# Anticancer effects of the flavonoid diosmetin on cell cycle progression and proliferation of MDA-MB 468 breast cancer cells due to CYP1 activation

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**Abstract.** Flavonoids constitute a large class of polyphenolic compounds with cancer preventative properties. We have examined the ability of the natural flavone diosmetin to inhibit proliferation of breast adenocarcinoma MDA-MB 468 and normal breast MCF-10A cells and found that this compound is selective for the cancer cells with slight toxicity in the normal breast cells. Diosmetin was metabolised to the structurally similar flavone luteolin in MDA-MB 468 cells, whereas no metabolism was seen in MCF-10A cells. Diosmetin caused $G_1$ arrest at 10 $\mu$M in MDA-MB 468 cells after 48-h treatment whereas this effect was not observed in MCF-10A cells. We suggest that diosmetin exerts cytostatic effects in MDA-MB 468 cells, due to CYP1A1 and CYP1B1 catalyzed conversion to the flavone luteolin.

## Introduction

Flavonoids are polyphenolic compounds present in plants with multiple modes of anticancer activity. Their chemopreventative properties have been explained, amongst others, by their ability to scavenge free radicals, induce apoptosis and inhibit cell signalling (1-3). A more detailed investigation of their versatile activity in cancer prevention has recently revealed a novel mechanism of action in cancer cell line models. Flavonoids such as eupatorin, daidzein and the stilbene resveratrol have been shown to be further activated to cytostatic and cytotoxic agents by the cytochrome P450 enzymes CYP1A1 and CYP1B1 (4-6). The differential expression of CYP1 enzymes in cancer cells has been proposed before as a target for cancer therapy (7). As a result flavonoids which are CYP1 substrates can in theory inhibit tumor growth, while sparing normal tissue.

Diosmetin is a flavone which is present in plants belonging to the genus *Teucrium* (Lamiaceae) and in Portuguese olive leafs (8,9). A recent study from our group has identified diosmetin as a CYP1 substrate (10). Metabolism of this compound in human breast adenocarcinoma MCF-7 cells, preinduced with the potent CYP1 inducer TCDD, caused enhanced inhibition of cell proliferation (10). In this pilot study the metabolism of diosmetin in human breast adenocarcinoma MDA-MB 468 cells is reported. The antiproliferative activity of diosmetin is further examined in MDA-MB 468 and normal breast MCF-10A cells, as well as the effect of the compound on cell cycle progression. The data suggest that diosmetin selectively inhibits the proliferation and cell cycle progression of MDA-MB 468 cells, as a result of CYP1 enzyme mediated metabolism.

## Materials and methods

*Materials*. Diosmetin was obtained from Indofine and luteolin from Lancaster (Heysham, Lancashire, UK). Salicylamide, propidium iodide, PBS, MTT, RNase were from Sigma (Poole, Dorset, UK) and HPLC grade acetonitrile and methanol from Fisher Scientific (Loughborough, UK). Media and cell culture reagents were from Sigma Chemical Co.

*Cell culture*. MDA-MB 468 cells were maintained in RPMI-1640 without phenol red, containing glutamine (2 mM) and 10% (v/v) heat-inactivated fetal calf serum. MCF-10A cells were maintained in DMEM at 37˚C, containing 2 mM glutamine, 10% (v/v) heat-inactivated fetal calf serum as above, EGF (20 ng/ml), insulin (10 $\mu$g/ml) and hydrocortisone (500 ng/ml). The cells were grown at 37˚C, 5% $CO_2$/95% air and passaged using Trypsin-EDTA.

