphthoquinone (1.0 g, 4.4 mmol) was dissolved in 80 ml ethanol at 60°C. M-Toluidine (1.88 g, 17.6 mmol) in 20 ml ethanol was added to the solution of 2,3-dichloro-1,4-naphthoquinone. The mixture was then re-fluxed at 100°C for 2 h. The precipitated product was filtered-off and re-crystallized from a mixture of ethanol/chloroform (1:1) to afford fine red needles. Yield: 1.22 g (yield 93%), melting point: 178°C.

(NMR) gave the following results; (500 MHz; CDCl₃-d₆) 1.62 (m, 4H); 2.26 (s, 3H); 3.48 (m, 4H); 6.53 (d, 2H, J=8.5 Hz); 6.81 (s, 1H); 7.01 (d, 2H, J=8.5 Hz); 7.60 (m, 2H); 7.99 (m, 2H).

HR-MS (CI in CH₄) (m/z): found: 332.1557, calculated for C₂₁H₂₀N₂O₂; 332.1524.

Purity was checked by HPLC-Eluent (ACN/ Water) (50%-50%, V/V). The compound was then dissolved in dimethyl sulfoxide (DMSO) at a stock solution of 20 mM and kept at –20°C. Working solutions were diluted in Phosphate Buffer saline (PBS).

Reagents and antibodies. Dichlorodihydrofluorescein diacetate (DCFH-DA) and 3, 3’-dihehexylcarbocyanine iodide (DiOC 6) were from Molecular Probes (Eugene, OR, USA). The MAPK inhibitors SB203580 and PD98059 and the caspase-3 specific inhibitor were obtained from Calbiochem (La Jolla, CA, USA). All antibodies against MAPKs, namely rabbit anti-human phospho p38 MAPK (Thr180/Tyr182), rabbit anti-human phospho-p44/42 MAPK (Thr202/Tyr204), anti-human polyclonal procaspase 3/9 antibodies recognizing either the 32 or 47 kDa proforms of human procaspase-3, procaspase-9 respectively, and antibodies raised against the de-naturated form of human cytochrome C (15 kDa) were all purchased from BD-Pharmingen (San-Diego, CA, USA).

Cell culture and drug treatment. Suspension cultures of U937 myeloid leukemia cells. Promyelocytic HL-60, as well as lymphoblastic CCRF-CEM cells, obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA), were grown at logarithmic scale in RPMI-1640 medium (GIBCO BRL, Life Technologies, Invitrogen corporation, CA, USA), supplemented with 10% fetal bovine serum (FBS) (Hyclone Laboratories Inc. UT, USA), 100 IU/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine (GIBCO) in humidified air at 37°C with 5% CO₂. Cells (3×10⁶ cells/ml) were treated with different concentrations of naphthoquinone or vehicle for different time periods.

Determination of cell viability, apoptosis and necrosis. Viability and apoptosis analysis of U937 cells exposed to TW-74 were performed as previously described (3, 11). Briefly, p38-stress kinase (SB203580) as well MEK (PD98059) inhibitors were dissolved in DMSO at 40 mM stock solution. U937 cells were seeded in 24-well plates then pre-incubated at a 10 μM final concentration, for 1 h before being treated with TW-74 for additional 24 or 48 h. Appropriate amounts of DMSO (final concentration less than 0.1%) were added to control cultures. Cell counts were determined using the trypan blue dye exclusion, while cell viability was determined using the XTT viability assay. For determination of apoptosis,
apoptotic cells were quantified using either Propidium Iodide (PI) for flow cytometry or acridine orange-ethidium bromide staining observed under a fluorescent microscope.

**Measurement of hydrogen peroxide and mitochondrial transmembrane potential (MMP).** The intracellular H$_2$O$_2$, as well as MMP were measured by using the fluorescent dyes, DCFH-DA and DiOC$_6$ respectively, as previously described (3, 11).

