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The genotoxicity and cytotoxicity assessments of andrographolide *in vitro*

Yusrizam Sharifuddin [a,*], Elizabeth M. Parry [a,1], James M. Parry [a,1]

[a] School of Medicine, Swansea University, Singleton Park, Swansea SA2 8PP, UK

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This paper is dedicated to the memory of James M. Parry, who passed away on 15th June 2010. A teacher, mentor and a friend, he shall be profoundly missed both professionally and personally, for his brilliant tutelage as well as his dedicated contributions to the field of genotoxicology.

Keywords:
Andrographolide
Micronuclei
Necrosis
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ABSTRACT

Andrographolide is a major phytoconstituent present in *Andrographis paniculata*, a plant used in traditional medicines in Asia for various ailments. This tropical shrub was reported to possess various pharmacological activities and has been marketed around the world including Europe, however the toxicological data especially potential genotoxicity assessment on the phytocompound is still lacking. This study was performed to assess the ability of andrographolide to induce chromosomal changes using the *in vitro* cytokinesis-blocked micronucleus assay with immunofluorescent labelling of kinetochores in metabolically-competent AHH-1 and MCL-5 human lymphoblastoid cell lines. Various cytotoxicity endpoints were also evaluated in this study. Andrographolide was found to cause a weak increase in micronuclei induction at 10–50 μM in both AHH-1 and MCL-5 cell lines, respectively which were within the historical range. Kinetochore analysis revealed that the micronuclei induced in MCL-5 cells due to andrographolide exposure originated via an aneugenic mechanism that was indicated by the relatively higher but non-significant percentage of kinetochore positive micronuclei compared to negative control. Andrographolide also elicited a dose-dependent cellular cytotoxicity, with cells dying primarily via necrosis compared to apoptosis. Here we report that andrographolide was not genotoxic at the doses tested and it induces dose-dependent necrosis *in vitro*.

1. Introduction

The advances in organic chemistry, biological and medical sciences in the 20th century had paved the way for the introduction and consumption of efficacious synthetic drugs, resulting in the decline of phytomedicines usage in modern medicine. However, the current revival of public interest in herbal supplements has caused the industry to start marketing an increasing amount of new herbal products to match the flourishing consumer demands. The assumption that all natural products are safe and without side-effects is popular in the public domain. Furthermore, distributing herbal products as supplements rather than as comprehensively evaluated medicines is relatively easy from the regulation point of view. This highlights the need to ensure the quality, efficacy and safety of herbal products as they do not often require prescription for the treatment of minor or chronic diseases or to be adapted in palliative care. Many herbs possess a long history of traditional use but most are of unproven efficacy by acceptable scientific standards. Moreover, a historical application of these herbs does not necessarily mean they are absolutely safe especially against subtle toxic or genotoxic effects that have no immediate and distinct clinical manifestations. One of the best examples is the traditional use of *Aristolochia* spp. in herbal preparations until aristolochic acid, the compound found in *Aristolochia* was shown to be a strong carcinogen in rats (Mengs et al., 1982, 1983) and was implicated in the Chinese herbs nephropathy (Vanherweghem et al., 1993; Vanhaelen et al., 1994; Vanherweghem, 1998).

Andrographis paniculata (Burm. f) Nees (Acanthaceae) is a shrub that is found throughout tropical and sub-tropical Asia and is locally known in Malaysia as 'Hempedu bumi' and 'Akar cerita bidara'. It is known that this plant was used in various traditional medicine systems to treat various ailments. *A. paniculata* is available to consumers in the UK as dried herb, in tablet or powdered forms as well as tea infusion and is normally referred to as Chuan Xin Lian in Chinese medicine shops. Extracts of this plant and andrographolide were shown to possess pharmacological activities such as immuno-stimulatory (Puri et al., 1993; Rajagopal et al., 2003; Kumar et al., 2004), anti-viral (Calabrese et al., 2000) and anti-bacterial (Singha et al., 2003).

Andrographolide is a major bioactive phytoconstituent found in various parts of *A. paniculata* particularly in the leaves. It is a colourless labdane diterpene harbouring a γ-lactone ring attached

Abbreviations: DAPI, 4′-6-diamidino-2-phenylindole; AHH, aryl hydrocarbon hydroxylase; MFO, mixed-function oxygenase; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; RI, replication index; WHO, World Health Organisation.

* Corresponding author. Present address: Program Sains Biokesihatan, Institut Sains Biologi, Fakulti Sains, Universiti Malaya, 50603 Kuala Lumpur, Malaysia. Tel.: +6 03 7967 6740; fax: +6 03 7967 6799.