*MTT assay*. MDA-MB 468 or MCF-10A cells were seeded at a density of $10^4$ cells/ml in 96-well flat-bottomed plates. After 24 h the medium was removed and diosmetin was added in quadruplicate in fresh medium at different concentrations and at a final concentration of not more than 0.1% (v/v) DMSO. The cells were allowed to grow for 96 h and the medium was removed. MTT (0.4 mg/ml) was added to each well for 3 h in

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*Abbreviations:* CDK, cyclin dependent kinase; CYP, cytochrome P450; DMBA, dimethylbenzanthracene; EGFR, epidermal growth factor receptor; EROD, 7-ethoxy resorufin-*O*-deethylase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5diphenyltetrazolium bromide; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin

*Key words:* antiproliferative activity, cell cycle arrest, CYP1 enzymes, diosmetin, luteolin

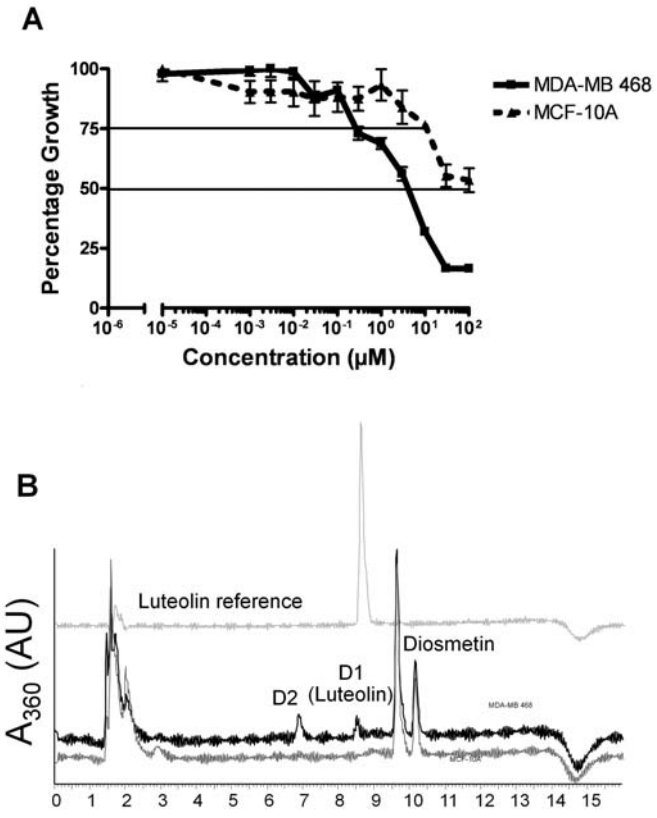


Figure 1. The bioactivation of diosmetin in MDA-MB 468 cells. (A), Cytotoxicity of diosmetin in MDA-MB 468 and MCF-10A cells. Cells were treated with different concentrations of compound for 96 h and cell viability was determined as described in Materials and methods. Error bars represent standard deviations for n=4 determinations. (B), Metabolic profile of diosmetin in MDA-MB 468 cells. MDA-MB 468 (grey) or MCF-10A (black) cells were incubated with diosmetin for 45 min and the samples analysed by HPLC. Luteolin standard was used as a reference for the identification of metabolite D1.

fresh medium. All medium was aspirated and the formazan product generated by viable cells was solubilised with DMSO (150 $\mu$l). Plates were shaken to dissolve MTT crystals and the absorbance at 540 nm determined using a Spectra Max M5/M5$^e$ microplate reader (Molecular Devices, Sunnyvale, USA). Results were expressed as the percentage of 100% (control) growth and the $IC_{50}$ and $IC_{25}$ calculated using Graphpad PRISM software. Dose range was in half log dilutions e.g. 100, 30, 10, 3, 1, 0.3, 0.1, 0.03, 0.01, 0.003, 0.001 $\mu$M.

*Metabolism of diosmetin in cells*. The experiment was performed as described previously (4).

