Toxicology xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Toxicology

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

The age factor for mitoxantrone's cardiotoxicity: Multiple doses render the adult mouse heart more susceptible to injury

José Luís Dores-Sousa [a,*], José Alberto Duarte [b], Vítor Seabra [c], Maria de Lourdes Bastos [a], Félix Carvalho [a], Vera Marisa Costa [a,*]

[a] REQUIMTE (Rede de Química e Tecnologia), Laboratório de Toxicologia, Departamento de Ciências Biológicas, Faculdade de Farmácia, Universidade do Porto, Porto, Portugal
[b] CIAFEL, Faculdade de Desporto, Universidade do Porto, Porto, Portugal
[c] Departamento de Ciências Farmacêuticas, CESPU, Instituto Superior de Ciências da Saúde do Norte, ISCS-N, Paredes, Portugal

ARTICLE INFO

Article history:
Received 22 November 2014
Received in revised form 4 January 2015
Accepted 8 January 2015
Available online xxx

Keywords:
Mitoxantrone
Noradrenaline
Age
Glutathione
Protein carbonylation

ABSTRACT

Age is a known susceptibility factor for the cardiotoxicity of several anticancer drugs, including mitoxantrone (MTX). The impact of anticancer drugs in young patients is underestimated, thus we aimed to evaluate the cardiotoxicity of MTX in juvenile and adult animals. Juvenile (3 week-old) and adult (8–10 week-old) male CD-1 mice were used. Each group was treated with a 9.0 mg/kg cumulative dose of MTX or saline; they were maintained in a drug-free period for 3-weeks after the last administration to allow the development of late toxicity (protocol 1), or sacrificed 24 h after the last MTX administration to evaluate early cardiotoxicity (protocol 2). In protocol 1, no adult mice survived, while 2 of the juveniles reached the end of the protocol. High plasma aspartate aminotransferase/alanine aminotransferase ratio and a high cardiac reduced/oxidized glutathione ratio were found in the surviving MTX-treated juvenile mice. In protocol 2, a significant decrease in plasma creatine-kinase MB in juveniles was found 24 h after the last MTX-administration. Cardiac histology showed that both MTX-treated populations had significant damage, although higher in adults. However, MTX-treated juveniles had a significant increase in fibrotic tissue. The MTX-treated adults had higher values of cardiac GSSG and protein carbonylation, but lower cardiac noradrenaline levels. For the first time, mature adult animals were shown to be more susceptible to MTX as evidenced by several biomarkers, while young animals appear to better adjust to the MTX-induced cardiac injury. Even so, the higher level of fibrotic tissue and the histological damage showed that MTX also causes cardiac damage in the juvenile population.

1. Introduction

Mitoxantrone (MTX) is an anthracenedione with a broad spectrum of antitumor activity. It is used in the treatment of breast and prostate cancer, acute leukemia, and lymphomas, and since 2000 it has been approved by the U.S Food and Drug Administration (FDA) as an immunomodulatory agent for reducing the neurological disability of worsening relapsing-remitting multiple sclerosis (Seiter, 2005). The initial purpose of the synthesis of MTX was to maintain or improve the antitumor activity of anthracyclines and reduce their cardiotoxic side effects. However, cardiotoxicity induced by MTX has been extensively reported (Scully and Lipshultz, 2010; Seiter, 2005), although the mechanisms are still poorly understood.

The cardiotoxicity of MTX depends on several factors, with age and life-time cumulative dose being two important factors (Seiter, 2005). Presently, the recommended maximum lifetime cumulative dose of MTX is 140 mg/m^2, with 2.6 to 13% of patients developing cardiac toxicity with that dose (Seiter, 2005).

Although childhood cancer represents less than 2% of human cancers, it is the most common cause of disease-induced death in children older than 1 year (Vassal et al., 2013). Throughout the years, research in the chemotherapy field has yielded important achievements regarding pediatric cancer treatment: before the

Abbreviation: ALT, alanine aminotransferase; AST, aspartate aminotransferase; ATP, adenosine 5′-triphosphate; CK-MB, creatine kinase MB; GSH, reduced glutathione; GSHt, total glutathione; GSSG, oxidized glutathione; HPLC, high-performance liquid-chromatography; h, hour; i.p., intraperitoneal; MDA, malondialdehyde; min, minute; MTX, mitoxantrone; •NO, nitric oxide; SD, standard deviation.

