In vitro cytotoxic potential of newly synthesized furo[3,2-*c*]pyran-4-one derivatives in cultured human lymphocytes

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Received: 17 August 2010 / Accepted: 19 April 2011 / Published online: 15 May 2011
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Abstract The in vitro cytotoxic potentials of Furo[3,2-*c*]pyran-4-one derivatives in human lymphocytes were investigated. Blood samples were obtained from six healthy donors, non-smoking volunteers, which were incubated and exposed to increasing concentrations (0.05, 0.1, 0.5, 1 and 2 mg/mL) of Furo[3,2-*c*]pyran-4-one derivatives which are methyl 2-methoxy-7-(4-methylbenzoyl)-6-(4-methylphenyl)-4-oxo-4*H*-furo[3,2-*c*]pyran-3-carboxylate (1a) and methyl 2-methoxy-7-(4-methoxybenzoyl)-6-(4-methoxyphenyl)-4-oxo-4*H*-furo[3,2-*c*]pyran-3-carboxylate (1b). Compounds 1a and 1b induced micronucleus, mitotic and replication indexes in human lymphocytes (1 and 2 mg/mL). The increases of micronucleus, mitotic and replication indexes show that compounds at high concentrations may become cytotoxic, genotoxic and carcinogenic.

Keywords Cytotoxic · Furo[3,2-*c*]pyran-4-one · Neo-tanshinlactone

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Introduction

Natural products have been the most important source of new medicinal leads (Balunas and Kinghorn 2005; Saklani and Kutty 2008; Vuorelaa et al. 2004). However, the structural complexity of natural products, such as intricate ring systems and numerous chiral centers, may lead to limited supplies and hamper mechanism of action studies and clinical development (Magedov et al. 2008). For this reason, structural simplification of natural products is a powerful and highly productive tool for lead development and analog design. Neo-tanshinlactone (Fig. 1) is a steroid-like tetracyclic natural product originally isolated from the traditional Chinese medicine Tanshen. Neo-tanshinlactone was reported as a highly selective inhibitor of the growth of breast cancer cell lines SK-BR-3 (HER2 over-expressing breast cancer) and ZR-75-1 (estrogen receptor positive breast cancer) (Wang et al. 2004; Wang et al. 2006). 6-Phenyl-4*H*-furo[3,2-*c*]pyran-4-one derivatives based on neo-tanshinlactone have shown potent inhibition against the SK-BR-3 breast cancer cell line (Dong et al. 2010).

Inoscavin A (Fig. 1) and Phelligridin F, which possess a 2,3-dihydrofuro[3,2-*c*]pyran-4-one skeleton, are natural products that were isolated from a fungus. There are several reports about these compounds as free radical scavengers with cytotoxic activities (Aramaki et al. 1995; Kim et al. 1999; Mo et al. 2003; Mo et al. 2004). Niveulone (Fig. 1), which also contains a

Fig. 1 Structures caused by neo-tanshinlactone, inoscavin A and niveulone

Neo-tanshinlactone

2,3-dihydrofuro[3,2-*c*]pyran-4-one moiety that is linked to a more traditional terpenoid part in a heterocyclic spiro compound, was isolated as one of several biologically active metabolites of the ascomycete *Dasyscyphus niveus*. It is weakly cytotoxic towards human cell lines and has a challenging structure with an interesting carbon skeleton (Parra et al. 2006). The phellifuropyranone, 2-(3,4-dihydroxyphenyl)-6-(2'-(3,4-dihydroxyphenyl)-*E*-ethenyl)furo[3,2-*c*]pyran-4-one, has antiproliferative activity against mouse melanoma cells and human lung cancer cells in vitro (Kojima et al. 2008).