*DNA analysis*. MCF-10A or MDA-MB 468 cells were passaged in Petri dishes at a density of $3x10^4$ cells/ml and left to grow for 24 h. The cells were then pretreated with diosmetin or 0.1% DMSO (negative control) for 48 h. The medium was removed and following trypsinisation cells were washed with cold PBS, fixed in 70% ethanol and stored at least 2 h in -20˚C. The cells were then resuspended in propidium iodide solution (70 $\mu$g/ml in PBS) containing 13 Kunitz units of RNase and incubated for 30 min at 37˚C. Fluoresence of propidium iodide was measured on a FACS Beckman Coulter (Beckman Coulter, Oakley Court, Buckinghamshire, UK) flow cytometer. Calibration and laser alignment were performed using fluorescent beads and a flow check protocol, before each sample was run with all PMTs producing a CV of less than 2.5 in the calibration. All the parameters required for the DNA analysis protocol, such as Voltage, Peak gain, Integrated gain and Discriminator were optimised in preliminary experiments. The data obtained from each histogram were analyzed using Multicycle Analysis 2.0 software.

## Results

In the present study the antiproliferative effects of diosmetin in MDA-MB 468 and MCF-10A cells were examined. Diosmetin was shown to strongly inhibit the proliferation of MDA-MB 468 cells ($IC_{25}$=0.4 $\mu$M, $IC_{50}\approx$4.5 $\mu$M) whereas it was considerably less active ($IC_{25}\approx$12 $\mu$M, $IC_{50}$=100 $\mu$M) in MCF-10A cells (Fig. 1A). Based on our previous report, a differential over-expression of CYP1A1 and CYP1B1

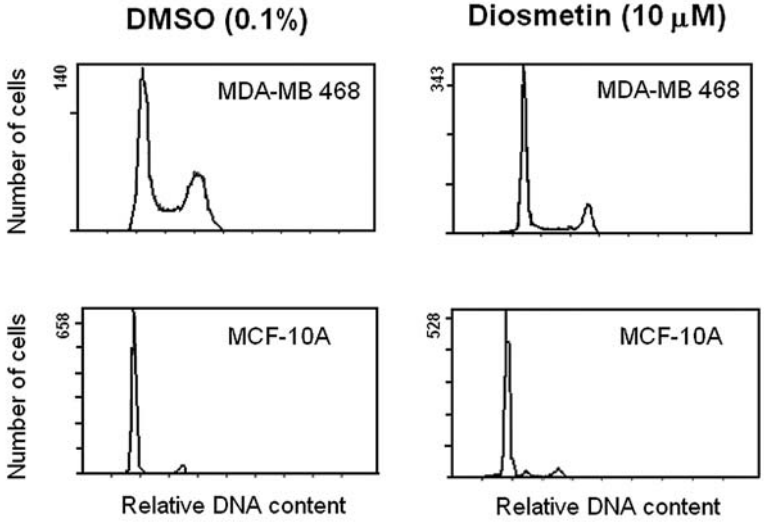


Figure 2. Cell cycle histograms of MDA-MB 468 and MCF-10A cells following 10 $\mu$M treatment of diosmetin for 48 h. The histograms are a representative trace of two independent experiments.