* Corresponding authors at: REQUIMTE, Laboratory of Toxicology, Faculty of Pharmacy, University of Porto Rua de Jorge Viterbo Ferreira, 228, 4050-313 Porto, Portugal. Tel.: +351 22 0428596.
E-mail addresses: jldsousa@ff.up.pt (J.L. Dores-Sousa), veramcosta@ff.up.pt (V.M. Costa).

http://dx.doi.org/10.1016/j.tox.2015.01.006
0300-483X/

1970s, the 5-year survival in children was lower than 50% (Scully and Lipshultz, 2010), while nowadays it reaches 70% in the USA and Western Europe (Lipshultz et al., 2008). In the USA, the number of long-term survivors of pediatric cancers is estimated to be more than 360,000 (Scully and Lipshultz, 2010). In a study from the Childhood Cancer Survivor Study, with 10,397 children diagnosed with cancer in the 70s and 80s and treated with anthracycline or non-anthracycline based regimens, it was observed that survivors of children cancer had a 15.1-fold higher rate of developing congestive heart failure and a 10.4-fold higher rate developing cardiovascular disease when compared with their siblings (Oeffinger et al., 2006).

MTX is used in pediatric population, both in cancer treatment and as a second line therapy for multiple sclerosis, providing an increase in overall survival (Kingwell et al., 2010). In a retrospective review by van Dalen et al. (2004), they estimated that the incidence of MTX-related symptomatic cardiotoxicity/clinical heart failure varies between 0 and 6.7% and asymptomatic cardiac damage varies between 0 and 80% in children (<18 years). In the treatment of advanced acute leukemia and solid tumors in 84 children, 6 developed cardiac dysfunction, including 3 cases of congestive heart failure, after administration of MTX (Ungerleider et al., 1985). It is expected that the number of pediatric MTX-users and survivors will largely increase in the coming decades, which will also be accompanied by higher incidence of MTX-induced cardiotoxicity. Despite the widespread use of MTX and the fact that it presents a toxic cardiac clinical profile similar to doxorubicin, the mechanisms of MTX-induced toxicity toward non-target tissues, namely the heart, are largely unknown. It is known that, in humans, youth (<4 years) is considered a risk factor for MTX-induced cardiotoxicity (Lipshultz et al., 2008), although, presently, no susceptibility factors regarding MTX-induced cardiotoxicity in young patients have been identified. To the best of our knowledge, the present study is the first to evaluate the impact of MTX-induced cardiotoxicity in a juvenile animal model when compared to an adult population. As the cumulative dose is the most predictive risk factor for cardiotoxicity, the same cumulative dose of MTX (9.0 mg/kg) was given in multiple administrations to juvenile and adult CD-1 mice and those administrations were interrupted by free-drug periods as to mimic human MTX-therapy. Two protocols were performed: one group was sacrificed one day and the other 3 weeks after the last MTX administration in order to evaluate time-dependent biomarkers of toxicity, mainly focusing on cardiotoxicity.

