Mutation Research 767 (2014) 37–43

Contents lists available at ScienceDirect

Mutation Research/Genetic Toxicology and Environmental Mutagenesis

journal homepage: www.elsevier.com/locate/gentox
Community address: www.elsevier.com/locate/mutres

A comparative study of the aneugenic and polyploidy-inducing effects of fisetin and two model Aurora kinase inhibitors

P. Gollapudi, L.S. Hasegawa, D.A. Eastmond*

Environmental Toxicology Graduate Program and Department of Cell Biology & Neuroscience, University of California, Riverside, CA 92521, USA

ARTICLE INFO

Article history:
Received 19 December 2013
Received in revised form 14 March 2014
Accepted 15 March 2014
Available online 26 March 2014

Keywords:
Aneuploidy
Polyploidy
Micronucleus
Flavonoid
Nutritional supplement

ABSTRACT

Fisetin, a plant flavonol commonly found in fruits, nuts and vegetables, is frequently added to nutritional supplements due to its reported cardioprotective, anti-carcinogenic and antioxidant properties. Earlier reports from our laboratory and others have indicated that fisetin has both aneugenic and clastogenic properties in cultured cells. More recently, fisetin has also been reported to target Aurora B kinase, a Ser/Thr kinase involved in ensuring proper microtubule attachment at the spindle assembly checkpoint, and an enzyme that is overexpressed in several types of cancer. Here we have further characterized the chromosome damage caused by fisetin and compared it with that induced by two known Aurora kinase inhibitors, VX-680 and ZM-447439, in cultured TK6 cells using the micronucleus assay with CREST staining as well as a flow cytometry-based assay that measures multiple types of numerical chromosomal aberrations. The three compounds were highly effective in inducing aneuploidy and polyploidy as evidenced by increases in kinetochore-positive micronuclei, hyperdiploidy, and polyploidy. With fisetin, however, the latter two effects were most significantly observed only after cells were allowed to overcome a cell cycle delay, and occurred at higher concentrations than those induced by the other Aurora kinase inhibitors. Modest increases in kinetochore-negative micronuclei were also seen with the model Aurora kinase inhibitors. These results indicate that fisetin induces multiple types of chromosome abnormalities in human cells, and indicate a need for a thorough investigation of fisetin-augmented dietary supplements.

1. Introduction

Fisetin is a plant flavonol commonly found in fruits, vegetables, nuts, and wine [1]. It is also frequently used as an additive in nutritional supplements due to its reported cardiprotective, anti-carcinogenic and antioxidant properties [2,3]. In addition, fisetin has been reported to have a number of potentially adverse cellular and biochemical effects including prevention of cell proliferation and angiogenesis *in vitro* as well as inhibition of critical enzymes such as cyclin-dependent kinases and topoisomerase II [4–10]. Earlier reports from our laboratory and others have indicated that fisetin has both aneugenic and, to a lessor degree, clastogenic properties in cultured cells [9,11,12]. Recently, fisetin has also been reported to target Aurora B kinase, a Ser/Thr kinase involved in ensuring proper microtubule attachment at the spindle assembly checkpoint [13].

Aurora kinases are critical for the proper passage of cells through several stages of the cell cycle. Aurora A kinase localizes to the centrosomes and spindle poles, and plays an important role in the development of the centrosomes and in bipolar spindle formation [14]. Aurora B kinase localizes along the chromosome arms and at centromeres in prophase, at the inner centromeric region during metaphase, at the central spindle and cortex during anaphase, and in the midbody in telophase [15]. It has been shown to play an important role in chromosome biorientation, destabilization of improper microtubule attachments, phosphorylation of histone H3, and cytokinesis [15]. A third kinase in this family, Aurora C, is thought to have overlapping functions with Aurora B kinase and acts primarily in germ-line cells.

