The flavonoid eupatorin inactivates the mitotic checkpoint leading to polyploidy and apoptosis


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ABSTRACT

The spindle assembly checkpoint (SAC) is a conserved mechanism that ensures the fidelity of chromosome distribution in mitosis by preventing anaphase onset until the correct bipolar microtubule–kinetochore attachments are formed. Errors in SAC function may contribute to tumorigenesis by inducing numerical chromosome anomalies (aneuploidy). On the other hand, total disruption of SAC can lead to massive genomic imbalance followed by cell death, a phenomena that has therapeutic potency. We performed a cell-based high-throughput screen with a compound library of 2000 bioactives for novel SAC inhibitors and discovered a plant-derived phenolic compound eupatorin (3',5-dihydroxy-4',6,7-trimethoxyflavone) as an anti-mitotic flavonoid. The premature override of the microtubule drug-imposed mitotic arrest by eupatorin is dependent on microtubule–kinetochore attachments but not interkinetochore tension. Aurora B kinase activity, which is essential for maintenance of normal SAC signaling, is diminished by eupatorin in cells and in vitro providing a mechanistic explanation for the observed forced mitotic exit. Eupatorin likely has additional targets since eupatorin treatment of premitotic cells causes spindle anomalies triggering a transient M phase delay followed by impaired cytokinesis and polyploidy. Finally, eupatorin potently induces apoptosis in multiple cancer cell lines and suppresses cancer cell proliferation in organotypic 3D cell culture model.
Introduction

The spindle assembly checkpoint (SAC, also called mitotic checkpoint) is a conserved cell cycle control mechanism that prevents improper chromosome segregation and aneuploidy [1]. The SAC inhibits activity of the mitotic ubiquitin ligase termed anaphase-promoting complex/cyclosome (APC/C) until all kinetochore–microtubule (MT) attachments are stabilized and all chromosomes have achieved bipolar orientation at the spindle equator. Satisfaction of the SAC leads to APC/C activation and proteasome-mediated degradation of anaphase inhibitors such as securin, and ultimately exit from mitosis. One key regulatory element of the SAC is the Aurora B kinase the activity of which is essential for the correction of improper kinetochore–MT attachments and maintenance of SAC signaling. Premature inactivation of the SAC can lead to changes in chromosome numbers (aneuploidy) in daughter cells. In animal and cell-based studies, low levels of aneuploidy have been associated with tumorigenesis while high levels induce cell death [2,3]. Therefore, induction of massive aneuploidy through inhibition of the SAC has raised interest as a new therapeutic opportunity to suppress cancer cell growth. Especially, regulatory SAC proteins that are highly expressed in cancer and whose loss of function causes high rates of aneuploidy are attractive targets currently under extensive research [4]. One such target is the Aurora B kinase; inhibition of its kinase activity causes tetraploidy, a phenotype associated with increased cell death in a variety of tumor cell lines [5–8].

Polyphenols are a diverse group of compounds that exist as secondary metabolites in plants. Based on the chemical structure, polyphenols can be classified into ten groups including the flavonoids which are further divided into six subgroups [9–11]. Polyphenols have been shown to possess anti-inflammatory as well as DNA- and cytoprotective properties which are potentially beneficial to human health [12]. Interestingly, dietary phytochemicals can modulate processes regulating the cell cycle likely via their action on key signaling pathways such as the mitogen-activated protein kinase (MAPK) and Akt kinase cascades [13–15].

Paclitaxel, which was isolated from the bark of Taxus brevifoli [16], is one of the first examples of phytochemicals approved for treatment of cancer [15]. At the moment there are other interesting phytochemicals, e.g. resveratrol, gingerol and myricetin, which modulate signaling cascades involved in induction of cancer cell death or inhibition of cell proliferation [15]. However, use of these agents as cancer therapeutics is restricted until their cellular mechanism of action has been determined. Identification of phytochemicals’ targets can be of a challenge, since they tend to affect multiple cellular events. However, this may also be beneficial as tumorigenesis is a complex process involving multiple signaling pathways. Therefore, identification of phytochemicals having specificity for multiple targets might be advantageous over synthetic chemicals that typically are designed to inhibit single proteins. Furthermore, anti-carcinogenic effects of polyphenols may be potentiated by combining them with currently used cancer drugs [17,18]. The low toxicity of phytochemicals encourages their use also for longer time periods possibly even as chemopreventive agents [15].

In a cell-based high-throughput screen (HTS), we have identified eupatorin (3′,5-di-hydroxy-4′,6,7-trimethoxyflavone, $M_w = 344.3$, Fig. 1C) was obtained from Extrasynthese. Other chemicals were from Sigma unless otherwise stated. Eupatorin was prepared as a 25 mM stock solution in DMSO and stored at $-20$ °C. Eupatorin was used in cell-based assays at 50 μM, staurosporine at 10 μM, hydrocortisone (5 μg/ml), HEPES (20 mM), non-essential amino acids (0.1 mM), dihydroartemisin (5 μg/ml), epidermal growth factor (20 ng/ml), cholera toxin (100 ng/ml) and 5% horse serum. PC3 prostate adenocarcinoma cells were grown in RPMI medium supplemented with glutamine (2 mM) and 10% FBS. A549 lung carcinoma and DU145 prostate carcinoma cells were grown in RPMI medium supplemented with glutamine (2 mM) and 10% FBS. LNCaP and 22RV1 prostate cancer cells were grown in RPMI medium supplemented with l-glutamine (2 mM), penicillin/streptomycin (0.1 mg/ml) and 10% FBS. All cell lines were cultured at 37 °C and with 5% CO₂.

Materials and methods

Cell culture

HeLa cervical adenocarcinoma and HeLa H2B-GFP (HeLa cells stably expressing histone H2B-GFP fusion protein [19]) cell lines were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with penicillin/streptomycin (0.1 mg/ml), glutamine (2 mM), non-essential amino acids (0.1 mM), HEPES (20 mM) and 10% fetal bovine serum (FBS). For HeLa H2B-GFP cells, basicolin (2 μg/ml) was added to the growth medium. MCF-10A non-tumorigenic breast epithelial cells were maintained in DMEM/HAM F-12 (1:1) supplemented with glutamine (2 mM), insulin (10 μg/ml), hydrocortisone (5 μg/ml), epidermal growth factor (20 ng/ml), cholera toxin (100 ng/ml) and 5% horse serum. PC3 prostate adenocarcinoma cells were grown in DMEM with glutamine (2 mM) and 10% FBS. A549 lung carcinoma and DU145 prostate carcinoma cells were grown in RPMI medium supplemented with glutamine (2 mM) and 10% FBS. LNCaP and 22RV1 prostate cancer cells were grown in RPMI medium supplemented with l-glutamine (2 mM), penicillin/streptomycin (0.1 mg/ml) and 10% FBS. All cell lines were cultured at 37 °C and with 5% CO₂.

