The red wine phenolics piceatannol and myricetin act as agonists for estrogen receptor α in human breast cancer cells

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Abstract

Previous epidemiological reports have suggested that red wine intake is associated with beneficial health effects due to the ability of certain phytochemical components to exert estrogen-like activity. It has been also documented that estrogens induce the proliferation of hormone-dependent breast cancer cells by binding to and transactivating estrogen receptor (ER) α , which in turn interacts with responsive DNA sequences located within the promoter region of target genes. In order to provide further insight into the positive association between wine consumption and the incidence of breast carcinoma in postmenopausal women, we have evaluated the estrogenic properties of two abundant wine-derived compounds, named piceatannol (PIC) and myricetin (MYR), using as model systems the hormone-sensitive MCF7 and the endocrine-independent SKBR3 breast cancer cells. On the basis of our experimental evidence PIC and MYR may contribute to the estrogenicity of red wine since: (1) they transactivate endogenous ER α ; (2) they activate the agonist-dependent activation function (AF) 2 of ER α and ER β in the context of the Gal4 chimeric proteins; (3) they rapidly induce the nuclear immunodetection of ER α ; (4) they regulate the expression of diverse estrogen target genes; (5) they compete with 17 β -estradiol for binding to ER α and ER β ; and – as a biological counterpart of the aforementioned abilities – (6) they exert stimulatory effects on the proliferation of MCF7 cells. Hence, the estrogenic activity of PIC and MYR might be considered at least as a potential factor in the association of red wine intake and breast tumors, particularly in postmenopausal women.

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Introduction

Piceatannol (PIC; 3,5,3',4'-tetrahydroxy-trans-stilbene) and myricetin (MYR; 3,3',4',5,5',7-hexahydroxyflavone) (Fig. 1) are phenolic compounds that occur naturally in grapes and red wine (MacDonald *et al.* 1998, Teugo *et al.* 1998). The total amount of PIC and MYR in red-grape wines has been reported to be up to 15 mg/l (MacDonald *et al.* 1998, Burns *et al.* 2000, Vuorinen *et al.* 2000, Cantos *et al.* 2003), however the biotransformation of the abundant red wine component named resveratrol contributes to increase PIC concentrations at tissue level (Piver *et al.* 2004). Previous studies have demonstrated that PIC is a natural tyrosine kinase inhibitor (Geahlen & McLaughlin 1989) exerting repressive effects in leukemia, melanomas and colorectal cancer cell lines (Wieder *et al.* 2001, Wolter *et al.* 2002, Larrosa *et al.* 2004), while MYR has been identified as a potent topoisomerase II repressor similar in activity to the epipodophyllotoxin widely used in cancer therapy (Markovits *et al.* 1989, Azuma *et al.* 1995, Chang *et al.* 1995). Several epidemiological investigations have

suggested that the consumption of red wine is associated with a variety of health benefits due to the estrogenic activity of certain bioactive components (Stampfer *et al.* 1988, Frankel *et al.* 1993, Bertelli *et al.* 1995, Goldberg *et al.* 1995, Gronbaek *et al.* 1995, Pace-Asciak *et al.* 1995). It has been largely reported that 17 β -estradiol (E2) mainly acts by binding to and transactivating estrogen receptor (ER) α and ER β , however different transduction pathways involved in the estrogen signaling have recently received a great deal of attention (Katzenellenbogen *et al.* 2000, Safe 2001, Maggiolini *et al.* 2004, Simoncini *et al.* 2004). A large body of evidence has demonstrated that E2 and E2-like compounds elicit stimulatory effects on breast cancer, the leading cause of tumor deaths among women in Western countries (Hoskins & Weber 1994, Eisen & Weber 1998, Lopez-Otin & Diamandis 1998, Maggiolini *et al.* 2001). Therefore, much effort has been addressed to ascertain the ability of natural compounds to interact with ER α and to trigger breast tumor progression. In this regard, it should be taken into account that wine consumption even in moderate amounts is directly associated with the

17 β -estradiol

Piceatannol

Myricetin

Figure 1 Chemical structures of 17 β -estradiol, PIC and MYC.

occurrence of breast carcinoma particularly in post-menopausal women (van den Brandt *et al.* 1995, Feigelson *et al.* 2001). Notably, in a multicentre Italian case-control study an increased risk of breast cancer was attributed to wine ingestion compared with other types of alcoholic beverages, explaining up to 12% of breast tumor cases in Italy (Ferraroni *et al.* 1998). In the present study, we have used as model systems the hormone-sensitive MCF7 and endocrine-independent SKBR3 breast cancer cells in order to provide new insight into the molecular mechanisms by which the wine-derived PIC and MYR behave as estrogen-like compounds. In a concentration range physiologically achievable with a moderate wine consumption, both phytochemicals elicited growth stimulatory effects in MCF7 cells as a biological counterpart of their agonistic activity for ER α .

Materials and methods

Reagents

E2, PIC, MYR and cycloheximide (Cx) were purchased from Sigma, while the ER antagonist ICI 182,780 (ICI) was obtained from Tocris Chemicals (Bristol, UK). Hydroxyflutamide (OHF) and ZK 98299 were a gift from Schering (Berlin, Germany). All compounds were solubilized in dimethylsulfoxide, except E2 which was dissolved in ethanol.