The micronucleus (MN), mitotic index (MI), and replication index (RI) analysis methods are cytogenetic tests that are used both in vivo and in vitro. Micronuclei may originate from acentric fragments (chromosome fragments lacking a centromere) or whole chromosomes that are unable to migrate with the rest of the chromosomes during anaphase in cell division (Fenech 2000). Because of its association with chromosomal aberrations, MN has been used since 1937 as an indicator of genotoxic exposition based on the radiation studies conducted by Brenneke and Mather (Heddle et al. 1983). The in vitro MN test started to be used in genotoxicity in the 1970s. A long experience of this system was first obtained from early studies on human lymphocytes (Fenech and Morley 1985; Norppa et al. 1993), and now the in vitro MN assay on human lymphocytes is widely used for biomonitoring studies and detecting clastogens and aneugens (Norppa et al. 1993). Investigations on MN frequencies support the widely accepted assumption that MN is a product of early events in human carcinogenic processes (Desai et al. 1996).

Alcohol consumption, smoking, exposure to X- and gamma-rays appear to be the most frequent exogenous risk factors for the development of cancer (Seitz et al. 1998). Carcinogenic substances pass into target cells and also systematically lead to decreased cell metabolism, producing a relative immune

Inoscavin A

deficiency (Carvalho 1997). Consequently, the MN test, currently known as the MN assay has been used for screening populations under the risk of mutagenic agents, especially for the identification of pre-clinical steps of the carcinogenic process (Ramirez et al. 1999).

The MI or the percentage of metaphases among harvested, fixed lymphocytes requires the addition of colchicine or colcemid to arrest the progression of cells from metaphase to anaphase ensuring a sufficient number of metaphases for cytogenetic analysis. The MI is used to characterize proliferating cells and identify compounds that inhibit or induce mitotic progression (Holland et al. 2002). The MI depends on two factors; first the proportion of the cell population that participates in the whole cycle of interphase leading to division; second the relative lengths of interphase and recognizable mitotic stages (Walker 1952). The RI measures cell division kinetics by counting the percentage of cells in the first, second, third or more metaphase (Holland et al. 2002).

We report a study of healthy donors, evaluating rates of MN, MI and RI in their peripheral blood lymphocytes with different concentrations of furo[3,2-*c*]pyran-4-one derivatives (compounds 1a, b) (Fig. 2), compared to a control group.

Materials and methods

Chemicals

Furo[3,2-*c*]pyran-4-one derivatives which are methyl 2-methoxy-7-(4-methylbenzoyl)-6-(4-methylphenyl)-4-oxo-4*H*-furo[3,2-*c*]pyran-3-carboxylate (1a) and methyl 2-methoxy-7-(4-methoxybenzoyl)-6-(4-methoxyphenyl)-4-oxo-4*H*-furo[3,2-*c*]pyran-3-carboxylate (1b) have been synthesized efficiently in good yields by multicomponent reaction of 2,3-furandiones with dimethyl acetylenedicarboxylate and triphenylphosphine (Koca et al. 2010; Özdemir et al. 2009). The test

Fig. 2 Structures of synthesized furo[3,2-c]pyran-4-one derivatives (Methyl 2-methoxy-7-(4-methylbenzoyl)-6-(4-methylphenyl)-4-oxo-4H-furo[3,2-c]pyran-3-carboxylate (1a) and

Methyl 2-methoxy-7-(4-methoxybenzoyl)-6-(4-methoxyphenyl)-4-oxo-4H-furo[3,2-c]pyran-3-carboxylate (1b)

agents were dissolved in glacial acetic acid (CAS No. 247K18855556, Merck) and filtered. The same range of concentrations of the test agent (0.05, 0.1, 0.5, 1 and 2 mg/mL) was used for MN, MI and RI assays. The concentrations were a result of trial and error. There was no previous literature we could resort to.

Peripheral blood (PB) karyotyping medium (CAS No. 01-201-1, Biological Industries), colcemid (CAS No. D1925, Sigma), cytochalasin-B (CAS No. 14930-96-2, Sigma) and giemsa stain (CAS No. 5400512, Merck) were used in peripheral blood culture. PB Karyotyping Medium was based on RPMI-1640 basal medium supplemented with L-glutamine (1%), fetal bovine serum (20%), antibiotic (gentamycin) (1%) and phytohemagglutinin (2%).