Chemicals

Eupatorin (3′,5-di-hydroxy-4′,6,7-trimethoxyflavone, $M_w = 344.3$, Fig. 1C) was obtained from Extrasynthese. Other chemicals were from Sigma unless otherwise stated. Eupatorin was prepared as a 25 mM stock solution in DMSO and stored at $-20$ °C. Eupatorin was used in cell-based assays at 50 μM, MG132 at 20 μM, nocodazole at 70 nM, 350 nM and 3 μM, taxol (Molecular Probes) at 600 nM, monastrol at 100 μM, vinblastin at 1 μM, ZM447439 (Tocris Bioscience) at 20 μM, staurosponine at 1 μM, and MLN8054 (Selleck) at 0.5 μM concentrations. Spectrum collection library used in the HTS was from MicroSource Discovery Systems.

Compound library screen

The HTS for small molecules that cause forced exit from a nocodazole-induced mitotic arrest in HeLa cells was performed as previously described [13].

Live cell microscopy

HeLa H2B-GFP cells were grown on 35 mm live cell chambers (MatTek Corp.). To study mitotic exit, the cells were pretreated with drugs inducing mitotic arrest (i.e. nocodazole, monastrol, vinblastin, taxol) for 8 h before addition of eupatorin and imaged using a Zeiss Axiovert 200 M microscope equipped with 63x (NA
1.4) objective, Orca-ER camera (Hamamatsu Photonics) and MetaMorph imaging software (Molecular Devices). Images were captured at 10 min intervals using transmitted light and FITC channels.

**Immunofluorescence, image acquisition and analysis**

Immunofluorescence was performed as described earlier [20]. We used primary antibodies against Aurora B/AIM1 (1:1000, Abcam and 1:1000 BD Biosciences), BubR1 (1:400, Abcam), CREST autoimmune serum (1:200, Antibodies Inc.), CenpA phosphorylated at Ser7 (pCenpA; 1:500, Millipore), pericentrin (1:2000, Abcam), survivin (1:600, Abcam), INCENP (1:500, Abcam), p-T232-AurB (1:1000, Rockland), p-T288-AurA (1:200, Cell Signaling), γ-tubulin (1:200, Abcam) and α-tubulin DM1A (1:400, Abcam). Secondary FITC, Cy3 or Cy5-conjugated antibodies were used at 1:600–1:800 (Jackson ImmunoResearch Inc.). Images of the fixed cells were acquired using a Zeiss Axiovert 200 M platform (see above) and MetaMorph software as Z-stacks with 0.3 μm step-size. Quantification of kinetochore protein signals was done using MetaMorph as described [20]. For each experiment, a minimum of 50 kinetochores was analyzed in five cells per condition. Statistical testing was performed with Student's t-test and P values <0.001 were considered significant.

**Monastrol washout**

Cells grown on coverslips were treated with monastrol for 4 h. Subsequently, cells were treated with MG132 for 1 h followed by a wash.

**Fig. 1** – Cell-based HTS for compounds inducing forced mitotic exit identified flavonoid eupatorin. A) The workflow of HTS for compounds inducing a premature mitotic exit. Cycling HeLa cell population was arrested in mitosis with nocodazole treatment overnight. Mitotic cells were harvested and re-plated on 384-well plates containing library compounds. The plates were incubated for 4 h in the continuous presence of nocodazole. After removal of loosely attached mitotic and apoptotic cells by washes, DNA was stained and the content of the wells was analyzed with a cell cytometer. Example micrographs showing a well with a forced mitotic exit causing drug (step 7; high fluorescence, cells exit from mitosis and re-adhere to the bottom) and a well with a non-effective library drug (step 8; low fluorescence, cells stay at mitosis and are washed away in step 5). The scale bar equals to 20 μm. B) Image showing fluorescence intensity of a 384-well plate from the secondary screen consisting of 34 effective compounds discovered in the primary screen. The cells were kept in medium supplemented with nocodazole and plated into wells containing the compounds at four different concentrations (60, 6, 0.6, 0.06 μM, lanes 3–22). Positive control (lane 1, black arrow, cells treated with an experimental drug Ro-31-8220) and negative control (lane 2, cells treated with nocodazole only) were included in each screen. Ro-31-8220 inhibits cyclin-dependent kinase 1 and induces a forced mitotic exit in nocodazole blocked cells. Blank wells contained culture medium without cells (lanes 23–24). Eupatorin induced premature exit from M-phase resulting in re-adherence of the cells to the bottom of the well and high fluorescence intensity. The arrow points to the well where mitotic cells were treated with 60 μM eupatorin (position M9). The effects of different concentrations of eupatorin on fluorescence intensity are shown in the inset. The middle graph shows quantification of the DNA fluorescence intensity converted into cell numbers for each well of the plate shown in the upper panel. The bottom histogram shows quantification of the cell number in wells with different eupatorin concentrations as well as negative (Neg) and positive control (Ro) wells (mean ± SEM). C) Chemical structure of eupatorin (3′,5-dihydroxy-4′,6,7-trimethoxyflavone).
addition of eupatorin for 2 h. The cells were fixed as described earlier using paraformaldehyde (PFA) and 0.2% glutaraldehyde [20]. Cells were released from monastrol block by repeated washes (3 × 10 min) with medium containing MG132. Subsequently, MG132 containing medium was supplemented with DMSO or eupatorin and the cells were incubated for 1 h before fixation.

Cold-calcium lysis

Cells growing on coverslips were rinsed twice with Pipes (0.1 M) followed by lysis for 5 min with Pipes (0.1 M), CaCl₂ (80 μM) and 1% Triton X-100 on ice. Finally, cells were rinsed twice with Pipes and fixed as described above in the presence of PFA and 0.2% glutaraldehyde.