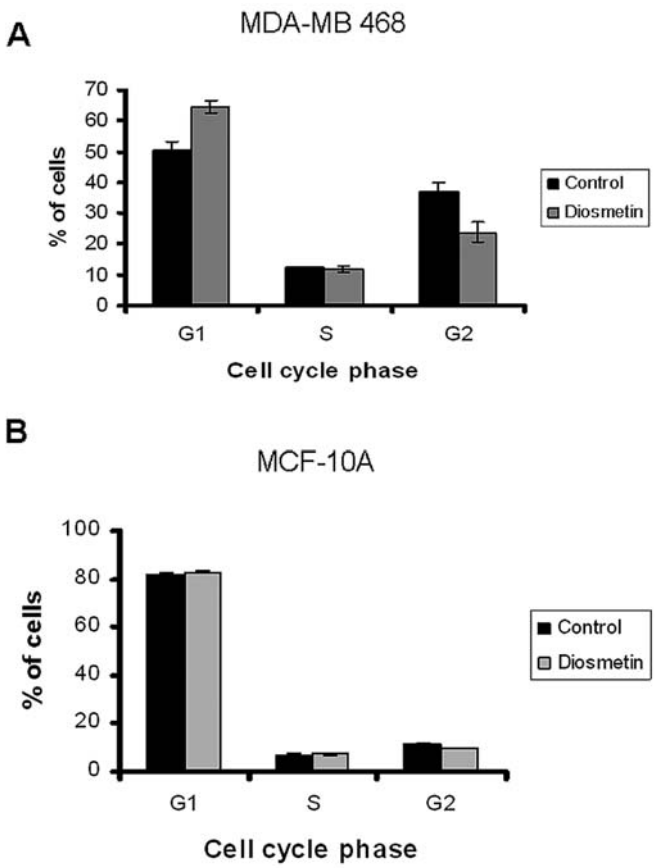


Figure 3. Percentage of cells in each phase of the cell cycle after treatment with diosmetin. MDA-MB 468 (A) or MCF-10A (B) cells were incubated with diosmetin (10 $\mu$M) for 48 h and analysed by flow cytometry as described in Materials and methods. Error bars represent min and max values for n=2 determinations.

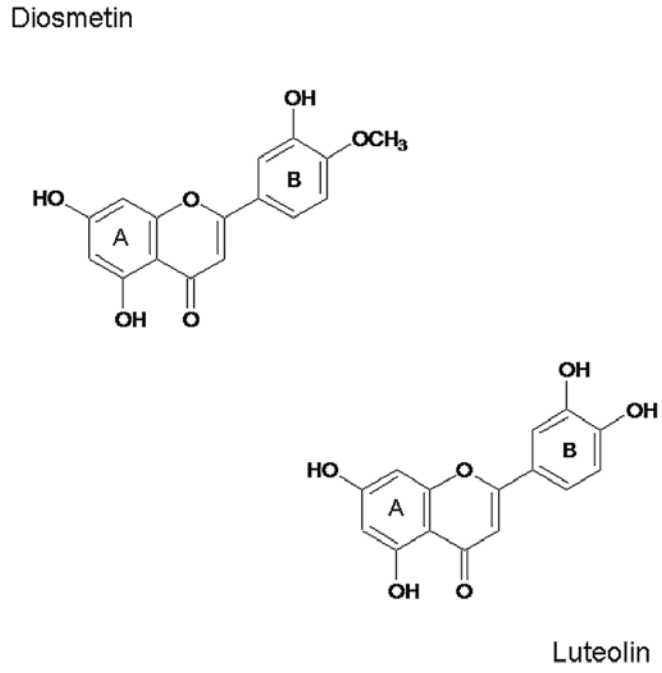


Figure 4. Chemical structures of the chemopreventative flavones diosmetin and luteolin.

enzymes in MDA-MB 468 cells has been demonstrated (4). Thus, the hypothesis that the activation of diosmetin in the latter cell line is due to metabolism of the compound to more active species by CYP1A1 and CYP1B1, was investigated. Diosmetin was indeed metabolised to two minor products assigned D1 and D2 eluting at approximately 8.7 and 7 min respectively, in MDA-MB 468 cells whereas no metabolism was seen in MCF-10A cells (Fig. 1B). Luteolin (3',4',5,7 tetrahydroxy-flavone) was identified as the metabolite D1 by comparison of UV spectrum and elution profile with a luteolin reference standard (Fig. 1B). The identity of the metabolite D2 was not found, because no authentic standard corresponding to its structure was available.

In the course of investigating further the antiproliferative mechanism of action of diosmetin in MDA-MB 468 and MCF-10A cells, the compound was subjected to DNA flow cytometric analysis. Diosmetin caused $G_1$ arrest in MDA-MB 468 cells, while no effect was seen in MCF-10A cells (Fig. 2). Specifically 48-h treatment with 10 $\mu$M diosmetin caused 13.5% of MDA-MB 468 cells to be arrested in $G_1$ phase of the cell cycle (Fig. 3).