2. Materials and methods

2.1. Chemicals

All chemicals and reagents were of analytical grade or of the highest grade available. Dimethyl sulfoxide (DMSO), ethylenediaminetetraacetic acid (EDTA), Folin–Ciocalteu reagent, Histosec paraffin pastilles, magnesium chloride, perchloric acid, sodium hydroxide, copper (II) sulfate, sodium carbonate, and disodium phosphate were purchased from Merck (Darmstadt, Germany). The fluorescent peptide substrates for caspase-3 (Ac-DMQD-AMC), for caspase-8 (Ac-IETD-AMC), and for caspase-9 (Ac-LEHD-AMC) were obtained from Peptanova (Sandhausen, Germany). Eosin 1% aqueous was obtained from Biostain (Traralgon, Australia). Harris hematoxylin was purchased from Harris Surgipath (Richmond, IL, USA) and Histofluid from Marienfeld (Lauda-Königshofen, Germany). ABX Pentra reagents were purchased from HORIBA (Kyoto, Japan). Bio-Rad DC protein assay kit was purchased from Bio-Rad Laboratories (Hercules, CA, USA). Horseradish peroxidase (HRP) conjugated anti-rabbit antibody, ECL chemiluminescence reagents, and 0.45 μm Amersham Protran nitrocellulose blotting membrane were purchased from GE Healthcare Bio-Sciences (Pittsburgh, PA, USA). Dinitropenhyl-KLH rabbit IgG antibody was purchased from Invitrogen/Life Technologies (Grand Island, NY, USA). All the other reagents used were purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Animals

Male CD-1 mice weighing 10–12 g and 38–40 g were obtained from Harlan (Udine, Italy). Animals were allowed to adjust to the environmental conditions for 4 days, before starting the experiments. The animals weighing 10–12 g, corresponding to a juvenile population, were approximately 3-weeks old, and the mature adult mice, weighing 38–40 g, were approximately 8 to 10 week-old. According to the available literature, the first group had not enter puberty (child) while the latter was in the beginning of adulthood (mature adults) (Flurkey et al., 2006). The animals were housed in a 1290D Eurostandard Type III cage in a temperature (22 ± 2 °C) and humidity-controlled environment and a 12 h light-dark cycle. Standard rodent chow 4RF21 GLP certificate diet (Mucedola, Settimo Milanese, Italy) and water were provided *ad libitum*. Housing and experimental treatment of the animals were in accordance with the guidelines defined by the European Council Directive (2010/63/EU). The animal experiments were licensed by the Portuguese General Directory of Veterinary Medicine (reference number 0421/000/000/2013) and approved by the Ethical Committee of Faculty of Pharmacy of the University of Porto (protocol number 7/03/2013).

2.3. Study design

The administration schedule of MTX was selected to mimic human MTX-therapy that consists of multiple administrations at separated time-points (Kingwell et al., 2010; Seiter, 2005). All injections were given in the afternoon to improve tolerability toward MTX, as reported in the literature (Levi et al., 1994). The administrations were done at Mondays and Thursdays, the same days of bed changing to avoid further animal stress.

During the entire experiment protocols, the animals' body weight, and food and water intake were registered twice a week. Animals were kept in a social environment as a group (Curfs et al., 2011) and, therefore, food and water consumption were assessed for the entire group and afterwards calculated as a function of the body weight of each animal.

The animals were divided into two groups (MTX-treated and saline-control) of adult and two groups of juvenile mice, formed by 6 animals in protocol 1, and 8 animals in protocol 2. MTX-treated animals were subjected to a total cumulative dose of 9.0 mg/kg MTX as a result of 6 intraperitoneal (i.p.) injections (2 *per* week). MTX dihydrochloride was dissolved in sterile 0.9% saline solution. Control animals were given 0.9% saline solution on the same schedule and at the same equivalent volume of MTX- injections. After the last MTX administration, the animals were maintained in a drug-free period for 20 days to allow the development of late toxicity, before sacrifice (protocol 1), or were sacrificed 24 h after the last injection (protocol 2) to evaluate early cardiac damage. This 9.0 mg/kg cumulative dose corresponds to 27 mg/m^2 of a mouse weighing 20 g (Reagan-Shaw et al., 2008), being similar to the dose of MTX used in the human therapy and about one-fifth of the maximum dose recommended in humans (140 mg/m^2) (Seiter, 2005).

2.4. Blood and tissue collection

Animals were anesthetized with isoflurane and sacrificed by exsanguination. Blood was taken from inferior vena cava into

Please cite this article in press as: Dores-Sousa, J.L., et al., The age factor for mitoxantrone's cardiotoxicity: Multiple doses render the adult mouse heart more susceptible to injury. Toxicology (2015), http://dx.doi.org/10.1016/j.tox.2015.01.006