In vitro kinase assay

The in vitro kinase assay to determine whether eupatorin inhibits Aurora B activity was performed as described previously [13].

Western blotting

Cells were arrested in mitosis with 70 nM nocodazole for 16 h. Cell culture medium was supplemented with MG132 1 h before addition of eupatorin, ZM447439 or DMSO for 2 h. Preparation of cell extracts, SDS-PAGE and immunoblotting were done as described elsewhere [20]. The blots were incubated with antibodies against p-T288-AurA (1:1000, Cell Signaling), cleaved PARP (1:1000, Cell Signaling) and GAPDH (1:30000, Advanced ImmunoChemical). IR Dye® Conjugated secondary antibodies (Rockland Immunochemicals Inc.) were used at 1:5000. Signals were detected using Odyssey Infrared Imaging System (LI-COR Biotechnology).

Fluorescent-activated cell sorting

To harvest all cells, including apoptotic cells not attached to the substrate, both culture medium and trypsinized cells were collected. Cells were then spun down and fixed in 70% ethanol (−20 °C). After incubation for at least 30 min at −20 °C, the cells were washed once with PBS before resuspension in 200 μl PBS containing 100 μg/ml RNase and 20 μg/ml propidium iodide. After incubation for at least 30 min at RT in the dark under constant agitation, FACs data was collected on the LSR II (BD Biosciences). The data was analyzed using FCS Express 3 (De Novo Software).

In vitro tubulin polymerization assay

Fluorescence based in vitro tubulin polymerization assay (BK011, Cytoskeleton Inc.) was performed according to the manufacturer’s instructions. Briefly, the reaction mixture contained PEM buffer, glycerol (13.8%), fluorescent reporter (5 μM), GTP (1 mM), porcine brain >99% pure tubulin (2 mg/ml) and eupatorin at 1, 5, 10 and 20 μM concentrations. Taxol (3 μM), vinblastin (3 μM), and DMSO were included as controls. Tubulin polymerization was recorded at 1-min intervals for 60 min at 37 °C with excitation at 355 nm and emission at 460 nm with Victor 1420 Multilabel HTS Counter (PerkinElmer).

3D organotypic cell culture and imaging

The 3D cell culture was performed as previously described [21]. Briefly, cells were plated between two layers of Matrigel on uncoated Angiogenesis μ-slides (Ibidi GmbH). The bottoms of wells were filled with 50% Matrigel in culture medium and allowed to polymerize at 37 °C for 1 h. LNCaP or 22RV1 cells were seeded at a density of 1000 cells/well. After attachment (1–2 h at 37 °C), cells were covered with another layer of 25% Matrigel in culture medium and allowed to polymerize for 3–4 h at 37 °C. Cell culture medium was changed every other day. Eupatorin (20 μM) or taxol (50 nM) was added after 4 day incubation and the cultures were maintained for 7 additional days. The treatments were performed in triplicate. The forming spheroids were monitored by live-cell imaging (Incucyte, Essen Instruments; 10 × objective). 3D structures were stained with Calcein AM live cell dye (Invitrogen, 1:5 from 1 mM stock). Confocal three-dimensional images were taken by using the Zeiss Axiovert 200 M with spinning disc confocal unit Yokogawa CSU22 and 5 × objective. Intensity projections were created by SlideBook 4.2.0.7. Images were further analyzed with VTT Acca software and box blots visualized with R.

Results

Eupatorin induces a forced mitotic exit dependent on proteasome activity

To identify small molecules inhibiting SAC function, we performed a cell-based high-throughput screen [13,22] with Spectrum Collection library of 2000 bioactive compounds including known drugs, experimental compounds and pure natural products. In short, HeLa cells were arrested in mitosis overnight with 350 nM nocodazole, harvested and replated in the presence of 70 nM nocodazole into 384-well plates containing the bioactives in four different concentrations (60, 6.0, 0.6 and 0.06 μM; Fig. 1A). Four hours later the loosely attached mitotic or apoptotic cells were washed out and remaining interphase cells which had escaped the nocodazole-induced mitotic arrest were fixed with paraformaldehyde including Sybr GOLD nucleic acid stain. Fluorescence intensity of the DNA was measured with Acumen cell cytometer (Fig. 1B). With 60 μM eupatorin, high fluorescence intensity of the DNA was observed due to increased amount of cells attached to the well. The fluorescence was weaker with 6 μM eupatorin and very low with the lowest concentrations. The wells were also checked by fluorescence microscopy showing the decondensation of chromosomes by eupatorin (data not shown). The structure of eupatorin (3',5'-dihydroxy-4',6,7-trimethoxyflavone) is shown in Fig. 1C.

To confirm that eupatorin overrides a chemically induced mitotic arrest, we arrested HeLa H2B-GFP cells in mitosis by incubating for 8 h with 70 nM nocodazole which hyperactivates the SAC without significantly depolymerizing microtubules [23] and then added 50 μM eupatorin or DMSO to the culture medium. The cells were subsequently followed by time lapse microscopy in the continuous presence of nocodazole. The majority (82%, n = 157) of nocodazole-arrested cells remained in mitosis for 4 h after addition of DMSO (Figs. 2A, B; Supplemental video 1). In contrast, most of the eupatorin-treated cells (80%, n = 179) changed
their round mitotic appearance into a flat interphase morphology and showed chromosome decondensation within two hours after eupatorin addition (Figs. 2A, B; Supplemental video 2). Eupatorin induced the same phenotype also in PC3 (metastatic prostate cancer cell line), MCF-10A (non-transformed mammary epithelial cell line), DU145 (hormone insensitive prostate cancer cell line),

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**Fig. 2** – The flavonoid eupatorin causes a forced exit from drug-induced mitotic arrest. A) HeLa H2B-GFP cells were pretreated with nocodazole (Noc; 70 nM) or vinblastin (1 μM) for 8 h before addition of eupatorin (Eup; 50 μM) or DMSO and followed immediately by time lapse microscopy. To block the proteasome activity, MG132 (20 μM) was added 1 h before eupatorin. The figure shows representative FITC and phase-contrast still images from the time lapse films of cell populations treated as indicated. The scale bar equals to 10 μm. B) Quantification of eupatorin-induced forced mitotic exit. Cells were pretreated with SAC hyperactivating drugs nocodazole (Noc; 70 nM or Noch; 3 μM), nocodazole and MG132 (Noc + MG132), taxol (Tx; 600 nM), monastrol (Mon; 100 μM), or vinblastin (Vbl; 1 μM), and followed by time lapse microscopy immediately after addition of eupatorin (50 μM) or DMSO. The number of cells that exit mitosis is indicated as a percentage of the total mitotic cell amount. The data represents mean ± SEM of two independent experiments.
A549 (lung adenocarcinoma cell line) and LNCaP cells (hormone sensitive prostate adenocarcinoma cell line) proposing that its mechanism of action was independent of the cell type (data not shown). In conclusion, the data indicates that eupatorin induces override of nocodazole block.

