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A Comparative Study of the Aneugenic and Polyploidy-inducing Effects of Fisetin and Two Model Aurora Kinase Inhibitors

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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.

Keywords

aneuploidy; polyploidy; micronucleus; flavonoid; nutritional supplement

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

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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 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.

Methods

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 hours after treatment. For time course experiments, the test media was removed at 24 hours and the cells were re-suspended in fresh media for an additional 6 to 24 hours.

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 hours 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 2–4 separate experiments.

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 hours prior to harvesting, which occurred at 24 hr (or later for the time-course experiments), and the cells were fixed in 70% ethanol. Fixed cells were then stained with phospho-histone 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 5–6 separate experiments for fisetin, and 2 experiments for each of the model Aurora kinase inhibitors.

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.

Results

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 hr 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 nonmonotonic 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 13.6–20 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 hours. Cells were then harvested at 12 and 24 hours 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 hours after initial treatment, consistent with a cell cycle delay. At the 36 hr. 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.

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 100nM for ZM-447439 (Fig 3a and Fig 3b). 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 Fig 3d). Somewhat surprisingly, no increase in hypodiploidy was detected after 24 hours 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 hr. with ~70% of the cells exhibiting polyploidy at the 25 nM concentration. Significant increases in hyperdiploidy were also seen. In contrast, ZM-447439 induced polyploidy, but no increase in hyperdiploidy was seen.

As a follow-up, extended time course studies were performed and consistent differences in numerical chromosomal aberrations were not seen between the 24, 36 and 48 hr time points (data not shown).

Discussion

The goal of the present study was to characterize the chromosome altering effects of fisetin, a natural compound found in plants that has recently been reported to inhibit Aurora B kinase, and compare the results to those of two model Aurora kinase inhibitors, VX-680 and ZM-447439. Our results showed that all three compounds were effective at inducing numerical chromosomal alterations although different patterns were seen with the different agents. At micromolar concentrations fisetin induced strong increases in K+ MNC indicative of chromosome loss, hyperdiploidy and polyploidy; the latter two effects were maximally detected at a later 36 hr harvest (12 hr after fisetin had been removed from the cultures) which allowed the cells to overcome a cell cycle delay. VX-680 caused a modest increase in K+ MNC as well as very large increases in polyploid and hyperdiploid cells. Similarly, ZM-447439 induced a modest increase in K+ MNC as well as a strong increase in polyploidy although no increase in hyperdiploidy was seen. Both model Aurora kinase inhibitors induced K− MNC indicating that they could also cause chromosome breakage.

The results of these experiments confirm previous reports that fisetin acts as an aneugen [9, 11], with strong increases in K+ micronucleated cells observed across the concentration range tested. We did not, however, observe any noticeable increases in micronuclei due to breakage after treatment with fisetin. While at first glance this appears to differ from our earlier results in which fisetin was reported to induce K− MNC, it should be noted that the major clastogenic effect seen in the previous study occurred at a 45 μM concentration which is above our highest test concentration of 30 μM, and where considerable cytotoxicity would be expected (Figure 1a and b) [9]. In comparison, VX-680 and ZM-447439 induced approximately even amounts of both K+ and K− micronucleated cells at concentrations where appreciable cytotoxicity was not seen. The similarity in the types of micronuclei formed after treatment with the two kinase inhibitors is of note and suggests that similar targets are being affected by these agents, even at the sub-micro molar concentrations used in our experiments. Previous studies have reported that both VX-680 and ZM-447439 are able to inhibit both Aurora A and Aurora B kinases [24–26].