Overexpression of Aurora A kinase leads to an early entry into mitosis due to hyperactive centrosomes and multipolar spindle formation, and can lead to chromosome instability [16]. Similarly, overexpression of Aurora B kinase is thought play a role in chromosomal instability by interfering with chromosome biorientation and the spindle checkpoint [14]. Overexpression of both Aurora A and B kinases has been associated with several types of cancer including breast, colorectal, ovarian, and pancreatic cancer among

* Corresponding author. Tel.: +1 951 827 4497; fax: +1 951 827 3087.
E-mail address: david.eastmond@ucr.edu (D.A. Eastmond).

http://dx.doi.org/10.1016/j.mrgentox.2014.03.004
1383-5718/

others [17–19]. As a result, both Aurora A and B kinases are thought to be promising targets for chemotherapeutic agents.

As a follow-up to the recent report on its Aurora B kinase inhibiting properties, we decided to more fully characterize the aneugenic and polyploidy-inducing effects of fisetin and compare them with those seen with two known small molecule model Aurora kinase inhibitors, VX-680 and ZM-447439, which act preferentially on Aurora A and Aurora B kinases, respectively. Disruption of the spindle assembly and inhibition of Aurora kinases could lead to segregation errors and aneuploidy, providing insights into the mechanisms by which these agents could induce aneuploidy and polyploidy. While some information is known about the ability of fisetin to induce micronuclei and aneuploidy *in vitro*, very little is known about the chromosome-altering effects of other Aurora kinase inhibitors such as VX-680 and ZM-447439.

2. Methods

2.1. Cell culture and treatments

The human lymphoblastoid cell line TK6 was maintained in RPMI 1640 medium (GIBCO; Carlsbad, CA) containing 10% iron-supplemented calf serum (Hyclone; Logan, UT) with 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (Fisher Scientific; Pittsburg, PA) at 37 °C in an atmosphere of 5% CO_2/95% air. Exponentially growing cells at a starting density of 2.5×10^5 cells/ml were treated with various concentrations of either fisetin (Sigma Aldrich; St Louis, MO), or the Aurora kinase inhbitors VX-680 or ZM-447439 (Cayman Chemical; Ann Arbor, MI) in a final dimethylsulfoxide (DMSO) concentration of 0.1%. Cells were harvested at 24 h after treatment. For time course experiments, the test media was removed at 24 h and the cells were re-suspended in fresh media for an additional 6–24 h.

2.2. In vitro micronucleus assay with CREST staining

The procedure for the *in vitro* micronucleus assay was performed as previously described with minor modifications [20]. Cytochalasin B was added 24 h prior to harvest to the treatment flasks designated for manual scoring of micronuclei. Aliquots of the cell suspension were centrifuged directly onto slides and then briefly air-dried and fixed in 100% methanol. Prepared slides were then stained with CREST primary antibody, followed by a FITC-conjugated secondary antibody (both obtained from Antibodies Inc., Davis, CA), with DAPI used as a DNA counterstain. Slides were then coded and 1000 binucleated cells per test concentration were scored for the presence of kinetochore-positive (K+) and kinetochore-negative (K−) micronuclei (MN), and analyzed and reported as micronucleated cells (MNC), K+ MNC and K− MNC, respectively. The means and standard error of the means (SEM) were calculated using data from 2 to 4 separate experiments.

2.3. Numerical chromosomal aberration assay by flow cytometry

For the detection of chromosomal abnormalities by flow cytometry, the staining, data acquisition and analytical methods previously described by Meuhlbauer and Schuler [21] were employed with one notable modification; A trapezoid-shaped gate was used to more efficiently exclude doublets and apoptotic cells in the hyperdiploid and polyploidy region. Colcemid was added to the cell cultures 2–3 h prior to harvesting, which occurred at 24 h (or later for the time-course experiments), and the cells were fixed in 70% ethanol. Fixed cells were then stained with phosphohistone H3 (Ser10) 6G3 monoclonal mouse antibody (Cell Signaling Technologies; Beverly, MA) followed by an Alexa-Fluor 488 conjugated goat anti-mouse IgG secondary antibody (Molecular Probes, Eugene, OR) to identify the mitotic cells. The DNA was then stained with propidium iodide (PI) and ploidy of the mitotic cells was measured. Hypodiploid cells were defined as mitotic cells with >2C but <4C DNA content. Cells with >4C but <8C DNA content were considered hyperdiploid, and cells with >8C DNA content were classified as polyploid. Data from 2000 mitotic cells per test concentration were acquired and analyzed using a Becton Dickinson FACSort flow cytometer and CellQuest software. The means and SEM were determined based on data from 5 to 6 separate experiments for fisetin, and 2 experiments for each of the model Aurora kinase inhibitors.