A plausible explanation for the override of nocodazole-induced mitotic arrest is that eupatorin interferes with normal SAC signaling. Since mitotic exit induced by satisfaction of the SAC is dependent on protein degradation by the proteasome, we first examined whether the flavonoid-induced escape from mitosis requires proteasome activity. Nocodazole-arrested HeLa H2B-GFP cells were treated with proteasome inhibitor MG132 (which arrests cells in metaphase downstream of the SAC) for 1 h before addition of eupatorin or DMSO and subsequent time-lapse imaging (Figs. 2A, B; Supplemental video 3). Only 7% of the nocodazole-MG132-pretreated mitotic cells (n = 224) escaped M phase within the next 6 h after addition of eupatorin, indicating that the flavonoid-induced forced mitotic exit is dependent on proteasome activity.

**Eupatorin overrides the SAC activated by lack of tension but not by unattached kinetochores**

Anaphase inhibiting SAC signals are thought to be generated in the presence of unattached kinetochore(s) and in the absence of proper interkinetochore tension [1]. Tension is created between sister kinetochores that are stably attached to microtubules from opposing spindle poles that causes centromeric chromatin and kinetochore structures to become stretched. To examine whether eupatorin-induced override of mitotic arrest is dependent on interkinetochore tension or microtubule attachments, we pretreated HeLa H2B-GFP cells with DMSO, vinblastin (1 μM), taxol (600 nM), nocodazole (70 nM or 3 μM), or monastrol (100 μM) for 8 h before addition of eupatorin to the culture medium and time-lapse filming. These drugs hyperactivate the SAC and in HeLa H2B-GFP cells typically induce mitotic arrest persisting over 16 h. Only 10% (13 cells out of 132) and 6% (7 cells out of 114) of cells arrested at M phase with MT destabilizing drugs vinblastin (1 μM) or nocodazole (3 μM, NoH) at concentrations that completely disrupt MTs [23] escaped from mitosis within 4 h after addition of eupatorin, respectively (Fig. 2B). In contrast, 81% (129 cells out of 159) of cells blocked at M phase with 0.6 μM taxol, a MT stabilizing drug that decreases tension between the sister kinetochores but preserves the attachments, escaped from mitosis in 4 h after addition of eupatorin. Monastrol is an Eg5 inhibitor known to cause M-phase arrest with a monopolar spindle [24] where the kinetochores are attached to MTs but lack interkinetochore tension. Similarly to taxol-treated cells, nearly all cells (97%, 66 cells out of 68) that were exposed to 100 μM monastrol exited from M phase within 4 h after addition of eupatorin. Together, these observations propose that eupatorin can overcome mitotic block caused by lack of tension but not by lack of MT-kinetochore attachment.

**Eupatorin interferes with localization of BubR1 and Aurora B, and inhibits Aurora kinase activity**

As proteins involved in SAC signaling are known to concentrate on unattached kinetochores in mitosis [25], we investigated whether eupatorin interferes with kinetochore targeting of key SAC proteins. For this purpose, HeLa cells blocked at M phase with 70 nM nocodazole (16 hour incubation) were treated for 1 h with MG132 prior to 3 hour incubation with eupatorin in the continued presence of nocodazole and MG132. The cells were subsequently fixed and immunostained for BubR1, Aurora B, Survivin, INCENP, pCenp-A and CREST auto-immune serum. Control cells blocked at mitosis with nocodazole were incubated for 4 h with MG132 before fixation, immunostainings, and measurement of the kinetochore fluorescence intensities of the immunostained marker proteins. BubR1 kinetochore accumulation was significantly reduced after eupatorin treatment (82% reduction, P < 0.001; Fig. 3A), potentially explaining the impaired SAC in these cells. Importantly, CREST signals at the kinetochores of eupatorin-treated cells were comparable to those in control cells (Fig. 3A), indicating that the general architecture of the kinetochores is not affected by the flavonoid.

Aurora B is required for correcting improper kinetochore–MT interactions and sustaining SAC signaling when sister kinetochores are attached to MTs but are not under tension [5,6]. In accordance with previous studies [26,27], Aurora B accumulated in inner centromeres in cells exposed to nocodazole and MG132 (Fig. 3B). The localization was confirmed by co-immunostaining with INCENP, a known inner centromeric protein (Fig. S1). Interestingly, addition of eupatorin on these cells led to mislocalization of Aurora B to the chromosome arms. To investigate if eupatorin interfered with Aurora B function, cells were stained with an antibody recognizing Cenp-A phosphorylated at S7 (pCenp-A), a specific target epitope for Aurora B [27]. A highly significant reduction in kinetochore accumulation of pCenp-A was observed in the eupatorin-treated cells (reduced by 99%, P < 0.001; Fig. 3A), which is comparable to the reduction in pCenp-A staining induced by the Aurora inhibitor ZM447439 [13]. Together these results demonstrate that eupatorin inhibits Aurora B activity in cells.

As Aurora B is part of the chromosomal passenger complex (CPC, [28]) we further studied whether eupatorin affects the localization of the other CPC subunits survivin and INCENP. We observed that in the presence of eupatorin also survivin and INCENP were mislocalized to the chromosome arms in the majority of cells as evaluated by immunofluorescence staining (Fig. S-B). This suggests that eupatorin abolishes the correct localization of the CPC at the inner centromeres.