The observation that VX-680 and ZM-447439 have both aneugenic and clastogenic effects is also interesting. Inhibition of Aurora kinases is thought to interfere mostly with spindle

formation and separation of sister chromatids. As a result, one would expect that treatment with Aurora Kinase inhibitors would lead to increases in segregation errors and thus increases in of K+ micronucleated cells. The observed increase in chromosome breakage and K− micronucleated cells at non-cytotoxic concentrations, however, was unexpected and the mechanisms underlying this effect are not as well understood. One possibility is that Aurora B kinase inhibition could result in lagging chromosomes that are broken by the cleavage furrow due to premature abscission and deregulated cytokinesis. In yeast, the Aurora B homolog, Ipl-1, has been shown to play a key role in the regulation of cytokinesis as part of the NoCut pathway [27]. Mutations that result in inactivation of Ipl-1 have been reported to cause premature cytokinesis in yeast cells, resulting in breakage of lagging chromosomes. While Aurora B in mammalian cells has also been shown to play an important role in controlling the timing of cytokinesis, to our knowledge, it has yet to be shown that a similar clastogenic effect occurs in human cells.

It is also unclear whether the clastogenic effects of the model Aurora kinase inhibitors are related to those that have been associated with fisetin. It has previously been reported that fisetin can directly inhibit topoisomerase II-alpha, providing a possible mechanism by which fisetin can induce chromosome breakage [9, 10, 28]. The breakage also occurred under cytotoxic conditions, which may have played a role in the breakage observed in the earlier fisetin study [29, 30]. Since both kinase inhibitors we tested are considered to be highly specific to the Aurora kinases and act at relatively non-toxic, nanomolar concentrations, it seems unlikely that treatment with either VX-680 or ZM-447439 would also lead to inhibition of topoisomerase II or that the breaks would be a consequence of cytotoxicity. As another possible explanation, it has previously been reported that sustained mitotic delay caused by compounds such as spindle disrupting agents can lead to chromosome breakage, likely as an early apoptotic stress response [31]. Since inhibition of Aurora kinases is known to disrupt the spindle assembly checkpoint, it is possible that the breakage observed here might be due to a similar type of stress response.

One unexpected finding was that much smaller increases in hypodiploidy were detected using the flow cytometry-based assay to detect numerical chromosomal aberrations despite the appearance of significant increases in K+ micronucleated cells following treatment with either fisetin or the Aurora kinase inhibitors. This was unexpected since previous studies in our laboratory using a variety of aneugenic agents have generally seen qualitatively similar results using the micronucleus and flow-based assays (data not shown). There are a number of possible explanations for the differences observed in this study. For instance, in their previous study detailing the use of this assay, Muehlbauer and Schuler [21] suggested that one potential limitation of the flow assay might be a limited capacity for detecting single chromosome loss events due to gating requirements. Manual scoring of binucleated cells treated with either fisetin or the two Aurora kinase inhibitors with the *in vitro* micronucleus assay with CREST staining showed that most cells exhibiting chromosome loss had only a single K+ micronucleus even at the higher concentrations, indicating that an inability to establish discriminating gating parameters could provide a possible explanation for the differences seen. Consistent with this explanation, when performing the flow assay with the

fisetintreated cultures, occasionally a separate cluster of cells was seen that was close to the hypodiploid gate but still within the normal gate settings for diploid cells (data not shown).

Other possible explanations for the somewhat disparate results include re-incorporation of the micronuclei or sub-optimal timing of the cell harvest. For example, while previous studies have generally assumed that micronuclei are either extruded or degraded, Crasta and associates provided data to indicate that chromosomes contained in the micronuclei can be reincorporated into daughter cells during mitosis [32]. It is therefore possible that if cells treated with either fisetin or the Aurora kinase inhibitors lose a chromosome during the first mitosis and if reincorporation of that chromosome occurs early during the second mitosis, then the cell could appear to be normal in the flow assay which measures ploidy during the second mitosis.

