Toxicology Letters 214 (2012) 137–144

Contents lists available at SciVerse ScienceDirect

Toxicology Letters

journal homepage: www.elsevier.com/locate/toxlet

Dibenzofuran induces oxidative stress, disruption of trans-mitochondrial membrane potential ($\Delta\Psi m$) and G1 arrest in human hepatoma cell line

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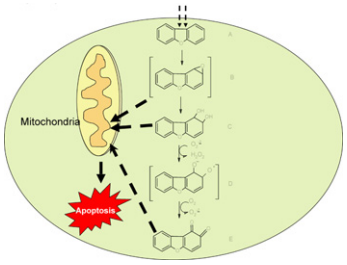
HIGHLIGHTS

- Dibenzofuran is widely distributed in environment.
- It is direct precursor molecule of extremely toxic halogenated dibenzofuran.
- The mechanism of dibenzofuran induced toxicity is proposed.
- The mechanism explains dibenzofuran forms quinone metabolite after its metabolism in HepG2 cells.
- It potentiates ROS formation, G-1 arrest and mitochondrial membrane potential dip and hence cell death.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 19 May 2012
Received in revised form 17 August 2012
Accepted 17 August 2012
Available online 23 August 2012

Keywords:
Dioxin
Dibenzofuran
HepG2
ROS
MMP

ABSTRACT

Dioxins are a class of extremely toxic environmentally persistent pollutant, comprised of halogenated dibenzo-p-dioxins, dibenzofurans and biphenyls. Despite significant human exposure via multiple routes, very little is known about toxicity induced by dibenzofuran (DF). Current study shed lights on the potential toxicity mechanism of DF using human hepatoma cell line (HepG2). It was observed that the exposure to DF potentiate oxidative stress, apoptosis and necrosis at 10 μM within 8 h in HepG2 cells. Interestingly, when we pre-incubated the cells with α-NF (1 nM) for 12 h, an aromatic hydrocarbon receptor antagonist, the IC_{50} of DF increased by 14 folds indicating the cytoprotective ability of α-NF from DF induced toxicity. Furthermore, three additional metabolites were observed while studying the metabolic profile of DF in HepG2 cells with and without pre-incubation with α-NF using chromatography–mass spectroscopy (GC–MS). Of these, two metabolites were characterized as dihydroxylated derivative of DF and third metabolite was characterized as quinone derivative of DF. By flow cytometry and confocal laser microscopy analysis we followed the ROS formation after DF (10 μM) exposure for 3 h. Significantly low ROS was generated in cells which were pre-incubated with α-NF than cells which were not pre-incubated with α-NF underlining the importance of metabolism in DF toxicity. The same pattern of protection was consistent while measuring mitochondrial membrane potential (MMP), i.e., less MMP dip was observed in 'with α-NF pre-incubated and DF (10 μM) exposed cells' than 'without α-NF pre-incubated but DF exposed cells'. In cell cycle studies, it was confirmed that cell population of HepG2 at G1 stage progressively increased in number (~74%) within 24 h. Thus, DF and its metabolites induce significantly higher cytotoxicity after metabolism in HepG2 cells than its parent compound (DF) by ROS formation, MMP dip and impaired cell cycle.

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http://dx.doi.org/10.1016/j.toxlet.2012.08.014

1. Introduction

Halogenated biaryl ethers, such as diphenylether, dibenzo-p-dioxin (DD) and dibenzofuran (DF) are collectively known as dioxins. These compounds are persistent organic pollutants (POPs) with extremely toxic properties such as carcinogenic, immunosuppressive, endocrine disruptive and teratogenic and can bind with aromatic hydrocarbon receptor (AhR) (Kaiser, 2000; Landvik et al., 2007; Ozeki et al., 2011). After binding with receptor, dioxins are translocated to the nucleus after forming a complex with aromatic hydrocarbon nuclear transfer protein. Dioxins bind to a dioxin-responsive element (DRE) and cause induction of several undesirable physiological disorders, including cancer and birth defects (Becher and Flesch-Janys, 1998; Durrin et al., 1987; Ilsen et al., 1996).