To study whether the dramatic decrease in Aurora B activity caused by eupatorin in cells is due to direct inhibition of Aurora B or modulation of its upstream regulators, we performed an *in vitro* kinase assay, where γ-32P-ATP incorporation to myelin basic protein (MBP) by recombinant Aurora B was determined in the presence of different concentrations of eupatorin. The flavonoid inhibited Aurora B *in vitro* at a concentration that causes escape from mitosis (60% reduced Aurora B activity at 50 μM) with an IC50 value of approximately 20 μM (Fig. 3C). *In vivo* result was verified by immunofluorescence analysis of auto-phosphorylated Aurora B (p-T232-AurB) showing notable reduction (53% activity remaining) of active Aurora B upon eupatorin treatment (Fig. 3C). As expected, in the presence of ZM447439 only residual Aurora B activity was observed. Together, the data suggest that eupatorin interferes with SAC signaling through direct inhibition of Aurora B activity.

Another member of the Aurora kinase family, Aurora A, has important roles in regulating of centrosome and spindle functions, as
well as in controlling the mitotic commitment of cells [29]. The kinase domain of Aurora A is very similar to that of Aurora B, and therefore compounds that inhibit Aurora B may also target Aurora A kinase. To determine the specificity of eupatorin, we analyzed if eupatorin has an effect on Aurora A activity in cells. The levels of Aurora A, auto-phosphorylated on Thr288, a marker for Aurora A activity [30], were slightly down-regulated in the mitotic eupatorin-treated cells compared to control cells as detected by Western blotting (Fig. 3D). The immunofluorescence staining for phosphorylated Aurora A confirmed this result (activity decreased...
Fig. 4 – The flavonoid eupatorin induces cytokinesis defects and delayed mitotic progression in HeLa H2B-GFP cells. A) Representative FITC and phase-contrast still images from the time lapse films of cell populations treated with DMSO or eupatorin (50 μM). The scale bar equals to 10 μm. B) Quantification of the eupatorin effects on cytokinesis in comparison to DMSO treated controls. The graphs show percentage of mitotic cells that exhibited normal cytokinesis or aborted cytokinesis resulting in cell fusion, or mitotic cell that showed no apparent signs of cytokinesis during the forced mitotic exit (no CK). The data represents mean ± SEM of three separate experiments (n = 47 eupatorin-treated and n = 18 control cells). C) Quantification of the length of mitosis in cell population treated with DMSO or eupatorin. The duration of mitosis was significantly longer in the presence of eupatorin (n = 16, average length 489 ± 156 min) when compared to controls (n = 23, average length 64 ± 28 min). Data represents mean ± SEM (***, P < 0.001).

Fig. 3 – Localization of SAC proteins to kinetochores is abolished and Aurora kinase activity is decreased in cells treated with eupatorin. A) Immunolocalization of BubR1 and pCenpA in control and eupatorin-treated HeLa cells blocked in mitosis with nocodazole. Staining with CREST autoimmune serum is shown as a control. DNA was stained with DAPI. MG132 was added to prevent exit from mitosis. The scale bars equal to 10 μm. The graph shows quantification of kinetochore fluorescence intensities of BubR1 and pCenpA (mean ± SEM; n = 10 in each of the 5 analyzed cells, ***, P < 0.001). B) Immunolocalization of Aurora B, INCENP and Survivin in control (DMSO) and eupatorin-treated HeLa cells blocked in mitosis with nocodazole. MG132 was added to prevent exit from mitosis. DNA was stained with DAPI. The scale bar equals to 10 μm. C) Determination of eupatorin effects on Aurora B activity in vitro and in cells. In vitro kinase activity assay of Aurora B. Incorporation of γ-32P-ATP on MBP by recombinant Aurora B in the presence of 0.4, 2, 10, and 50 μM of eupatorin is shown in the autoradiograph. Control reaction was performed in the absence of eupatorin. The graph shows the quantification of normalized Aurora B activity from a representative experiment. The IC50 value for Aurora B inhibition is approximately 20 μM. The micrographs below show representative images of pAurora B (T232), γ-tubulin and CREST immunostained cells in control (DMSO) and eupatorin treated populations. DNA was stained with DAPI. The scale bar equals to 10 μm. The graph at the bottom shows quantification of the fluorescence intensity of pAurora B in control (DMSO), eupatorin, or ZM447439 (ZM) treated mitotic cells (whole cell level, mean ± SEM; n = 10–14 cells per group). D) Determination of eupatorin effects on Aurora A activity in cells. The amount of Aurora A phosphorylated on Thr288 (pAurA) was measured by Western blotting in nocodazole arrested cell populations treated with vehicle (DMSO), eupatorin (Eup) or MLN8054 (MLN) for 3 h. GAPDH protein levels are shown as control for equal loading of samples. The graph shows quantification of the normalized pAurora A band intensities in the blots. The micrographs show representative images of pAurora A (Thr288) and γ-tubulin immunostained cells in control (DMSO) and eupatorin treated populations. DNA was stained with DAPI. The scale bar equals to 10 μm. The graphs at the bottom show quantification of active Aurora A at whole cell level (mean ± SEM; n = 18) and at the spindle poles (mean ± SEM; n = 39) in control (DMSO) and eupatorin treated mitotic cells (***, P < 0.001).
by 41% in whole cells and by 58% at poles) proposing that not only Aurora B but also Aurora A activity is inhibited at M phase by eupatorin. Importantly, as detected by Western blotting, the specific Aurora A kinase inhibitor MLN8054 [31] greatly abolished Aurora A phosphorylation in the nocodazole-arrested cells, as expected (activity decreased by 94%, Fig. 3D).