Another possible explanation for the differing results is that fisetin as well as the Aurora kinase inhibitors may cause a cell cycle delay. The main difference between the MN assay and the flow-based aneuploidy assay is the stage of the cell cycle in which the cells are evaluated. With the *in vitro* micronucleus assay, cells have completed mitosis and are back in interphase, while the flow-based aneuploidy assay generally detects cells during their second metaphase. If there is an effect that causes a cell cycle delay that prevents cells from progressing to metaphase, then it is possible that the chromosome loss will not be detected. As indicated above, to examine this possibility, initial follow-up time course experiments were performed with chemical washout at 24hrs, looking at aneuploidy at time points of 24, 36, and 48 hours after initial treatment. With Aurora kinase inhibitors, there was no difference when the later time points were compared to the initial 24 hr treatment. With fisetin, large increases in hyperdiploidy and polyploidy were observed 36 and 48 hr (12 and 24 hours after chemical washout), with a profile resembling more closely those of cells treated with VX-680 and ZM-447439. A related possibility is that we may have missed a transient wave of hypodiploid cells because the 12 hr sample collection window between 24 and 36 hr was too wide. To further investigate this possibility, we conducted an additional time course experiment using a smaller dose range and an intermediate harvest time of 30 hr (6 hours after chemical washout). While a small increase in hypodiploidy was seen at the 13.4 μM concentration, it was not seen at the 20 μM concentration (data not shown) suggesting that an inadequate sampling time was unlikely to explain the differences seen.

In addition to the similarities seen between fisetin and the model Aurora kinase inhibitors such as the induction of K+ MNC, hyperdiploidy, and polyploidy, there was one particularly notable difference. Our time course experiments indicate that treatment of cells with fisetin caused a substantial cell cycle delay whereas there was no evidence of a cell cycle delay seen with the model Aurora kinase inhibitors. While both VX-680 and ZM-447439 have previously been shown to be fairly selective inhibitors of the Aurora kinases, fisetin has been reported to have a broader range of targets within the cell. Among the known targets of fisetin are cyclin-dependent kinases, which when inhibited can cause cell cycle arrest [7, 8]. Inhibition of these cyclin-dependent kinases could be the mechanism underlying the cell cycle delay seen with fisetin.

These results also underscore the challenges in trying to identify multiple types of numerical chromosomal alterations at a single harvest time. For the Aurora kinase inhibitors, a single harvest at 24 hrs was sufficient to detect significant increases in micronuclei, hyperdiploidy (for VX-680) and polyploidy. In contrast, while a substantial increase in micronuclei was seen with fisetin, only modest increases in the other ploidy measures were seen at the 24 hr harvest time. Following removal of the test agent and with a 12 hr recovery, a much larger increase in hyperdiploidy and a very large increase in polyploidy were seen. These numerical changes would likely have been regarded as only minor effects if only the 24 hr harvest period had been used. By evaluating the later time points, fisetin was shown to be a potent inducer of hyperdiploidy and polyploidy. One of the advantages of the flow cytometry method is that the cell cultures can be rapidly and simultaneously evaluated for hypodiploidy, hyperdiploidy and polyploidy, thereby allowing multiple time points to be evaluated if there is a concern about cell cycle delay. Previous studies from our laboratory have shown a generally good qualitative concordance between the K+ MNC frequency and hypodiploidy as measured by the flow assay. However, in a number of circumstances, such as in this case with fisetin, manual scoring of MNC appears to be more sensitive at detecting chromosome loss than the detection of hypodiploidy as measured by flow cytometry.

In conclusion, our results clearly demonstrate that fisetin, VX-680 and ZM-447439 are effective aneuploidy and polyploidy-inducing agents *in vitro*, and indicate that numerical chromosomal alterations result from the inhibition of Aurora kinases in human cells. Given that these effects were seen at nanomolar concentrations with VX-680 and ZM-447939, these types of chromosomal changes would likely to contribute to potential anti-neoplastic effects of these Aurora kinase inhibitors. With fisetin, the induced chromosomal alterations were seen at much higher micromolar concentrations. If similar effects occur *in vivo*, this would be a concern as fisetin is currently being used at high concentrations in dietary supplements [13], which typically receive little or no testing prior to marketing.

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Highlights

- Fisetin, a plant flavonol that is added at high levels to some nutritional supplements, has recently been reported to inhibit Aurora B kinase.
- Chromosomal alterations induced by fisetin were characterized and compared to those induced by two model Aurora kinase inhibitors.
- All three agents were highly effective in inducing aneuploidy and polyploidy in cells in culture.
- Only fisetin induced a significant cell cycle delay suggesting that it has cellular targets in addition to Aurora B kinase.