DF, an oxygen heterocyclic polycyclic aromatic hydrocarbon (PAH), is one of the most important direct precursors for dioxin formation (Luthe and Berry, 1996). In addition, it is also an unintentional by-product of various industrial, agricultural, thermal insulation, municipal incinerations and many more anthropogenic activities (Cains et al., 1997; Fiedler et al., 1990). Despite being widely distributed in the environment, DF has not been a subject of intensive toxicological examination (Chang et al., 1988; Landvik et al., 2007). It has been reported recently that DF potentiates decreased cell proliferation and MTT reduction in A549 human lung cells (Duarte et al., 2011). After exposure to DF, ATP starts depleting in cells that result in energetic failure. The lysosomal vacuole content and up-regulating proteins such as microtubule-associated protein 1 light chain 3B or LC3 protein involved in autophagy starts increasing. Furthermore, an increase in number of dysfunctional mitochondria and impairment in the energetic status maintenance after DF exposure results in cell death (Duarte et al., 2012). Despite of these studies conducted on DF induced toxicity, many other aspects are yet to be explored such as metabolism.

It has been already reported that DF binds to AhRs and induces the cytochrome P-450 monooxygenases activity (CYP), especially CYP1A2 activities in HepG2 cells (Chaloupka et al., 1994, 1995). CYP super family has principle enzymes that are responsible for xenobiotic metabolism and lead to bioactivation or bioattenuation (Sims et al., 1974). CYP is a large and diverse group of enzymes that are mostly involved in oxidation of aromatic compounds. CYP can oxygenate PAH via one of the following three pathways (Park et al., 2008). CYP1A1/1B1 may catalyze one or two sequential monooxygenation and form diol-epoxide that may cause G- to T-transversion (Conney, 1982); or CYP peroxidases convert aromatic compound to radical cations that can either form depurinating adduct or cause G- to T-transversion (Cavalieri and Rogan, 1995); or PAH activated by NAD(P)H dependent oxidation of trans-dihydrodiol forms electrophilic PAH-o-quinones and these PAH-o-quinones undergo enzymatic reduction to reform catechols resulting in futile redox cycling and amplification of ROS at the expense of NAD(P)H that may lead to prooxidant cellular state (Penning et al., 1999).

Many oxygenated PAH can enter the mitochondria and reach the inner membrane via calcium-, voltage- and pH sensitive-permeability transition pore (PTP). Some of these oxygenated PAH can uncouple the mitochondrial membrane, leading to inner mitochondrial membrane potential (MMP) ($\Delta\Psi m$) collapse. Oxygenated PAH may induce accumulation of Ca^{2+} in mitochondrial matrix. PTP causes mitochondrial swelling, outer membrane rupture that may start releasing cytochrome C (proapoptotic signals) to form apoptosome which interacts with Apaf-1, dATP/ATP, procaspase-9 and downstream effectors caspases (Xia et al., 2004).

Human liver cell lines differ markedly in the ability to metabolize small heterocyclic compound to toxic species that may induce apoptosis (O'Brien et al., 2002). In optimum conditions, hepatocyte cells live with a balance of free radical production, scavengers and damage repair mechanism which is disturbed by the free radical producing chemicals. Hepatic carcinoma cell line (HepG2) model has been widely used for in vitro toxicity studies due to its similarity to in vivo hepatic cells in terms of biosynthetic and biotransformation activities (Mattsson et al., 2009). Also, it has been used as a cell line model for bioactivation related studies, including antagonist (such as alpha-naphthoflavone) based inhibition of CYP1A-dependent xenobiotic metabolism (Chang et al., 1988; Wei et al., 2009). Furthermore, middle-sized heterocyclic compounds, such as DF, are biologically active molecules that can inhibit cell division by blocking cell cycle progression at the G1 phase, resulting in cell death (Ding et al., 2006).