**Immunoblot assay.** Whole-cell lysates for analysis for cytochrome C, and caspase activity were lysed by (RIPA) buffer. For the analysis of phospho proteins, cells were lysed in a buffer containing (30 mM Tris-Base pH 6.8), 2% (SDS), 2.88 mM β-mercaptoethanol, and 10% glycerol, 1 mM whole-cell extracts were fractionated by SDS-polyacrylamide gel electrophoresis in 15% polyacrylamide mini-gels and transferred to Immobilon™-P transfer membranes (Millipore Corp., Medford, MA) as previously described (3, 11, 12).

**Isolation of mitochondria, and determination of Ca$^{2+}$ accumulation and mitochondrial swelling.** Ca$^+$ uptake by freshly-prepared liver mitochondria (0.5 mg/ml) isolated from rat liver, was assayed according to procedures reported previously (3, 11-13).

**Statistical analysis.** Results are expressed as mean of ±SEM of three experiments, performed in duplicate. The significance of differences obtained between tested experimental conditions was determined using the two-tailed Student’s t-test and Prism version 3.0 software (Graph Pad software, San Diego, CA, USA).

$p$-Values <0.05 were considered significant: *$p$-Value 0.05, **$p$-Value <0.01 and ***$p$-Value <0.001.

**Results**

**TW-74 reduces cell viability and induces cell death of U937 myeloid leukemia cells.** We have previously shown that introduction of a methyl group at the para position of the aromatic ring of (2-chloro-3-amino-phenyl)-1,4 naphthoquinone (TW-69) renders the compound relatively inactive in apoptosis induction (3). In the present study, a novel derivative was used, in which the methyl group was inserted at the meta (m) (TW-74) as compared to the ortho (o) (TW-91) position of the phenyl ring (Figure 1). The antileukemic activity of these compounds was studied using the human myeloid leukemic cell line U937. As shown by the results, TW-74 causes a significant reduction of cell viability, assessed by the XTT method, at 3 μM concentration (IC$_{50}$=2.576 μM) (Figure 2a), while TW-91 with a methyl group at the o-position on the benzoic ring, was inactive at this concentration, indicating that a methyl group at the m-position is the most active (>10 μM).

This is supported by our earlier observation that TW-69, which has a methyl group at the para (p) position is also inactive at low concentrations (IC$_{50}$=8.046 μM (3)).

TW-74 was further tested to determine the means of cell death induction. Following cell staining with propidium iodide (PI) and cell-cycle phase analysis, a hypoploid sub-G$_1$ peak was evident after treatment with 3 μM TW-74. This finding indicates that TW-74 induces cell death at micromolar concentrations. At 3 μM concentration, TW-74 induced cell death mostly by apoptosis, as shown by the presence of a pre-G$_1$ peak (Figure 2b), as compared to control cells (Figure 2a). Of note, TW-91 and (TW-69), were inactive at the tested concentrations (data not shown). The pro-apoptotic action of TW-74 was studied on additional leukemic cell lines. The promyelocytic HL-60 cell line was less responsive (data not shown), while TW-74 elicited a mixed form of cell death of the lymphoblastic CCRF-CEM cell line (data not shown). Our mechanistic studies were performed with U937 cells since efficient separation of cell
populations undergoing either apoptosis or necrosis, as dependent on the compound concentration used, was achieved using this cell line. Moreover this cell line comprises a sensitive model for apoptosis induction.

**TW-74 induces loss of MMP, mitochondrial swelling, release of cytochrome C into the cytosol, followed by caspase activation and BCL2-/BAX protein modulation.** One of the early events in the mitochondrial pathway of apoptosis is the alteration of mitochondrial membrane integrity. The effect of TW-74 on mitochondrial permeability transition (Δψm) of U937 cells was evaluated. As shown in Figure 3a, the fluorescence intensity of DiOC6 shifted to the left as early as 30 min, demonstrating the efficacy of TW-74, at a concentration of 3 μM to depolarize the mitochondrial membrane. Next, we investigated the direct effect of TW-74 on rat liver mitochondrial swelling. Mitochondria incubated in the presence of Ca^{2+} (200 μM) exhibited a marked decrease in absorbance at 540 nm, indicative of mitochondrial swelling as a result of Ca^{2+} accumulation, which was then followed by irreversible depolarization of mitochondria over the next 5 min. A rapid and large decrease in the absorbance was observed in mitochondria treated with 3 μM TW-74, suggesting swelling of mitochondria (Figure 3b). Treatment with vehicle or buffer- alone did not alter the absorbance of mitochondrial suspensions.