Figure 1a

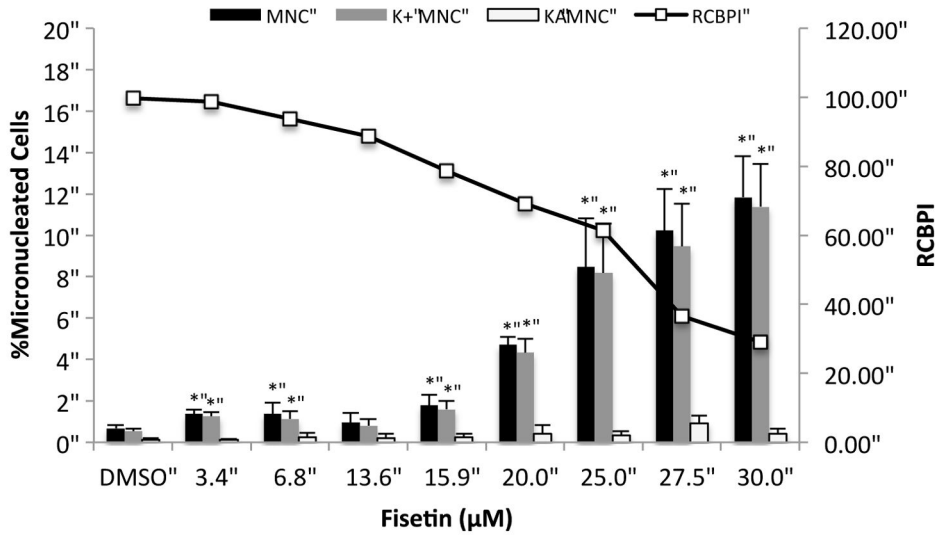

Figure 1b

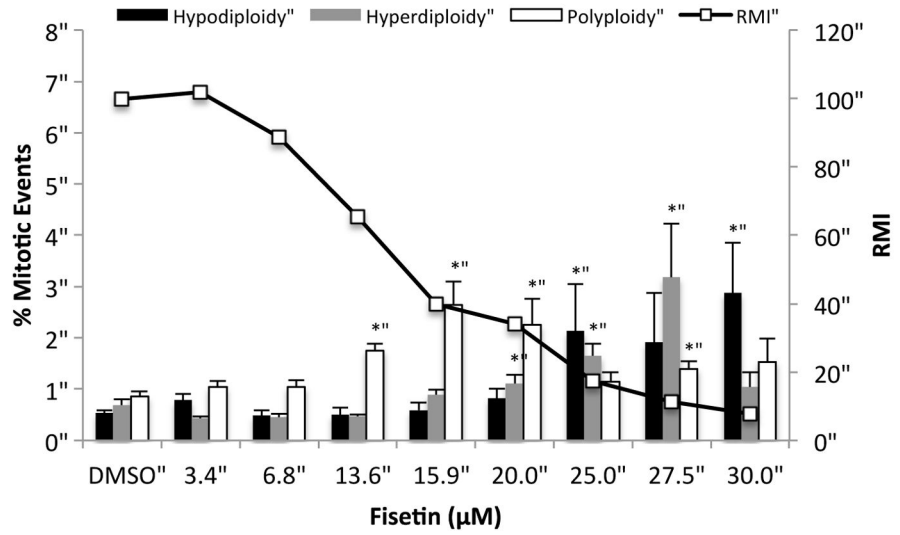

Figure 1.

a) Frequencies of micronucleated cells (MNC), kinetochore-negative micronucleated cells (K-MNC), and kinetochore-positive micronucleated cells (K+ MNC) in TK6 cells treated with fisetin. 1000 binucleated cells were scored per test concentration and the means and standard error of the means (SEM) from 2–4 separate experiments are shown. The relative cytochalasin B proliferation index (RCBI), a measure of cytotoxicity, for each test concentration is also shown. *Statistically significant *vs.* the DMSO controls (Fisher's exact test; $P \leq 0.05$). b) Numerical chromosomal aberrations measured by flow cytometry. Aberrations were computed as a percentage of 2000 gated mitotic events in TK6 cultures treated with fisetin for 24 h. The means and SEM from 5–6 separate experiments are shown. The relative mitotic index (RMI) is also shown. *Statistically significant *vs.* the DMSO controls (Mann-Whitney U test; $P \leq 0.05$).

