Tangeretin inhibits extracellular-signal-regulated kinase (ERK) phosphorylation

Séverine Van Slambrouck[a], Virinder S. Parmar[b], Sunil K. Sharma[b], Bart De Bondt[a], Fleur Foré[a], Peter Coopman[c], Barbara W. Vanhoecke[d], Tom Boterberg[a], Herman T. Depypere[d], Guy Leclercq[e], Marc E. Bracke[a,*]

[a] *Laboratory of Experimental Cancerology, Department of Radiotherapy and Nuclear Medicine, Ghent University Hospital, B-9000 Ghent, Belgium*
[b] *Bioorganic Laboratory, Department of Chemistry, University of Delhi, Delhi, India*
[c] *CNRS UMR 5539, University of Montpellier II, 34095 Montpellier, France*
[d] *Department of Gynaecological Oncology, Ghent University Hospital, B-9000 Ghent, Belgium*
[e] *Laboratory J-C Heuson of Mammary Cancerology, Bordet Institute, B-1000 Brussels, Belgium*

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Abstract Tangeretin is a methoxyflavone from citrus fruits, which inhibits growth of human mammary cancer cells and cytolysis by natural killer cells. Attempting to unravel the flavonoid's action mechanism, we found that it inhibited extracellular-signal-regulated kinases 1/2 (ERK1/2) phosphorylation in a dose- and time-dependent way. In human T47D mammary cancer cells this inhibition was optimally observed after priming with estradiol. The spectrum of the intracellular signalling kinase inhibition was narrow and comparison of structural congeners showed that inhibition of ERK phosphorylation was not unique for tangeretin. Our data add tangeretin to the list of small kinase inhibitors with a restricted intracellular inhibition profile.

Keywords: Tangeretin; Methoxyflavone; Citrus; Extracellular-signal-regulated kinases 1/2; Mammary cancer

1. Introduction

Tangeretin (4′,5,6,7,8-pentamethoxyflavone), a natural compound from citrus fruits, was found to inhibit a number of cell activities such as growth of mammary cancer cells in vitro [1] and cytolysis by natural killer (NK) cells in vivo [2]. For both activities a pivotal role of the mitogen-activated protein kinase (MAPK)/extracellular-signal-regulated kinase (ERK) pathway has been reported [3,4]. A variety of peptide growth factors and steroid hormones are able to activate ERK via upstream activation of cytoplasmic kinases, including Ras, Src, Raf, and MAPK kinase (MKK) [5]. The synthetic methoxyflavone PD98059 was identified as an inhibitor of MKK [6] and hence of MAPK-driven cell proliferation and NK-cell activity [7]. Inspired by its structure similarity with PD 98059 and its ability to suppress cell proliferation and NK-activity as well, we investigated the effect of tangeretin on the MAPK signalling cascade. The model we used consisted of human T47D breast cancer cells stimulated with estradiol [8] and treated with tangeretin. Human breast cancer cell lines exhibit elevated activation levels of ERK after estradiol treatment, and this activation, mediated by plasma membrane-bound estrogen receptors, involves transactivation of ErbB tyrosine kinase receptors [9]. The dual tyrosine/threonine phosphorylation status of ERK was selected as an activation marker for this signalling pathway.

2. Materials and methods

2.1. Cell culture

T47D cells are derived from a pleural effusion of a human mammary ductal carcinoma. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Merelbeke, Belgium), supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml amphotericin B, and 10% heat-inactivated fetal bovine serum (FBS) (Greiner Bio-one, Wemmel, Belgium) at 37 °C in an atmosphere of 10% CO_2 in air.

2.2. Chemicals and antibodies

Tangeretin was kindly provided by Dr. Bill Widmer (Department of Citrus, Winter Haven, FL), and dissolved in ethanol as a stock solution of 10^{-2} M. 17-β-Estradiol (E2) was from Sigma (St. Louis, MO, USA), PD 98059 from Calbiochem (La Jolla, CA, USA), and recombinant heregulin-β1 (rHRG-β1) from R&D Systems (Abingdon, UK). The primary antibody for Western blotting was a rabbit polyclonal anti-phospho-p42/44 MAPK (Thr202/Tyr204) from Cell Signalling Technology (Beverly, MA, USA). The secondary anti-rabbit antibody was from Amersham Pharmacia Biotech (Amsterdam, The Netherlands). The monoclonal mouse-anti-Syk antibody (clone 4D10) was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA), while the polyclonal rabbit-anti-Src CST.1 antibody was kindly provided by Dr. S. Roche (CNRS FRE2593, Montpellier, France). RU 58668 from Roussel Uclaf (Romainville, France), 4-hydroxytamoxifen (4-OH-TAM) from Zeneca Pharmaceuticals (Macclesfield, UK) and [^{3}H]estradiol (±95 Ci/mmol) from Amersham Pharmacia Biotech were used for estrogen receptor α-mediated luciferase induction and ligand binding assays.