Cytochrome C release into cytosol is a key feature of the mitochondrial pathway of apoptotic cell death. Figure 3c shows that a marked increase in the cytosolic cytochrome C

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Figure 3. TW-74 potentiates apoptotic cell death of leukemia cells by mitochondrial damage through reduction in mitochondrial membrane potential and cytochrome C release, followed by caspase activation. a: U937 cells were exposed to naphthoquinones for 30 min and then assayed for loss of mitochondrial membrane permeability by determining the percentage of cells exhibiting low levels of DiOC6. b: The effect of TW-74 on mitochondrial (PT) pore opening. Rat liver mitochondria (0.5 mg/ml) were assayed for swelling and PT pore opening in the presence or absence of naphthoquinone compounds. The reaction was initiated by addition of either Ca^{2+} (100 μM) as a positive control, while TW-74 treatment, was added after 20 s. All assays were conducted at room temperature for 5 min. The experiment was repeated three times, yielding similar results each time. Western analysis was carried out for cytosolic levels of cytochrome C (c), as well as for degradation/activation of pro-caspase-3 (d), in extracts from U937 cells after exposure to naphthoquinone compounds, for different times. These data are representative of one out of three independent experiments that yielded similar results. e: TW-74 down-regulated expression of (BCL2) transcripts as shown by western blot analysis for the levels of BCL2. Cells were left untreated or naphthoquinone-treated (3 μM) for the indicated times. Total protein extracts were obtained and BCL2 was detected by western blotting. Down-regulation of BCL2 became evident at 48 h, coinciding with the onset of cell death. As a control for protein loading, β-actin levels are also shown. Results are representative of two independent experiments with similar results.
level was induced by TW-74 administration, a result consistent with the view that TW-74 activates the mitochondrial pathway of cell death.

We next assessed activation of caspases in the cell death response induced upon TW-74 treatment. Procaspase-3 down-regulation was observed within 1 to 3 h of incubation with TW-74 (Figure 3d), indicating involvement of caspase function in TW-74-mediated action. To determine whether administration of TW-74 modified the expression of apoptotic regulatory proteins, the expressions of BCL2 and BAX were assayed by western blots. Pre-incubation of U937 cells with 3 μM TW-74 produced no change in BAX expression levels (data not shown). In contrast, down-regulation of BCL2 expression was found 24 h after TW-74 treatment (Figure 3e). These observations suggest that TW-74-induced apoptosis is derived, at least in part, from reduced expression of the anti-apoptotic protein BCL2.

In summary, the present results indicate that TW-74-induced apoptotic cell death in U937 cells is mediated through the mitochondrial pathway.

**TW-74-mediated apoptosis is abrogated by Antioxidant.** It has been suggested that reactive oxygen species (ROS) might play an important role in the regulation of cell death. Therefore, we examined the effects of the antioxidants N-acetyl cysteine (NAC) and lypophylic vitamin E on TW-74 induced apoptotic death. The addition of 10 mM NAC significantly inhibited apoptosis induced by TW-74, while the addition of 300 μM vitamin E did not inhibit TW-74-mediated cell death (Figure 4). These findings indicate that the modulatory effect on cell death of this compound is based on its oxidative potential.