Human lymphocyte cultures and cell harvesting

Heparinized blood samples (0.4 mL), which were obtained from six healthy donors with the permission of Local Ethic Committee, were placed in sterile culture tubes containing 5 mL of PB karyotyping medium. Then, the compounds (1a, 1b) were added to obtain five final concentrations (0.05, 0.1, 0.5, 1 and 2 mg/mL). However, the compounds were not added to the tubes of control groups. The contents of each tube were mixed by gentle shaking, and then the tubes were incubated in a slanted position at 37 °C for 72 h. Cytochalasin-B was added at 44 h of incubation at a final concentration of 6 µg/mL to block cytokinesis for MN and RI. After 70 h of incubation, 0.1 mL of colcemid solution (10 µg/mL) was added to each tube and the contents were mixed again by shaking the tubes gently. After 72 h of incubation, cells were harvested by centrifugation, by

giving hypotonic treatment (0.075 M KCl) and they were fixed in fresh fixative solution (methanol:acetic acid, 3:1). This fixation step was repeated three times. Slides were air-dried and stained with giemsa (Eroğlu et al. 2009).

Staining and examination of micronucleus

The MN staining was performed according to Eroğlu et al. (2009). The slides were scored by a single observer. 500 cells were examined at 600× magnification from each slide and when MN cells were located they were examined at 1000× magnification (Yavuz et al. 2010). The criteria suggested by Scarpato and Migliore (1996) for recognizing micronuclei were followed. Dead or degenerating cells were excluded from evaluation. Nuclear blebbing (MN-like structure connected with the main nucleus with a bridge) were not considered. Only micronuclei equal to or smaller than one-fifth of the main nucleus were considered. Multimicronucleated cells were also scored but not included in the evaluation of MN frequency.

Examination of mitotic index

The MI was calculated as the proportion of metaphase for 2,000 cells for each donor and concentration.

Examination of replication index

The cell proliferation kinetics was defined as the proportion of first (M_1), second (M_2) and third (M_3) mitotic division and it was scored each drug in 500 consecutive metaphases per culture, concentration and donor. The RI was calculated according to Arslan

et al. (2008) by the formula: $RI = (1 \times M_1 + 2 \times M_2 + 3 \times M_3)/500$.

Statistical analysis

The computer software program SPSS 10.0 was used to analyze the data. The statistical significance of the effects of furo[3,2-*c*]pyran-4-one derivatives on the MN, MI and RI was assessed using repeated measures of the analysis of variance (ANOVA) and the differences between groups were determined by the least significant differences (LSD) test with $p < 0.01$ were considered significant. Correlation and regression coefficients were calculated in order to determine the dose–response relationships between MI and RI.

ANOVA: 1 ($P < 0.01$)

ANOVA: 2 ($P = 0.01$)

Fig. 3 Micronucleus changes of compound 1a and b. MN rates were increased by the compound 1a and b. These increases were shown to be dose-dependent. MN rates of control and concentrations of compound 1a (0.05, 0.1, 0.5, 1 and 2 mg/mL) were found 1.40 ± 0.80 , 1.80 ± 1.11 , 2.40 ± 0.91 , 2.46 ± 0.64 , 2.93 ± 0.75 and $3.00 \pm 1.05\%$, respectively. MN rates of control and concentrations of compound 1b (0.05, 0.1, 0.5, 1 and 2 mg/mL) were found 2.00 ± 0.91 , 3.13 ± 0.94 , 3.26 ± 1.22 , 3.46 ± 0.83 , 3.80 ± 0.91 and $3.86 \pm 0.98\%$, respectively. MN frequencies at concentrations of 1 and 2 mg/mL were approximate 2 times higher than the control. ANOVA: 1 ($p < 0.01$), ANOVA: 2 ($p = 0.01$)

Results

Micronucleus

According to the Fig. 3, significant changes were detected in the percentage of MN for 1 (compound 1b) and 2 mg/mL (compound 1a and b) ($p < 0.01$) but not for other extract concentrations (0.05, 0.1 and 0.5 mg/mL). These concentrations did not induce significant changes in MN frequencies compared to untreated group (control) ($p = 0.01$).