Fig. 5 – Eupatorin perturbs normal spindle structure and centrosome separation. A) Quantification of the spindle defects in HeLa cell populations treated with various drug combinations followed by detection of spindle poles and satellite astral foci using antibodies against α-tubulin and pericentrin. The cells were either incubated with MG132 for 3 h, or treated with eupatorin for 3 h in the presence of MG132 for the last 1 h (Eup + MG132), or first arrested at metaphase with 1 hour long treatment with MG132 followed by co-treatment with eupatorin for 2 additional hours (MG132 + Eup). The graph shows percentage of mitotic cells having one, two or more spindle poles with or without additional satellite foci. The micrographs show representative cells from populations treated with the indicated drugs, fixed, and immunostained for tubulin and pericentrin. DNA was stained with DAPI. The scale bar equals to 10 μm. The graph on the right hand side shows the percentage of mitotic cells having one, two, or more pericentrin-positive MT nucleating foci in cell populations treated as indicated. B) Quantification of the spindle defects in HeLa cell populations treated with Eg5 inhibitor monastrol followed by detection of the spindle poles and satellite astral foci using antibodies against α-tubulin and pericentrin. The cell populations were either incubated in the presence of monastrol for the whole duration of the assay or the drug was washed off at 4 hour time point and the cells were released into MG132 containing medium with or without eupatorin for 1 additional hour. The graph shows percentage of mitotic cells having one, two, or more spindle poles with or without additional satellite foci. The micrographs show representing cells from populations exposed to the indicated treatments, fixed, and immunostained for tubulin and pericentrin. DNA was stained with DAPI. The scale bar equals to 10 μm. The graph on the right hand side shows the percentage of mitotic cells having one, two, or more pericentrin-positive MT nucleating foci in cell populations treated as indicated. C) In vitro MT polymerization assay. The fluorescence signal of tubulin polymerization is shown in the presence of indicated eupatorin concentrations or in the presence of MT stabilizing drug taxol (Tx) or MT destabilizing drug vinblastin (Vbl). DMSO was used as a negative control. Tubulin polymerization was monitored at 1 min intervals for 60 min at 37 °C with excitation at 355 nm and emission at 460 nm using Victor 1420 Multilabel HTS Counter.
Eupatorin has profound effects on mitotic progression in cycling cell populations

In normal mammalian cells, the SAC is active from early prometaphase until all chromosomes have stable bipolar attachments with spindle MTs [1]. To study whether eupatorin interferes with mitotic progression in unperturbed cells we filmed cycling HeLa H2B-GFP cell populations immediately after addition of DMSO or eupatorin. All DMSO-treated mitotic cells (100%, n = 18) progressed normally through cell division (Figs. 4A, B). In contrast, the vast majority of cells that were exposed to eupatorin at M phase exited mitosis prematurely with defective cytokinesis. In 30% of the cells (14 out of 47) no signs of cleavage furrow formation were observed. In rest of the cells, cleavage furrow formation was initiated but cytokinesis was soon aborted resulting in fusion of two daughter cells into one polyploid cell (Figs. 4A, B). These results indicate that eupatorin impairs normal SAC signaling and inhibits normal cytokinesis when applied on otherwise unperturbed mitotic cells, which correlates with the phenotype induced by Aurora B kinase inhibitors [5,6].

Surprisingly, in the same assay we noticed that cells that were exposed to eupatorin before they entered mitosis exhibited a mitotic delay upon entry to M phase instead of undergoing a premature mitotic exit. All the cells that entered M phase in the presence of the flavonoid (n = 41 cells monitored) were delayed at mitosis for at least 270 min before abnormal exit, the average length of mitosis being 489 ± 156 min (Fig. 4C). This is, however, an underestimate of the extent of the delay as many cells (n = 23) were still at mitotic arrest at the end of the filming session. The average length of mitosis in DMSO-treated control cells was 64 ± 28 min (n = 23, Fig. 4C). This phenotype resembles the situation observed in cells pretreated with high concentration of nocodazole/vinblastine as those cells were resistant to eupatorin-induced forced mitotic exit even when Aurora B became inhibited. This suggests that the flavonoid has additional target(s) whose inhibition leads to prolonged mitosis.

Eupatorin affects spindle formation, spindle integrity and centrosome separation

To understand why the cells exposed to eupatorin at G2 were delayed in mitosis we investigated if the flavonoid interferes with the spindle dynamics, structure and/or MT polymerization. First we treated cycling cell population with eupatorin for 2 h, long enough to force all mitotic cells to exit the M phase. Then we added MG132 to the culture medium to prevent further exit from M phase and continued the incubation in the presence of eupatorin for 1 h before fixation and immunostaining for α-tubulin and pericentrin (marker of centrosome region). The majority of cells that were exposed to eupatorin at late G2 exhibited multipolar spindle structure with several small satellite poles (73%, 22 cells out of 30, Fig. 5A) at M phase. A smaller fraction of the cells in the population had bipolar spindle with satellite poles (17%, 5 cells out of 30). Moreover, multiple pericentrin positive centrosomes were detected in the majority of eupatorin-treated cells (70%, 21 cells out of 30, Fig. 5A). As expected, control cells treated with MG132 had bipolar spindle with two pericentrin-positive centrosomes (90%, 27 cells out of 30, Fig. 5A). Together this indicates that exposure of late G2 cells to eupatorin causes defects in spindle formation.

To study the effect of eupatorin on spindle maintenance, we treated MG132 blocked metaphase cells with eupatorin for 2 h in the continued presence of MG132. In this condition, eupatorin induced multipolarity (41%, 13 cells out of 32, Fig. 5A) that was frequently accompanied with formation of small satellite poles. Rest of the cells in the population had bipolar spindle (59%, 19 cells out of 32) but with several satellite poles. However, despite of their multipolar appearance, the majority of eupatorin-treated cells had two pericentrin positive centrosomes (83%, 27 cells out of 32, Fig. 5A) proposing that eupatorin induces acentrosomal pole formation.

To examine if eupatorin can perturb spindle dynamics at M phase, we tested the cells’ ability to convert the spindle architecture from monopolar to bipolar structure. Cells were blocked in mitosis with monastrol which induces monopolar spindles due to Eg5 inhibition [24]. Thereafter the cells were released either into normal or eupatorin-containing culture medium both supplemented with MG132 to prevent anaphase onset. After the release, the cells were incubated for 1 h before fixation and immunostaining for α-tubulin and pericentrin. As expected, the majority of cells treated with monastrol for 4 h exhibited monopolar phenotype (93%, 28 cells out of 30, Fig. 5B). Release of the cells into normal culture medium allowed formation of bipolar spindles (22 cells out of 30) with two pericentrin-positive centrosomes (20 cells out of 30) in the majority of cells.