**Activation of ERK and p38 kinase pathways is necessary for TW-74-mediated cell death of U937 cells.** We further investigated whether MAPKs were activated in response to TW-74 treatment and whether MAPKs contribute to TW-74-induced cell death. Treatment of U937 cells with TW-74 (Figure 5a) rapidly activated both ERK and p38 kinase, as evidenced by the immunoblotting of phosphorylated ERK1/2 and p38. The activation process occurred after 5 min of incubation and remained relatively constant for 1 h. These results imply that TW-74 is a potent inducer of MAPK activity. To further investigate whether MAPKs play a regulatory role in TW-74-mediated cell death, the effects of a selective MEK inhibitor, PD98059, as well as p38 inhibitor SB203580, on cell death were examined. Exposure to the PD98059 (10 μM)-alone had a minimal effect on cell death; however, this inhibitor increased the apoptotic activity elicited by TW-74 administration at 24 h of incubation, (Figure 5b). These results are consistent with the inhibitory action of ERK on the apoptotic process by activation of survival pathways. However, p38 MAPK inhibitor SB203580, which alone had no effect, attenuated the apoptotic cell death induced by TW-74. These results imply that p38 plays a role in the TW-74 induction of the apoptotic process (Figure 5c).

**TW-74 induces apoptosis of B-CLL cells in a dose-dependent manner.** TW-74 was shown to induce cell death in U937 leukemia cells as well as in CCRF-CEM cells in vitro. To determine whether TW-74 exerts the same action on B-CLL cells, primary cells obtained from three patients with CLL were incubated with varying concentrations of naphthoquinones for 72 h. Thereafter, cell viability was measured using trypan blue exclusion and cell death was assayed using acridine orange/ethidium bromide staining. The effect of TW-74 on mononuclear cells (PBMCs) obtained from healthy donors was studied in parallel. The results show that B-CLL cells were more susceptible to the cytotoxic effects of naphthoquinones for 72 h. Thereafter, cell viability was measured using trypan blue exclusion and cell death was assayed using acridine orange/ethidium bromide staining. The effect of TW-74 on mononuclear cells (PBMCs) obtained from healthy donors was studied in parallel. The results show that B-CLL cells were more susceptible to the cytotoxic effects of TW-74, as compared to healthy donors’ PBMCs (Figure 6b vs. 6d). Moreover, our results show that TW-74 exerts a selective proapoptotic effect on B-CLL cells (Figure 6a vs. 6c).

**Discussion**

In the present study we report on the antileukemic effect of a novel compound, TW-74, a 2-chloro-3-amino-phenyl-1,4 naphthoquinone. This compound carries a methyl group at the meta position of the naphthoquinone. Other 2-chloro-3-amino-phenyl-1,4 naphthoquinones with methyl substitution on the phenyl group were studied as well (TW-69 (3) and present study). Our findings show the functional importance of the site of the methyl substitution on the amino-phenyl ring. TW-74, with a methyl group at the m-position of the phenyl ring exhibited higher antileukemic activity compared to the compounds TW-91 and TW-69 with o- and p-substitutions respectively (3).
In the present study, we have shown that the in vitro antileukemic activity of TW-74 appears to be dependent on a combination of two factors, namely concentration and duration of exposure, respectively. This new naphthoquinone exhibited marked anti-leukemic activity at the 3 μM concentration, shown by (i) reduction in cell viability by XTT assay, (ii) induction of cell apoptosis as evident by a pre-G₁ apoptotic peak detected by flow cytometric analysis, (iii) morphological apoptotic changes detected by acridine orange/ethidium bromide, and (iv) activation of caspase-3.

It is well-documented that the activity of naphthoquinones is mediated through redox cycling activity (14, 15). Consistent with this observation, we showed that the apoptotic action of TW-74 is mediated, at least partly, by oxidative stress, since the apoptotic effect of the compound was inhibited by the addition of NAC. The inability of the lipid-soluble antioxidant vitamin E, acting at the level of cellular membranes to inhibit TW-74-induced cell death, suggests that the ROS source may not be of lipidic origin.

In our study, we observed a rapid and prolonged activation of ERK and p38 MAPK upon naphthoquinone treatment. Pre-treatment of monocytic U937 leukemia cells with the MEK inhibitor PD98059 further potentiated apoptosis by TW-74, confirming the pro-survival and antiapoptotic action of ERK kinase. Furthermore, TW-74-induced apoptosis was reduced by the specific p-38 inhibitor SB203580. This finding indicates that p38 MAPK plays a positive role in apoptosis mediation.

