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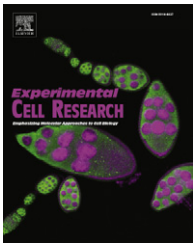

Research Article

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

Anna-Leena Salmela[a,b,c,1], *Jeroen Pouwels*[a,1], *Anu Kukkonen-Macchi*[a], *Sinikka Waris*[c], *Pauliina Toivonen*[c], *Kimmo Jaakkola*[a], *Jenni Mäki-Jouppila*[a,c,d], *Lila Kallio*[a,*], *Marko J. Kallio*[a,c,e]

[a]*VTT Technical Research Centre of Finland, Medical Biotechnology, P.O. Box 106, Turku, Finland*
[b]*Turku Graduate School of Biomedical Sciences, Turku, Finland*
[c]*Turku Centre for Biotechnology, P.O. Box 123, University of Turku, Finland*
[d]*Drug Discovery Graduate School, University of Turku, Finland*
[e]*Centre of Excellence for Translational Genome-Scale Biology, P.O. Box 106, Academy of Finland, Finland*

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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 pre-mitotic 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.

* *Corresponding author at:* VTT Technical Research Centre of Finland, Medical Biotechnology, Itäinen Pitkäkatu 4C, Pharmacity Bldg, 4th Floor, P.O. Box 106, FI-20521 Turku, Finland. Fax: +358 20 7222840.
E-mail address: lila.kallio@vtt.fi (L. Kallio).

Abbreviations: SAC, the spindle assembly checkpoint; M phase, mitotic phase; 3D cell culture, 3-dimensional cell culture; APC/C, anaphase-promoting complex/cyclosome; MT, microtubule; MAPK, mitogen-activated protein kinase; HTS, high-throughput screen; FACS, fluorescent-activated cell sorting; DMSO, dimethylsulfoxide; MBP, myelin basic protein; PARP, poly ADP-ribose polymerase; CYP1, Cytochrome P450 subfamily I; Rb, retinoblastoma protein.

[1] These authors contributed equally to the manuscript.

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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 the flavonoid eupatorin (3′,5-dihydroxy-4′,6,7-trimethoxyflavone) as an anti-mitotic compound. We show that eupatorin affects spindle integrity and increases polyploidy and apoptosis. We provide evidence that eupatorin forces mitotically arrested cells out of M phase *via* premature inactivation of the SAC by targeting the Aurora B kinase activity. The forced mitotic exit by eupatorin is dependent on proteasome activity and kinetochore–MT attachments. Interestingly, with respect to the flavonoids' clinical potential, the compound suppresses the tumorigenic properties of prostate cancer cells as demonstrated using an organotypic 3D prostate cancer cell model.

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, blasticidin (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% CO_2.

Chemicals

Eupatorin (3′,5-dihydroxy-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, staurosporine 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 63× (NA