We have used HepG2 cells to gain insight about the potential molecular mechanisms of DF induced toxicity. This current study includes metabolism of DF, its capability of causing oxidative stress at varying concentrations and several endpoints. The effect of DF exposure on cell morphology, MMP, cell cycle and markers of oxidative stress were also studied to further elucidate its potential toxicological mechanism.

2. Materials and methods

2.1. Chemicals and reagents

DF, alpha-naphthoflavone (α-NF), and dimethyl sulfoxide (DMSO), 2,7-dichlorofluorescein diacetate (DCFH-DA), and 3′,3-dihexyloxa-carbocyanine iodide ($DIOC_6$) were purchased from Sigma–Aldrich (India). All cell culture-related reagents, i.e., Dulbecco's minimal essential medium (DMEM), fetal bovine serum, sodium pyruvate, non-essential amino acids, penicillin, streptomycin and trypsin/EDTA solutions were purchased from Sigma–Aldrich (India).

The stock solutions of the test compounds were maintained in DMSO solution and were added directly to the cell culture medium as stocks to give the desired final concentration. All organic solvents and solid chemicals were of analytical reagent grade.

2.2. Cell culture

The HepG2 cells were cultured at 37 °C in a 5% CO_2 incubator with DMEM (Sigma), supplemented with 10% fetal calf serum, L-glutamine (2 mM), penicillin (100 units/ml), and streptomycin (100 mg/ml). The cells formed a monolayer at confluence. Trypsin (0.25% (v/v) of media) with EDTA solution was used to detach the cells from the culture flask for plating and passing the cells as described previously (Jahroudi et al., 1990). The HepG2 cell cultures were maintained for 12 h in a 75-cm^2 surface area, 250 ml tissue culture flasks in 5% CO_2 incubator at 37 °C before treatment with DF. In case of α-NF (1 nM) pretreatment, cells were treated with α-NF for 12 h at time of culturing before the DF treatment.

2.3. Cell viability assay

Cell viability was assessed using MTT assay as described (Hansen et al., 1989). Briefly, live cells convert tetrazolium salt (MTT) to colored formazan, unlike dead cells. The viability of HepG2 cells was determined by adding MTT to the cell cultures to reach a final concentration of 1 mg/ml. The dark crystals formed after 2 h of incubation at 37 °C were later dissolved by adding an equal volume of extraction buffer (20% sodium dodecyl sulfate, 50% N,N-dimethyl formamide, pH 4.7). The cells were then incubated overnight at 37 °C and absorbance was measured at λ_{570} nm after transferring 100 μl aliquots to 96-well plates.

2.4. DF treatment of HepG2 cells

DF (1 mM) was dissolved in DMSO (0.25 mM) to prepare a stock solution. HepG2 cells were seeded into 25-ml flasks at appropriate densities (5×10^6 cells/ml). DF (10 μM) dissolved in DMSO (0.25 mM) was incubated with and without α-NF (1 nM) pretreated HepG2 cells at 37 °C for 8 h. Extraction of metabolites was performed as described (Dehennin et al., 1984; Iida et al., 2002). Briefly, after incubation, mediums were collected from flasks and mixed with double volumes of ethyl acetate and hexane (1:1) at pH 2. Samples were centrifuged at 14,000 × *g* for 15 min. Parent compound and biotransformation products were extracted from supernatant after evaporation and dissolved in acetonitrile. The supernatants were transferred into conical glass tubes and extracted with ethyl acetate at pH 2.0 and later evaporated to dryness under N_2 gas at 30 °C. The residues reconstituted in 50 μl of acetonitrile were analyzed on a GC–MS system. Metabolites were then derivatized by adding 10 μl of bis-(trimethylsilyl) trifluoroacetamide (BSTFA) (Sigma Inc.) in the air-tight glass tubes at 70 °C for 45 min. Derivatization of metabolites was necessary to prevent