The opening of permeability transition pores (PTP) in a high conductance state is a critical event in the initiation of the intrinsic pathway of apoptosis, resulting in the release of pro-apoptotic factors such as cytochrome C and Apoptosis Inducing Factor (AIF) from the intermembrane space to the cytosol (16-19). We found that our compound directly targets the mitochondria by interacting with the PTP, causing its opening, as evidenced by the decrease in absorbance following TW-74 treatment of isolated mitochondria, indicating swelling.

Mitochondrial function in apoptosis is regulated by the balance of pro- and anti-apoptotic BCL2 proteins (20). An increased BCL2/BAX ratio is responsible for resistance of U937 cells to apoptosis, induced by different chemotherapeutic agents (21). TW-74 caused down-regulation of BCL2 protein, as seen after 24 h of treatment, suggesting that BCL2 down-regulation is an additional late pro-apoptotic effect of this compound.

B-CLL is a disease characterized by the accumulation of B-cells resistant to apoptosis. A high BCL2/BAX ratio expression is considered to contribute to the pathogenesis of
B-CLL (22, 23). The observation that BCL2 is overexpressed in most B-CLL cells (9, 24), has led to the suggestion that disturbances in the balance between pro-apoptotic and anti-apoptotic proteins may be critical in determining the susceptibility of such cells to apoptotic signals (10, 25). In accordance with this hypothesis, the present study showed that TW-74 induces dose-dependent apoptotic cell death of B-CLL, showing a frank apoptotic effect at 3 μM which was found to be minimally toxic to normal PBMCs, indicating strong selectivity of TW-74 towards CLL cells.

Of importance was the weak toxic effect of TW-74 on normal PBMCs, as compared to TW-92 (3), while TW-92 had a stronger effect on U937 cells and was active against AML cells compared to TW-74, it was toxic to some extent against normal PBMCs (3). On the other hand, TW-74 was not toxic when tested against healthy PBMCs and revealed specificity for cells. The mechanism of action of the two naphthoquinones in induction of cell death is also different. While both compounds induced the intrinsic apoptotic pathway, TW-92 down-regulated MCL1 and up-regulated BAX expression, while TW-74 down-regulated that of BCL2. How these differently structured compounds exert selective effects on leukemia cells warrants further investigation.

The results indicate that TW-74 is a potent antileukemic naphthoquinone that is effective against CLL cells at micromolar concentrations. The data presented here also indicate that treatment of TW-74-alone, in the presence of the pharmacological MEK 1/2 inhibitor PD98059, promotes apoptosis of leukemia cells. Taken in conjunction with observations involving myeloid leukemia cells (26), these findings raise the possibility that malignant hematopoietic cells may be particularly susceptible to a therapeutic strategy based

Figure 6. TW-74 induces cell death of peripheral blood mononuclear cells obtained from patients with CLL. Primary human CLL blasts obtained from three patients, and normal peripheral blood mononuclear cells (PBMCs) from three healthy donors were suspended in culture medium at a density of 1×10⁶cells/ml, then incubated for 72 h with different concentrations of TW-74. Cell death induced by TW-74 in CLL cells (a) and PBMCs (c) was monitored using acridine orange/ethidium bromide staining. A concomitant experiment was performed to determine viability of malignant CLL (b) and normal PBMCs (d) using trypan blue dye exclusion. Results represent the mean±SEM for triplicate determinations. p-Value *<0.05, and **<0.01 compared to control.
on apoptosis/necrosis-inducing agents combined with agents that can interrupt cytoprotective signaling pathways.

The present results add to our knowledge on the mechanisms underlying the cytotoxic effect of naphthoquinone derivatives exhibiting selective activity against different leukemia cells. The search for novel drugs that combine selectivity for antiapoptotic proteins with minimal toxic side-effects may be of value in cancer therapy. This strategy may provide a new approach to overcome resistance to chemotherapeutic agents and may ultimately reinforce the efficacy of chemotherapy for leukemia.

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