In contrast, the majority of cells released into eupatorin-containing medium remained monopolar with satellite poles (73%, 22 cells out of 30, Fig. 5B). Also cells that were bipolar had several satellite poles (17%, 5 cells out of 30). In the majority of eupatorin-treated cells multiple pericentrin positive centrosomes were observed (67%, 20 cells out of 30) and only 10% of cells recovered normally and exhibited two pericentrin positive centrosomes (Fig. 5B). Rest of the cells had only one centrosome (23%). In addition, the chromosome orientations were unorganized in eupatorin-treated cells. Interestingly, eupatorin does not induce formation of multiple centrosomes in the absence of Eg5 activity (Fig. S2). Cold calcium buffer treatment abolished all the satellite foci from these cells proposing that these MT foci did not contribute to formation of stable kinetochore-MT attachments and chromosome movement (Fig. S3). In conclusion, our data demonstrates that eupatorin interferes with reformation of bipolar spindle upon reactivation of Eg5 suggesting that the flavonoid has profound effects on spindle dynamics in mitosis.

To investigate whether eupatorin directly targets MTs, we performed an in vitro MT polymerization assay with 1, 5, 10, and 20 μM concentrations of eupatorin. The assay was conducted twice with similar results. In contrast to control drugs taxol and vinblastin which stabilize or destabilize MTs, respectively, eupatorin did not have any apparent effect on the MT polymerization (Fig. 5C) indicating that eupatorin affects spindle integrity indirectly.

Eupatorin induces polyploidy and apoptosis in several cell lines and suppresses tumorigenic property in a 3D prostate cancer cell model

To examine the fate of the eupatorin-treated cells we incubated several cell lines with DMSO or 50 μM eupatorin for 1 or 3 days,
Fig. 6 – Eupatorin treatment induces polyploidy and apoptosis, and suppresses formation of prostate cancer spheroids in an organotypic 3D cell culture model. A) FACS analysis of A549, DU145, HeLa, MCF-10A and PC3 cells treated with DMSO or eupatorin for 1 and 3 days. The cell populations were gated into apoptotic (sub-G1; Apo), G1 (2N), G2/M (4N) and polyploid (4N after first abnormal mitosis without cytokinesis, 8N, 16N) categories. B) Representative micrographs of the LNCaP prostate cancer cell spheroids formed during organotypic 3D cell culture in the presence of vehicle only (DMSO), eupatorin (Eup, 20 μM) or positive control drug taxol (Tx, 50 nM). Calcein AM was used for the visualization of the live cells. The scale bar equals 200 μm. The graphs show quantification of the measured average areas of LNCaP and 22RV1 prostate cancer cell spheroids in the presence of indicated drugs (mean±SEM, ***, P<0.001).
after which cells were harvested and analyzed using fluorescent-activated cell sorting (FACS). As expected, eupatorin caused severe polyploidy in A549, DU145 and PC3 cells, as indicated by the increase in 4N and 8N cell populations at both time points (Fig. 6A). Also a 16N cell population was observed in the PC3 cells (Fig. 6A). The 4N peak in the FACS profile of HeLa and MCF-10A cells after one day treatment with eupatorin was partly due to mitotic arrest as evaluated by microscopic analysis (data not shown). As a marker for apoptosis we used the percentage of cells in the sub-G1 peak appearing due to fragmentation of the genomic DNA during apoptosis. Apoptosis was verified in HeLa cells by blotting with an antibody against cleaved PARP (Fig. S4). Eupatorin elevated the frequency of apoptosis in all cell lines tested. The effect was most pronounced in HeLa cells whereas A549 and PC3 cells were less sensitive to eupatorin-induced apoptosis (Fig. 6A).

The cytotoxic and anti-proliferative properties of eupatorin were further analyzed using the organotypic 3D prostate cancer cell culture model [21]. The spheroid formation capacity of LNCaP and 22RV1 cells was tested in 3D matrigel cultures after treatment of cells with 20 μM of eupatorin for 7 days. The effects were studied by microscopy and the different morphological features of spheroids were analyzed using the Acca imaging software. As shown in Fig. 6B, eupatorin significantly decreased the area of LNCaP prostate cancer cell spheroids (decreased by 33%, P<0.001) and 22RV1 cell spheroids (decreased by 26%, P<0.001) indicating that the flavonoid suppresses the growth potency of prostate cancer cells in the organotypic culture model.

**Discussion**

Eupatorin is a natural compound found in several plants used for medical purposes [32]. It belongs to the group of flavones and is known to possess anti-inflammatory, anti-proliferative and cytotoxic properties in non-human cancer models and in human cell lines [32–35]. The anti-proliferative effects of eupatorin have been proposed to result from its hydroxylation by CYP1 family enzymes resulting in formation of bioactive eupatorin metabolites in a breast cancer cell line but not in normal breast cell line not expressing CYP1 [32]. The present study proposes a novel mechanism for eupatorin-induced anti-carcinogenic function. We show that unperturbed mitotic cells as well as cells arrested at M phase with drugs that reduce MT-mediated interkinetochore tension are rapidly forced out of mitosis without normal cytokinesis upon eupatorin treatment and as a consequence polyploid daughter cells are formed. The forced mitotic exit is dependent on proteasome activity, which suggests that the mitotic target of the flavonoid is involved in SAC signaling. Indeed, the activity of Aurora B, which is crucial for the maintenance of normal SAC function and proper execution of mitosis, was significantly reduced upon eupatorin treatment. In contrast, pre-mitotic cells exposed to the flavonoid exhibited defects in spindle architecture and centrosome function that resulted in a mitotic delay. This observation points to the possibility that the flavonoid has additional targets in the pre-mitotic phase of the cell cycle. This possibility may also hamper the use of eupatorin as a new research tool to explore mitotic processes. However, we demonstrate that in an organotypic 3D cancer cell culture model eupatorin acts as a growth inhibitor and in monolayer cell culture suppresses cellular viability in a cell-type independent manner, which points to anti-carcinogenic properties of the flavonoid.