## Discussion

The flavonoid diosmetin is a well known chemopreventative agent with multiple modes of action. It has been demonstrated to inhibit proliferation of SCC-9 human oral squamous carcinoma cells, block DMBA-induced DNA adduct formation and cytotoxicity in MCF-7 cells, as well as inhibit CYP1A1 and CYP1B1 EROD activity (11-13). According to our previous findings diosmetin was metabolized to luteolin and another unidentified metabolite by microsomes expressing human CYP1 enzymes (10). In addition luteolin was the main conversion product of the metabolism of diosmetin, in MCF-7 cells pretreated for 24 h with the potent CYP1 inducer TCDD (10). In agreement with these findings, the present study revealed that diosmetin is also metabolized to luteolin in MDA-MB 468 cell cultures. The metabolism enhanced the antiproliferative action of the compound, in a similar way as was previously noted in MCF-7 cells (10). A second unidentified metabolite was detected, which may have resulted from further hydroxylation of the A-ring (Fig. 4). Similar substitution patterns have been reported from CYP1B1 hydroxylation of the stilbene resveratrol (6).

Diosmetin was further shown to induce $G_1$ arrest of MDA-MB 468 cells. We suggest that this effect is mainly due to the metabolic product of diosmetin and luteolin. The effect of luteolin on the cell cycle progression of cancer cells has been examined previously. This compound was shown to inhibit cyclin dependent kinase CDK4 and CDK2, resulting in a $G_1$ arrest with a concominant decrease of phosphorylation of retinoblastoma protein in HT-29 human colon cancer cells, as well as inhibiting directly CDK2 in a cell free system (14). Luteolin also caused $G_1$ arrest in OCM-1 melanoma cells through direct inhibition of CDK2 and upregulation of $p27^{KIP1}$ and $p21^{CIP1}$ to a lesser extent (15). Furthermore, it was shown that luteolin possesses antiproliferative activity in human hepatoma PLC/PRF/5 cells via $G_0/G_1$ arrest and induction of apoptosis through caspase-3 and Bax activation (16). In addition luteolin was shown to inhibit the autophosphorylation

of the EGFR (17). MDA-MB 468 cells express EGFR as described previously (18). $G_1$ arrest of the cell cycle would be expected from an EGFR inhibitor, since the progression of $G_1$ to S phase is mainly dependent on mitogenic signals and the transcription of the cyclin D1 gene is promoted through the Ras/Raf/ERK pathway. Indeed treatment of DU145, PC3 and LNCap prostate cancer cells with the potent EGFR inhibitor Iressa™ caused $G_1$ arrest (19). In CAL33 head and neck cancer cells treatment of Iressa also caused a $G_1$ block (20). This supportive evidence provides more insight into the chemopreventative action of diosmetin in MDA-MB 468 cells. The dual function of luteolin as an EGFR and CDK inhibitor is mainly responsible for the $G_1$ arrest observed when diosmetin was incubated with MDA-MB 468 cells. However, the contribution of the metabolite D2 must not be neglected. Importantly, a $G_1$ block was not observed in MCF-10A cells, which indicates that the activation of diosmetin to luteolin is selective for the breast cancer cells.

In summary, the results presented in this study provide more insight into the chemopreventative mechanism of action of the natural flavone diosmetin. The latter is shown to inhibit proliferation of human breast cancer cells and cause a selective block in the $G_1$ phase of the cell cycle, as a result of cytochrome P450 CYP1 bioactivation, while this effect is not observed in the cell cycle of normal breast cells. Since the conversion product of the metabolism of diosmetin, mainly responsible for the cytostastic action, is the structurally related flavone luteolin, a well known chemopreventative agent, the findings provide additional evidence on a possible tumor suppressing role of the CYP1 family enzymes.

## Acknowledgements

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