2.4. Statistical analysis

Dose-related increases in micronucleated and aneuploid/polyploid cells were determined using the Cochran–Armitage test for trend in binomial proportions [22]. Following a positive response in the trend test, a one-tailed Fisher's exact test was used to compare individual treatments against the respective DMSO-treated controls [23]. In the flow experiments with fisetin, unusually high variability was seen, particularly at the higher test concentrations. As a result, linear regression or ANOVA was used to determine if there was a dose-related increase in ploidy, and positive results were followed by a Mann–Whitney *U* test to compare individual treatments with DMSO treated controls. For all studies, critical values were determined using a 0.05 probability of type I error.

3. Results

3.1. Fisetin

As reported previously, treatment with fisetin resulted dose-related increase in the formation micronucleated cells in TK6 cells (Fig. 1a). Strong and significant increases in K+ micronucleated cells, indicating chromosome loss were seen, confirming our previous report that fisetin acts as an aneugen [9]. To further investigate its aneugenic and polyploidy-inducing properties, numerical chromosomal aberrations induced by fisetin were also assessed using flow cytometry. At the same 24 h time point at which strong, up to 20-fold, increases in K+ MNC were observed following treatment with fisetin, more modest 2- to 5-fold increases in hypodiploidy were seen by flow cytometry ($r^2 = 0.2381$, $p = 0.0002$) (Fig. 1b). Similarly, smaller 2- to 4-fold increases in hyperdiploidy were also observed ($r^2 = 0.2183$, $p = 0.0003$). There also appeared to be a modest increase in polyploidy at some of the concentrations tested. Because the dose-response curve was non-monotonic with a reduced increase in polyploidy at the highest concentrations, ANOVA was used to analyze the experimental results and showed that a significant increase had occurred ($p \leq 0.05$). Subsequently, pair-wise comparisons using the Mann–Whitney *U* test as a *post hoc* test indicated that modest, but significant, 2- to 3-fold increases in polyploidy were induced at concentrations between 13.6 and 20 μM.

The unusual pattern and variability of the results raised the possibility that treatment with fisetin may have triggered a cell cycle delay, hindering cells from progressing to a second metaphase and therefore preventing chromosome loss from being detected in the flow-based assay. To explore this possibility, a time course experiment was performed with washout of the fisetin after 24 h. Cells were then harvested at 12 and 24 h after the washout to allow the treated cells to overcome a cell cycle delay. In this extended time course study (Fig. 2), fisetin at the 20 μM and higher concentrations induced large increases in hyperdiploidy and polyploidy at time points 36 and 48 h after initial treatment, consistent with a cell cycle delay. At the 36 h harvest time, there was an ~6-fold increase in hyperdiploidy and a very large ~50-fold increase in polyploid cells observed in the cultures. Interestingly, additional increases in hypodiploidy were not observed.

3.2. Model Aurora kinase inhibitors

For comparison, similar studies were performed with the model Aurora kinase inhibitors VX-680 and ZM-447439. Similar to fisetin, both VX-680 and ZM-447439 induced significant dose-related increases in micronucleated cells with significant approximately four-fold increases seen at concentrations as low as 25 nM for VX-680 and 100 nM for ZM-447439 (Fig. 3a and b). Whereas fisetin induced primarily K+ micronucleated cells, both model kinase inhibitors led to a significant increase in K− as well as K+ micronucleated cells, indicating that they induced both chromosome breakage and loss at concentrations that did not cause appreciable cytostatic effects. As before, the flow-cytometry based assay was used to look at numerical chromosomal aberrations after treatment with the two Aurora kinase inhibitors (Fig. 3c and d). Somewhat surprisingly, no increase in hypodiploidy was detected after 24 h with the Aurora kinase inhibitors despite the observed increase in K+ micronucleated cells. Treatment with VX-680 caused a very large increase in polyploidy at 24 h with ~70% of the cells exhibiting polyploidy at the 25 nM concentration. Significant increases in hyperdiploidy were also seen. In contrast,