Mitotic index

When the potential cytotoxicity of the extracts of compounds 1a and b in lymphocyte cultures were analyzed through MI evaluation, the significant increases were found for 1a (1 and 2 mg/mL— $p < 0.01$) and 1b (2 mg/mL— $p < 0.01$). The other extract concentrations (0.05, 0.1 and 0.5 mg/mL) did not affect the MI frequencies ($p = 0.01$) (Table 1).

Replication index

When the RI was analyzed, no modifications were detected for extract concentrations of the compounds ($p = 0.01$). As shown in Table 2, changes in RI are not significant statistically in spite of increasing with as dose–response.

Discussion

The newly synthesized furo[3,2-*c*]pyran-4-one analogs 1a, b were evaluated for in vitro cytotoxic activity in cultured human lymphocytes. Inoscavin A (Fig. 1) and Phelligrudin F, which possess a 2,3-dihydrofuro[3,2-*c*]pyran-4-one skeleton, are natural products that were isolated from a fungus. There are several reports about these compounds as free radical scavengers with cytotoxic activities (Aramaki et al. 1995; Kim et al. 1999; Mo et al. 2003, 2004). Therefore, it is important to determine the cytotoxic effects of newly synthesized furo[3,2-*c*]pyran-4-one derivatives used in this study. In vitro three different cytogenetic parameters (MN, MI and RI) were used.

According to the MN results (Fig. 3), compound 1a and b induced increase of MN frequencies. There are many factors affecting the MN frequency in

Table 1 Mitotic index (%) (mean $\pm$ SDs) in human lymphocyte cultures exposed to different concentrations of compound 1a and b

Derivatives	Concentrations (mg/mL)	Total counted cells	Total number: dividing cells	Mean $\pm$ SDs (%)
Compound 1a	Control	12,000	284	2.36 $\pm$ 0.70
	0.05	12,000	326	2.71 $\pm$ 0.67
	0.1	12,000	340	2.83 $\pm$ 0.22
	0.5	12,000	336	2.80 $\pm$ 0.39
	1	12,000	388	3.23 $\pm$ 0.22*
	2	12,000	394	3.28 $\pm$ 0.10*
Compound 1b	Control	12,000	236	1.96 $\pm$ 1.11
	0.05	12,000	288	2.40 $\pm$ 0.95
	0.1	12,000	316	2.63 $\pm$ 0.68
	0.5	12,000	380	3.16 $\pm$ 0.98
	1	12,000	456	3.80 $\pm$ 1.22
	2	12,000	462	3.85 $\pm$ 1.16*

ANOVA: * $p < 0.01$
(significantly different from control)

Table 2 Replication index (mean $\pm$ SDs) in human lymphocyte cultures exposed to different concentrations of compound 1a and b

Concentrations (mg/mL)	Furo[3,2-c]pyran-4-one derivatives	
	Compound 1a	Compound 1b
Control	1.168 $\pm$ 0.115	1.192 $\pm$ 0.041
0.05	1.198 $\pm$ 0.101	1.228 $\pm$ 0.052
0.1	1.201 $\pm$ 0.110	1.199 $\pm$ 0.057
0.5	1.211 $\pm$ 0.090	1.240 $\pm$ 0.050
1	1.230 $\pm$ 0.098	1.264 $\pm$ 0.048
2	1.236 $\pm$ 0.095	1.273 $\pm$ 0.060

$p = 0.01$ (not different from control)

lymphocytes: age, gender, smoking, alcohol consumption, viral infection, and exposure to X-ray and gamma-ray (Müller 1996). The donors chosen for this study did not smoke or consume alcohol. They had not been exposed to X-ray and gamma-ray and they did not have any viral infections. An increase in the MN may result from the interaction of a great variety of cytotoxic and genotoxic agents with chromosomal damage. The MN is an extremely valuable and highly relevant endpoint for the detection of potential carcinogens. The results of this study show an increase in the percentage of MN (Fig. 3), suggesting a strong interaction between the extracts (1 and 2 mg/mL) of compounds 1a, b and chromosomal damage, which could be responsible for the observed cytotoxicity.