2.3. Western blotting

All lysates were made from cells at approximately 70% confluency. For phosphorylation experiments, cells were washed three times with phosphate-buffered saline (PBS), serum-starved overnight, washed again three times with PBS and treated in serum-free medium. Before making lysates, the cells were washed again three times with PBS. Cells were lysed with PBS containing 1% Triton X-100, 1% Nonidet P-40 and the following protease inhibitors: aprotinin (10 μg/ml), leupeptin (10 μg/ml), phenylmethylsulfonyl fluoride (1.72 mM), NaF (100 M),

*Corresponding author. Fax: +32 9 240 4991.
E-mail address: brackemarc@hotmail.com (M.E. Bracke).

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$NaVO_3$ (500 μM), and $Na_4P_2O_7$ (500 μg/ml). After incubation at 4 °C for 5 min, the samples were sonicated for 15 s. After clearing the lysates by centrifugation at 14 000 rpm for 10 min, protein concentration was determined using the Rc Dc Protein Assay (Bio-Rad, Hercules, CA, USA) and samples were prepared in such a way that equal amounts of protein were loaded.

Sample buffer [10] with 5% 2-mercaptoethanol and 0.012% bromophenol blue was added, followed by boiling for 5 min and separation of proteins by gel electrophoresis on a 10% polyacrylamide gel and transfer onto a nitrocellulose membrane (Amersham Pharmacia Biotech). Quenching and immunostaining of the blots was done in 4% bovine serum albumin in PBS containing 0.2% Tween-20. The membranes were quenched for 1 h, incubated with primary antibody for 1 h, washed four times for 10 min, incubated with horseradish peroxidase-conjugated secondary antibody for 45 min, and washed six times for 10 min. Detection was done using Enhanced Chemiluminescence reagent (Amersham Pharmacia Biotech) as a substrate. Tubulin and total p42/p44 immunostaining was used as a loading control, and variations were less than 5%. After scanning the autoradiogram, bands quantification (the sum of p42 and p44) was performed using the Quantity One® software (Bio-Rad). Experimental results were confirmed by obtaining concordant data from experiments performed at least in duplo.

2.4. Phosphorylation status analysis of cytoplasmic signalling proteins

Kinetworks™ technology (Kinexus, Vancouver BC, Canada) permits the quantitative detection of the phosphorylation status of cytoplasmic proteins. The Kinetworks™ KPSS 1.3 Phosphoprotein Screen was performed as described by Pelech et al. [11] (http://www.kinexus.ca). The screen required 350 μg of protein, and for some kinases results obtained with different antibodies are presented.

For the measurement in vitro of Syk and Src tyrosine kinases, Simian Cos1 cells were transiently transfected with the pCAF1-Syk [12] or the pSG5-Src cDNA (kindly provided by Dr. S. Roche) using the SuperFect reagent (Qiagen Inc, Venlo, The Netherlands). Two days after transfection, cells were lysed and cleared lysates were used for immunoprecipitation with protein A-agarose beads linked to antibodies against Syk or Src for 3 h at 4 °C under constant rotation. Then the beads were washed in lysis buffer and used for Western blot or autophosphorylation testing. For in vitro kinase assays, the beads were re-suspended in 50 μL reaction buffer (10 mM Tris–HCl, pH 8, 10 mM $MgCl_2$, and 10 mM $MnCl_2$). Serial dilutions from a 10^{-2} M tangeretin stock solution or piceatannol, a specific inhibitor of Syk kinase activity (Calbiochem), were made in reaction buffer and added to the beads together with 10 μCi [γ-^{32}P] ATP (4500 Ci/mmol, ICN Biomedicals) and agitated for 20 min at room temperature. After washing in reaction buffer, beads were boiled in Laemmli sample buffer, loaded on 10% SDS–PAGE gels, dried, exposed and autophosphorylated Syk or Src kinase levels were detected and quantified using phosphor-image analysis (Molecular Dynamics, Sunnyvale, CA).