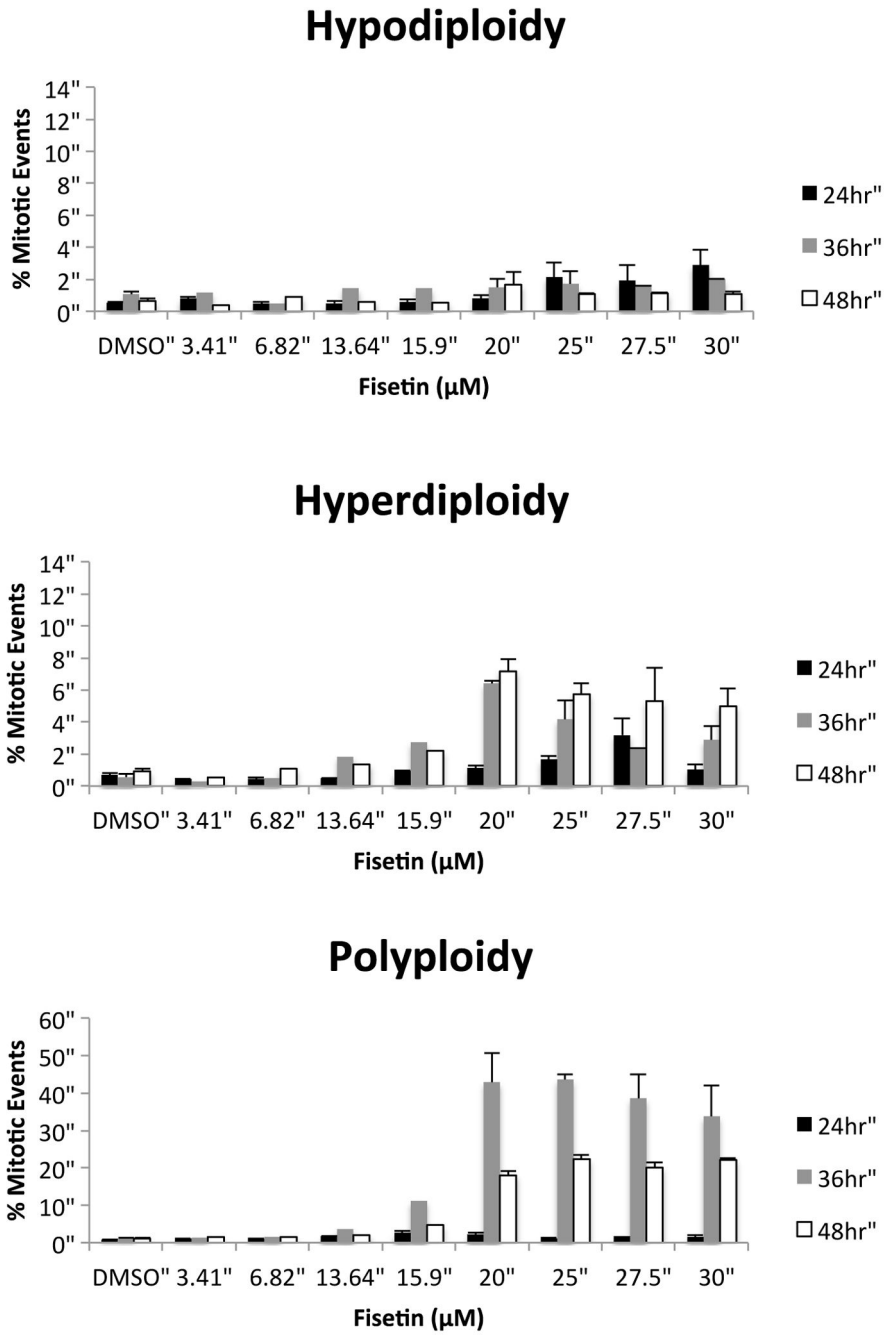

Figure 2.