E-mail addresses: yusrizam@hotmail.com, yusrizam@um.edu.my (Y. Sharifuddin).

[1] Present address: Safechem Consultants, 7 Cedarmount, Lyndhurst SO43 7ED, UK.

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to a decahydronapthalene ring system via an unsaturated C-2 moiety. As shown in Fig. 1, it contains an α-alkylidene γ-butyrolactone moiety as well as three hydroxyl groups located at C-3, C-14 and C-19 with these being secondary, allylic and primary respectively, which are considered to contribute to the reported bioactivities of andrographolide (Nanduri et al., 2004). Andrographolide is regarded to be the most active constituent present in *A. paniculata* extract (Chakravarti and Chakravarti, 1951; WHO, 2004; Kumar et al., 2004; Nanduri et al., 2004).

Realising the wide applications of *A. paniculata* especially in traditional medical systems and the current lack of genotoxic data, evaluating possible genotoxic potential of andrographolide is crucial. This study was carried out in two different human cell lines possessing different Phase I metabolic capabilities. The *in vitro* cytokinesis-blocked micronucleus assay was employed to quantify chromosomal damage due to andrographolide exposure, and was accompanied by kinetochore labelling using the CREST antibodies in order to determine the type of damage induced whether it is clastogenic or aneugenic in nature. The cytotoxicity and characterisation of cell death induction by andrographolide were also assessed in this study to gain more information in elucidating the phytocompound's possible mode(s) of toxicity.

2. Materials and methods

2.1. Chemicals and reagents

RPMI 1640 medium, L-glutamine and bovine serum albumin were purchased from Gibco-Invitrogen (UK). DPX mounting medium was purchased from Fisher Scientific (UK), 4′-6-diamidino-2-phenylindole (DAPI) was purchased from Cambio (UK), Vectashield antifade solution was purchased from Vector (UK). All other chemicals and reagents were obtained from Sigma–Aldrich (UK) and the sterile plasticware used were manufactured by Nunc®.

2.2. Cell lines

The cell lines used in this study were AHH-1 and MCL-5, both are genetically engineered metabolically-competent human lymphoblastoid cell lines.

2.2.1. AHH-1 cell line (Gentest™)

The AHH-1 $TK^{+/-}$ is a human B lymphoblastoid immortal cell line that is derived from the RPMI-1788 cell line (Freedman et al., 1979a,b) which was isolated from a healthy male donor transformed with the Epstein-Barr virus. The AHH-1 cell line has stable aryl hydrocarbon hydroxilase (AHH) activity, expressing mixed-function oxygenase (MFO) activity that is essential in oxidative xenobiotic metabolism. Mediated gene transfer of cytochrome P450 (CYP450) genes/cDNAs is a preferred method in the construction of metabolically competent cells (Crespi et al., 1990). AHH-1 is a homogenous, clonal derivative designed to constitutively express relatively high levels of CYP1A1 and not other CYP enzymes (Crespi and Thilly, 1984).

2.2.2. MCL-5 cell line (Gentest™)

MCL-5 cells are derived from a subpopulation of AHH-1 cells, the L3 cells which expresses an increased level of CYP1A1 activity (Crespi et al., 1991; Woodruff et al., 2001). The MCL-5 cell line was also transfected with two plasmids with the first plasmid harbouring a copy of *CYP2E1* complementary DNA (cDNA) and two copies of *CYP3A4* cDNA, whereas the second plasmid harbours one copy each of *CYP1A2*, *CYP2A6* and microsomal epoxide hydrolase cDNA (Crespi et al., 1991; Woodruff et al., 2001) allowing this cell line to stably express all five cDNAs. The cDNAs transfected in both AHH-1 and MCL-5 cell lines were of human origin. MCL-5 with its enhanced metabolic capacity has been demonstrated to be more sensitive than the parent AHH-1 cell line and had been used successfully in detecting genotoxic properties of many pro-mutagens and pro-carcinogens (Crespi et al., 1991; Doherty et al., 1996). These cell lines are heterozygous for p53 but undergo normal DNA repair and apoptotic responses upon exposure to genotoxins (Guest and Parry, 1999).

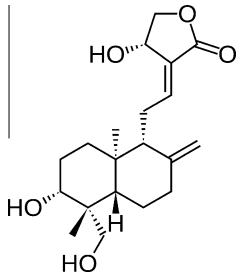

Fig. 1. Structure of andrographolide.