The Aurora kinases are often overexpressed or amplified in many human tumors leading to chromosomal abnormalities [2,3,36]. For these reasons, Aurora kinases are considered potent targets for anti-cancer therapeutics. Several small molecules targeting Aurora kinases are currently undergoing phase I and II clinical studies [37,38]. Many of our observations reported here support the notion that eupatorin-induced override of the SAC and abrogation of cytokinesis involve inhibition of Aurora B. First, the effects of eupatorin on mitotic cells were found to mimic those caused by Aurora B inhibitors [6,7,39], i.e. the cells were rapidly forced out of taxol-induced mitotic arrest and the cytokinesis was perturbed. There is evidence that Aurora B is crucial to maintain SAC function in the absence of tension [40]. Inhibition of Aurora B with ZM447439 or hesperadin causes a rapid override of the SAC in the presence of taxol-stabilized MTs but not when MTs are depolymerized with nocodazole [5,6,39]. Similarly, eupatorin-treated cells remained arrested in mitosis when cells were exposed to high concentrations of nocodazole or vinblastine which activate SAC by abolishing kinetochore–MT attachments. Moreover, phosphorylation of the Aurora B target epitope Ser7 on CenpA [27] was significantly decreased by the flavonoid. Finally, the activity of Aurora B kinase in vitro was compromised by eupatorin indicating direct binding of the flavonoid to the kinase.

We observed that the cells exposed to eupatorin at G2 exhibited multipolarity, satellite poles and delayed mitosis. In addition, eupatorin induced formation of satellite poles also when added during recovery from monastrol-induced Eg5 inhibition. When the flavonoid was present at G2 or during Eg5 reactivation, cells formed multiple centrosomes. In sharp contrast, centrosomes were not affected in metaphase cells or in cells where Eg5 activity was constantly inhibited despite formation of satellite poles in those cells. Our data on spindle and centrosome effects of eupatorin indicate that eupatorin interferes with formation of bipolar spindle and maintenance of the mitotic spindle structure. Furthermore, the observations that a short exposure to the flavonoid induces abnormal centrosome number only if present before the centrosomes have separated and when Eg5 is active suggest that eupatorin affects centrosome separation but does not have major effects on centrosome integrity.

Interestingly, actinomycin D has been shown to induce displacement of Aurora B protein complex (CPC) from inner centromeres to chromosome arms, a phenotype that is accompanied with SAC override and likely is a consequence of intercalation of the compound into DNA [41]. As we observed a similar mislocalization of the CPC in response to eupatorin, we cannot exclude the possibility that eupatorin might intercalate into DNA or cause direct DNA damage.

Spindle defects and mitotic delay are phenotypes typically associated with the loss of Aurora A function [31]. This raises a question whether the flavonoid also targets another member of the Aurora kinase family. Based on our results this is indeed the case since Aurora A phosphorylated on Thr288, an autoactivation site of the kinase, was slightly down-regulated by eupatorin. Therefore, we hypothesize that the spindle-perturbing effect of the flavonoid is possibly due to inhibition of Aurora A kinase. We conclude that in mitotic cells eupatorin targets directly Aurora B kinase.
whose inhibition can mechanistically explain the observed forced mitotic exit and erroneous cytokinesis. Inhibition of Aurora A by eupatorin, on the other hand, may explain the observed spindle assembly defects. Inhibition of both Aurora kinases A and B is not unexpected, taken the high structural conservation of the catalytic site of Aurora kinases. These results do not exclude the possibility that in the premitotic cells the flavonoid has other targets whose inhibition withstands the loss of Aurora kinase function at M phase. Cell-based screening of large chemical libraries or selected kinase inhibitor sets for discovery of low molecular weight compounds that override mitotic arrest by inactivating the SAC has been successfully used earlier [22,42]. Interestingly, also these screens have identified compounds that inhibit the activity of Aurora kinases that strengthens a notion that Aurora B is the main druggable target within the SAC. From a methodological point of view, use of cell-based screening is advantageous as it ensures that the identified compounds are cell membrane permeable and taken up by the cells. However, identification of the target protein(s) of the hit compounds can be laborious and the possibility for existence of multiple cellular targets remains high.

At the moment the identity of potential other targets of eupatorin remains speculative. They could be components of the centrosome whose functional perturbation can indirectly explain the observed induction of multipolarity. It is known that the structure and function of centrosomes and spindle involves integrated action of various proteins such as MT motors and MT-associated proteins. Whether eupatorin can modulate these protein functions remains, however, to be resolved. A highly potential target for eupatorin is tubulin, the interference of which could explain most of the observed spindle defects. The mode-of-action of MT-targeting drugs currently in clinical use is based on suppression of normal MT dynamics which prevents execution of mitosis and ultimately activates cell death pathways [43]. Furthermore, flavonoids have been shown to perturb MT polymerization via tubulin binding [44–47]. Interestingly, CYP1 enzymes have been shown to create eupatorin metabolites with 3′,4′-dihydroxy groups that are proposed to have binding affinity to the colchine-binding site in tubulin [32]. It should be noted, however, that our data from the in vitro tubulin polymerization assay indicates that the flavonoid eupatorin itself does not directly influence MT polymerization. Whether eupatorin metabolites could directly bind tubulin remains to be investigated.

Evidence is accumulating that flavonoids affect the cell cycle by targeting various cellular regulatory pathways including mitogenetic signaling, cell cycle effector proteins such as CDKs and cyclins, and tumor suppressive pathways e.g. Rb proteins and p53 [48]. It has also been shown that flavonoids can interfere with tubulin polymerization and halt the normal progression of cell cycle in mitosis [49–52]. To our knowledge, the present results and our recently published data on the dietary flavonoid fisetin [13] are among the first studies that illustrate phenomena of flavonoid-induced forced mitotic exit and indicate that flavonoids can perturb the SAC signaling with Aurora B as the target. With respect to potential clinical use of polyphenols, it has been shown that they have very low oral bioavailability in human due to rapid metabolism via glucuronidation and sulfation pathways [53]. Interestingly, there is data indicating that methoxylated or polymethoxylated flavones are more resistant to metabolizing enzymes resulting in higher bioavailability and enhanced potential to function in the target tissue [53]. Evaluation of the oral bioavailability of eupatorin which belongs to the polymethoxylated flavones, awaits further studies. The clinical potency of eupatorin and other anti-mitotic flavonoids can be determined only after identification of the cellular mechanisms of actions and analysis of their long term biological effects.

In conclusion, our study reveals a novel anti-proliferative mechanism for eupatorin. Premature inactivation of the mitotic safety mechanism is caused by Aurora B kinase inhibition that also leads to polyploidy via impairment of normal cytokinesis. Further evidence supporting the anti-carcinogenic property of eupatorin is provided in the organotypic 3D model where eupatorin suppresses the tumorigenic property of prostate cancer cells.

Conflict of interest

The authors declare no conflicts of interest.

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