Time-course evaluation of hypodiploidy, hyperdiploidy, and polyploidy in TK6 cells treated with fisetin for 24 h. Cells were re-suspended in fresh media and numerical chromosomal aberrations were monitored at 0, 12 and 24 hours after the end of treatment. The results from the 24 hr treatment with immediate harvest are the same as those presented in Fig. 1b.

Figure 3a

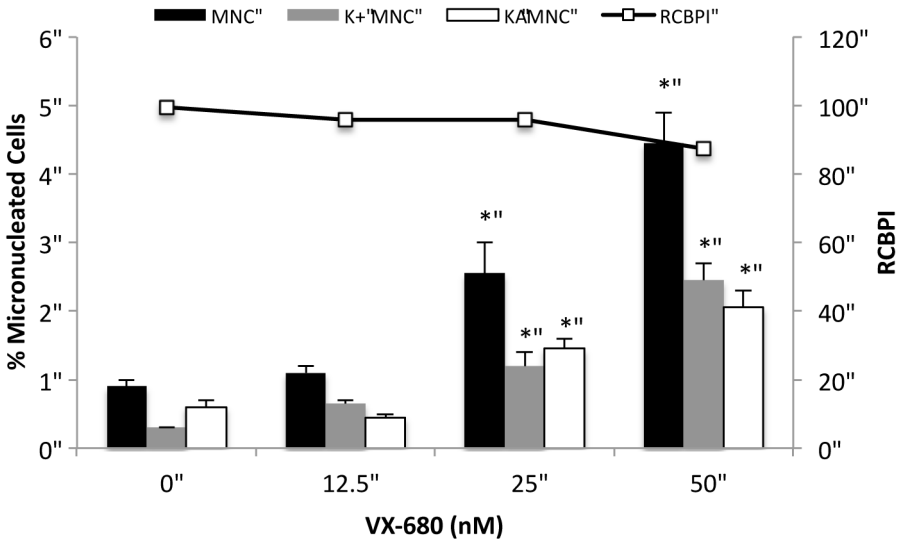

Figure 3b

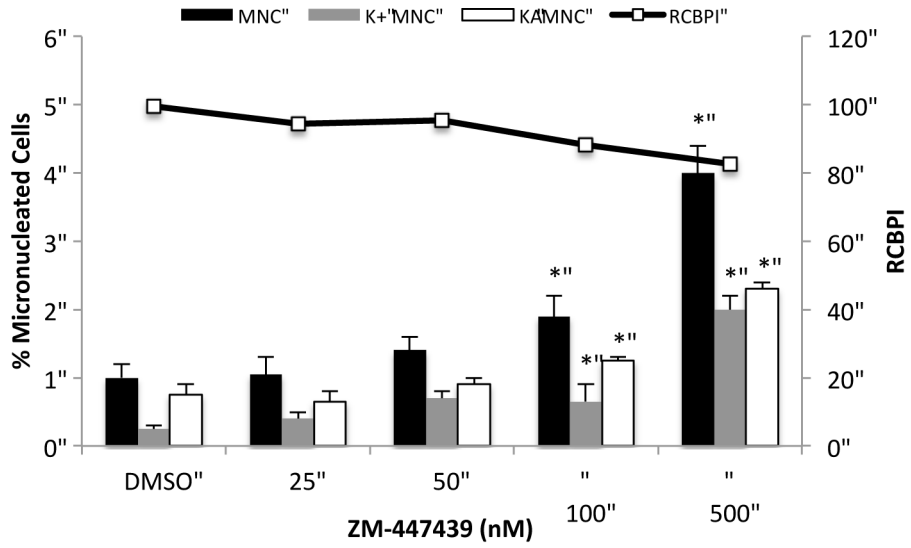

Figure 3c

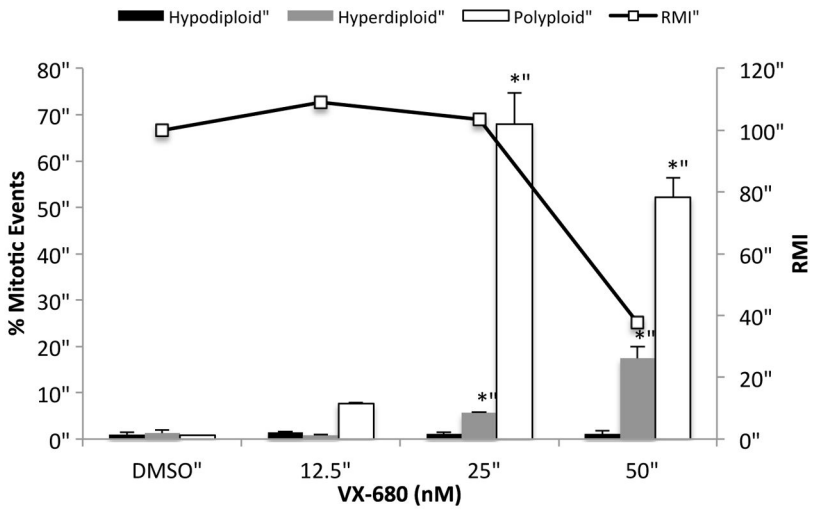

Figure 3d

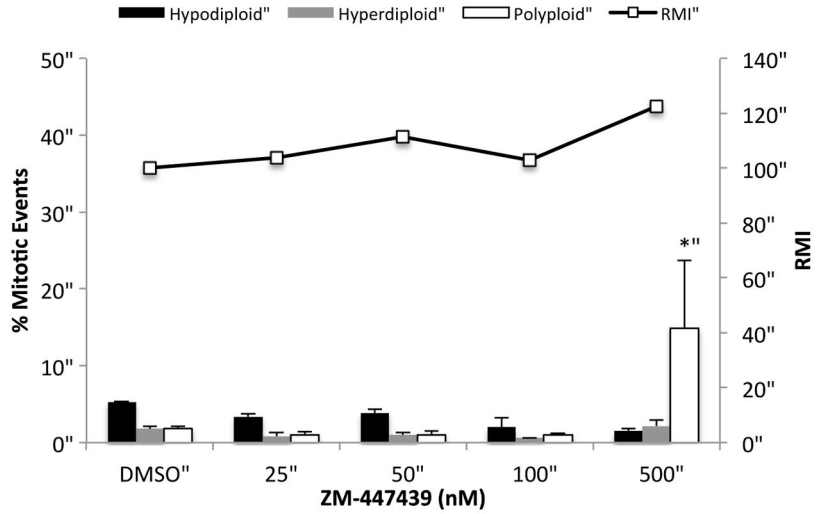

Figure 3.

a–b) Frequencies of micronucleated cells (MNC), kinetochore-negative micronucleated cells (K-MNC), and kinetochore-positive micronucleated cells (K + MNC) in TK6 cells treated with the Aurora kinase inhibitors (a) VX-680 or (b) ZM-447439 for 24 h. 1000 binucleated cells were scored for micronuclei per test concentration and the means and SEM from 2 separate experiments are shown. The relative cytochalasin B proliferation index (RCBI) for each test concentration is also shown. c–d) Numerical chromosomal aberrations measured by flow cytometry. Aberrations were computed as a percentage of 2000 gated mitotic events in TK6 cultures treated with (c) VX-680 or (d) ZM-447439 for 24 h. The means and SEM from 2–3 separate experiments are shown. The relative mitotic index (RMI) is also shown. *Statistically significant *vs.* the DMSO controls (Fisher's exact test; $P \leq 0.05$).