2.2.3. AHH-1 and MCL-5 cell culturing

The AHH-1 cell line was cultured in RPMI 1640 supplemented with 9% donor horse serum and 1% L-glutamine. The MCL-5 cell line was cultured in the same medium type as AHH-1 but with the addition of 200 μg/ml hygromycin-B (CAS number: 31282-04-9). Cells were cultured at 37 ± 1 °C with 5% CO_2 in air and were sub-cultured routinely every 2–3 days depending on confluency to ensure the cell concentration is at 1.5×10^5 cells/ml. Both cell lines have a cell cycle time of 22–24 h (Crespi et al., 1991) and they were routinely tested and have been consistently found to be free from mycoplasma in the laboratory.

2.3. In vitro cytokinesis-blocked micronucleus assay

Exponentially growing cell cultures were seeded into 25 cm^2 flasks to 1.5×10^5 cells/ml in 10 ml of fresh medium and incubated at 37 ± 1 °C with 5% CO_2 in air for 24 h. The following day, each was dosed (in duplicate) with andrographolide (CAS number: 5508-58-7; 98% purity) in DMSO (CAS number: 67-68-5, ~99.5% purity) giving 10, 30, 50, 70 and 90 μM final concentrations in all cell lines respectively. Fresh stock solution of andrographolide was used for each repeat experiment. Cytochalasin-B (CAS number: 14930-96-2) was also added to the cell cultures with a final concentration of 6 μg/ml for 24 h (one cell cycle) which is a standard practice in the laboratory. Untreated cells and those subjected to DMSO at 90 μM final concentration were used as negative controls whereas cells challenged with 0.2 μg/ml mitomycin-C (CAS number: 50-07-7) were used as positive control. Following incubation, the cell suspensions were centrifuged, the cell pellet was re-suspended in phosphate-buffered saline (PBS; pH 7.4) and gently vortexed using a Whirlimixer. Subsequently, aliquots of cells were harvested onto pre-polished slides using a cytocentrifuge and at least two replicate slides were prepared per culture. The slides were briefly air-dried before fixation in 90% ice cold methanol for 10 min followed by another air-drying session. The slides were then stained with 20% Giemsa (CAS number: 51811-82-6) and subsequently mounted with DPX prior to microscopy examination.

2.4. Indirect immunofluorescent labelling of kinetochore proteins

The indirect immunostaining of kinetochores was conducted as described by Ellard et al. (1991) with some minor modifications, which is a standard approach in the laboratory (OECD, 2010). The slides were dipped in 0.5 μg/ml primulin solution in PBS followed by washing and rewashing of the slides with PBS. CREST antibody was diluted in PBS at a 1:1 ratio and 50 μl of the diluted antibody was applied onto the cytodots of each slide. The slides were then incubated in humidified chambers at 37 °C for 45 min. After the incubation, unbound antibody was removed via a series of washings with bovine serum albumin in PBS and an aliquot of 50 μl of the second antibody, fluorescein isothiocyanate (FITC)-conjugated goat anti-human IgG in PBS was applied on the slides followed by 45 min incubation in humidified chambers. Again, the slides were washed twice with bovine serum albumin in PBS followed by a final wash with distilled water to remove salt. The slides were then left to air-dry in the dark. Finally, the slides were mounted with 4′-6-diamidino-2-phenylindole (DAPI) in Vectashield antifade solution and viewed using Olympus BH2-RCF fluorescent microscope with triple band pass filter permitting simultaneous observation of the DAPI and fluorochromes signals of kinetochore; whereas single band pass filters for blue and green spectra permit the DAPI and kinetochore signals to be separately viewed, respectively.

2.5. Microscopy and slide scoring

2.5.1. Micronucleus assay

The Giemsa-stained slides were coded and scored at 1000× magnification under oil immersion using an Olympus BH-2 light microscope. The scoring procedure was performed as described by (Fenech, 2000). The cells were categorised into mono-, bi-, tri-, tetra-, and multinucleated cells. In this study, micronuclei were scored in 2000 binucleated cells per dose (1000 binucleated cells per culture, 2 cultures per dose); micronuclei in other cells were not taken into account. Whereas the criteria followed for scoring necrotic and apoptotic cells stained with 20% Giemsa were as recommended by Fenech et al. (1999).

2.5.2. Kinetochore scoring

Kinetochore-labelled slides were scored for the presence or absence of kinetochores using an Olympus BH2-RCF fluorescent microscope with 1000× magnification. A total of 60 micronuclei were scored per dose, per experiment for the presence or absence of kinetochore proteins signals and a micronucleus was only scored if relatively good kinetochore signals were observed in the parent nuclei.