Both MI and RI frequencies were increased by compound 1a and b (Tables 1, 2). The significant

Fig. 4 The positive correlation between MI and RI for compound 1a

increases were found for MI frequencies. But, no modifications could be detected as statistically significant for RI ($p > 0.01$). MI and RI are used as indicators of adequate cell proliferation biomarkers. MI frequencies and RI values increased with increasing concentrations of the compounds. The results indicate the cytotoxic effects as well as proliferative effects and suggest that the extracts of the compounds exhibit cytotoxic properties as well as mitotic and proliferative properties. A positive correlation was observed between MN induction and cell proliferation; namely the higher the MN frequency was detected in exposed individuals, the higher the values of nuclear division progression were expressed as RI. Likewise, we detected a positive correlation between

Fig. 5 The positive correlation between MI and RI for compound 1b

MI and RI (Figs. 4, 5); namely the higher the MI frequencies were detected in exposed individuals, the higher the values of nuclear division progression were expressed as RI. The increases of MI and RI rates and the positive correlation between MI and RI could result from uncontrolled cell division and cytotoxic effects of the compounds. MI measures the proportion of cells in the M-phase of the cell cycle and its induction could be considered as uncontrolled cell division in the cell proliferation kinetics.

In the present study, we found that furo[3,2-*c*]pyran-4-one derivatives induced the MN, MI and RI in human lymphocytes. The increases of MI and RI in the peripheral blood lymphocytes indicate that compounds 1a and 1b may act as a proliferative and mitotic agent. Besides, the rises of MN show that compounds 1a and b at high concentrations may become carcinogenic and cytotoxic. Our compounds present a chemical structure of natural products as neo-tanshinlactone, inoscavin A, phelligridin F, niveulone. Many results of the investigation were reported regarding these natural products. Especially neo-tanshinlactone is a natural product known with anti-proliferative properties. Dong et al. (2010) reported anticancer properties of 6-Phenyl-4H-furo[3,2-*c*]pyran-4-one derivatives based on neo-tanshinlactone. Their compounds 10-13, 23, 25, and 27 showed potent inhibition against the SK-BR-3 breast cancer cell line. Neo-tanshinlactone was reported as a highly selective inhibitor of the growth of breast cancer cell lines SK-BR-3 (HER2 over-expressing breast cancer) and ZR-75-1 (estrogen receptor positive breast cancer) (Wang et al. 2004,

2006). The phellifuropyranone, 2-(3,4-dihydroxyphenyl)-6-(2'-(3,4-dihydroxyphenyl)-E-ethenyl)-furo[3,2-*c*]pyran-4-one, has antiproliferative activity against mouse melanoma cells and human lung cancer cells in vitro (Kojima et al. 2008). Mo et al. (2004) reported furo[3,2-*c*]pyran-4-one showed in vitro selective cytotoxicity against a human lung cancer cell line (A549) and a liver cancer cell line (Bel7402). Niveulone (Fig. 1), which also contains a 2,3-dihydrofuro[3,2-*c*]pyran-4-one moiety that is linked to a more traditional terpenoid part in a heterocyclic spiro compound, was isolated as one of several biologically active metabolites of the ascomycete *Dasyscyphus niveus*. It is weakly cytotoxic towards human cell lines (Parra et al. 2006).

Natural products may include furo[3,2-*c*]pyran-4-one skeleton. Further studies should be carried out to synthesize new furo[3,2-*c*]pyran-4-one derivatives and evaluate the cytotoxic, proliferative and antiproliferative effects on the MI, RI and MN.

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