2.5. Assay for growth of T47D cells in vitro

T47D cells were seeded in microtiter plates at an initial density of 1.5×10^4 cells in 200-μl culture medium. After an incubation period of 4 days, the amount of cell protein in each well was estimated with the sulforhodamine B assay [13].

2.6. Luciferase induction assay

MVLN cells are human MCF-7 mammary carcinoma cells, stably transfected with the pVit-tk-LUC reporter plasmid. These cells allow quantification of the transcriptional activity from estrogen receptor α-mediated estrogen responsive elements (ERE), in accordance with the luciferase induction assay by Pons et al. [14]. They were cultured in 35 mm diameter plastic petri dishes (80 000 cells/dish) in DMEM containing 10% dextran coated charcoal-treated FBS for 3–4 days. 250 μl of 5-fold diluted lysis solution (Promega E153A, Promega, Madison, WI, USA) was then added to the dishes and maintained under mild agitation for 20 min to extract luciferase. Lysed cells were subsequently detached with a scraper and centrifuged for 5 s at $10\,000 \times g$ to clarify the extracts. Finally, 20 μl of extracts was mixed at room temperature with 100 μl of a luciferase reactant medium (Promega E151A/E152A) prepared in accordance with the manufacturer's protocol and induced light was measured with a luminometer.

2.7. Statistics

Statistical evaluation of the data was performed with the Student's *t* test.

3. Results

3.1. Dose- and time-dependent effect of tangeretin on the phosphorylation status of ERK

Western blots were performed to determine whether tangeretin could inhibit the E2-induced ERK phosphorylation. T47D cells were treated with 10^{-9} M E2 for 20 min, in the absence or presence of varying concentrations of tangeretin (10^{-7} to 10^{-4} M), and assayed for ERK phosphorylation by immunostaining with an anti-phospho-MAPK antibody that detects the dual phosphorylation state (Thr202/Tyr204) of ERK 1/2 (p42 and p44). No activation was observed in solvent (ethanol 0.1%) treated cells as compared to untreated cells, but addition of E2 induced the phosphorylation of ERK 1/2. Tangeretin was able to inhibit this E2-induced phosphorylation in a dose-dependent manner (Fig. 1A).

To determine the kinetics, T47D cells were treated with E2 (10^{-9} M) and tangeretin (10^{-4} M), and cell lysates were prepared after various incubation periods (Fig. 1B). ERK phosphorylation inhibition by tangeretin was evident after 10–30 min.

Since E2 can stimulate the MAPK pathway via ErbB receptors, direct stimulation by rHRG-β1, a ligand of ErbB3 and ErbB4 [15], was applied as an alternative method to phosphorylate ERK [16]. To confirm the responsiveness of T47D cells to rHRG-β1, different concentrations (0.1–10 ng/ml) were initially tested and 1 ng/ml for 10 min proved to be an optimal stimulus. Tangeretin effectively reduced this rHRG-β1 stimulated ERK phosphorylation to 24% at a concentration of 10^{-4} M (Fig. 1C).

3.2. Effect of tangeretin on growth T47D mammary cancer cells

Tangeretin inhibited the growth of estradiol-stimulated human T47D cells in the sulforhodamine B assay. The effect was dose-dependent and could be demonstrated at concentrations from 10^{-4} down to 10^{-7} M (Fig. 2).

3.3. Phosphorylation screening of other signalling molecules

To further investigate the specificity of the kinase inhibition by tangeretin, a screening of the phosphorylation status of different cytoplasmic proteins (KPSS 1.3, Kinexus) was performed. Three samples pretreated with 10^{-9} M E2 for 20 min were analysed: one was solvent-treated, a second one was treated with 10^{-4} M tangeretin and the third one with 2.5×10^{-5} M PD98059 (Table 1). The results showed that tangeretin is indeed able to inhibit phophorylation of ERK 1/2 and of MAPK kinases. In contrast to PD98059, tangeretin is also able to inhibit phosphorylation of other proteins like adducin α and γ, protein kinase Cδ, signal transducer and activator of transcription (STAT) 1 and 3, and stress-activated protein kinase (JNK). The phosphorylation of other proteins such as JUN, protein kinase Bα (Akt1), and retinoblastoma 1, however, were increased. Overall the spectrum of inhibition by tangeretin differed from the one by PD98059.

Quercetin and epigallocatechin-3-gallate were recently found to down regulate the activity of the Syk kinase and