Plasmids

Firefly luciferase reporter plasmids used were XETL (Bunone *et al.* 1996) and TFF1/pS2-ERE (Berry *et al.* 1989) for the ERs and GK1 (Webb *et al.* 1998) for the Gal4 fusion proteins. The reporter plasmid XETL carries firefly luciferase sequences under the control of an estrogen response element (ERE) upstream of the thymidine kinase promoter. TFF1/pS2-ERE (a gift from V. Giguère, McGill University, Quebec City, Canada) contains the ~1050 bp TFF1/pS2 promoter preceding the luciferase reporter of pGL3 (Berry *et al.* 1989). As an internal transfection control, we co-transfected the plasmid pRL-CMV (Promega) that expresses renilla luciferase enzymatically distinguishable from firefly luciferase by the strong cytomegalovirus enhancer/promoter. Gal4 chimeras Gal-ER α , Gal-ER(V), Gal-ER(R), Gal-ER(L), Gal-ER(543/4A), Gal-ER(Δ F) and Gal-ER β were expressed from plasmids GAL93.ER(G), GAL93.ER(V), GAL93.ER(R), GAL93.ER(L), GAL93.ER(ML543/4AA), GAL93.ER(Δ F) and GAL93.ER β respectively. They were constructed by transferring the coding sequences for the hormone-binding domain (HBD) of ER α (amino acids 282–595) from HEG0 (Bunone *et al.* 1996), pCMVh ERG400 V (Aumais *et al.* 1997), pCMVhERG521R (Ekena *et al.* 1996), pCMVhERL525A (Ekena *et al.* 1996), a PCR-

mutagenized intermediate with the point mutations M543A-L544A, a PCR fragment lacking the coding sequences for the F domain, and for the ER β HBD (C-terminal 287 amino acids) from plasmid pCMV5-hERbeta (a gift from J.-Å. Gustafsson, Karolinska Institute, Huddinge, Sweden) respectively, into the mammalian expression vector pSCTEVGal93 (Siepel *et al.* 1992).

Cell culture

Wild-type ER α -positive MCF7 human breast cancer cells were a gift from E. Surmacz (Sbarro Institute for Cancer Research and Molecular Medicine, Philadelphia, USA), the ER-negative SKBR3 human breast cancer cells and the ER α -containing human uterine Ishikawa cells were a gift from D. Picard (Department of cell biology, University of Genève, Switzerland). MCF7 and Ishikawa cells were maintained respectively in Dulbecco's modified Eagle's medium (DMEM) or DMEM/F12 both without phenol red supplemented with 10% fetal bovine serum (FBS) (Invitrogen); SKBR3 cells were maintained in RPMI 1640 without phenol red supplemented with 10% FBS (Invitrogen).

Transfections and luciferase assays

A total of 10^5 MCF7, SKBR3 and Ishikawa cells were transferred into 24-well plates with 500 μ l of regular growth medium/well the day before transfection. Medium lacking phenol red as well as serum was used on the day of transfection, which was performed by Eugene6 reagent (Roche) with a mixture containing 0.5 μ g reporter plasmid, 5 ng pRL-CMV and 0.1 μ g effector plasmid where applicable. After 6 h, treatments were added and cells were incubated for a further 18–20 h. Luciferase activity was then measured with the Dual Luciferase Reporter Assay System (Promega) according to the manufacturer's recommendations. Firefly luciferase activity was normalized to the internal transfection control provided by renilla luciferase activity. Luciferase activity of cells receiving vehicle was set as 1-fold induction, upon which the results of treatments were calculated.

RT-PCR

MCF7 cells were grown in 10 cm dishes to about 60% confluence in regular growth medium and then were switched to medium lacking phenol red as well as serum for 24 h. Thereafter, treatments were added for 24 h and mRNA expression was evaluated by semi-quantitative RT-PCR as we have previously described (Maggiolini *et al.* 1999). The primers for ER α , estrogen-inducible trefoil factor TFF1/pS2 (Brown *et al.* 1984), cathepsin-D (Cavaillès *et al.* 1991), estrogen-responsive RING finger

protein (EFP) (Ikeda *et al.* 2000) as well as for the internal control acidic ribosomal phosphoprotein 36B4 (Laborda 1991) were: 5'AATTCAGATAATCGACGCCAG3' (ER α forward) and 5'GTGTTTCAACATTCTCCCT CCTC3' (ER α reverse);

5'TTCTATCCTAATACCATCGACG3' (pS2 forward) and

5'TTTGAGTAGTCAAAGTCAGAGC3' (pS2 reverse); 5'AACAACAGGGTGGGCTTC3' (cathepsin-D forward) and 5'ATGCACGAAACAGACTGTGC3' (cathepsin-D reverse);

5'CGTGTGGATTTGTGTGTGGACG3' (EFP forward) and 5'CCCCGAGGTGGAAGTGAACC3' (EFP reverse);

5'CTCAACATCTCCCCCTTCTC3' (36B4 forward) and

5'CAAATCCCATATCCTCGTCC3' (36B4 reverse); these primers yielded products of 345, 210, 303, 162 and 400 bp with 20, 15, 20, 20 and 15 PCR cycles respectively.

Western blotting

MCF7 cells were grown in 10 cm dishes to about 60% confluence in regular growth medium and then were switched to medium lacking phenol red as well as serum for 24 h. Thereafter, treatments were added for 24 h before lysis. In the case of Cx, cells were pretreated for 3 h prior to the addition of ligands. Cells were lysed with 300 μ l of 50 mM HEPES (pH 7.5), 150 mM NaCl, 1.5 mM MgCl₂, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 1% SDS, and a mixture of protease inhibitors containing 1 mM aprotinin, 20 mM phenylmethylsulfonyl fluoride and 0.2 M sodium orthovanadate. Equal amounts of whole protein extract were resolved on a 10% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane (Amersham), probed overnight at 4 °C with the antibodies F-10 against ER α and β -actin (both from Santa Cruz Biotechnology, CA, USA), and then revealed using the ECL System (Amersham).

Immunocytochemical staining

MCF7 cells were maintained in medium lacking phenol red as well as serum for 3 days, treated for 1 h and then fixed in fresh paraformaldehyde (PFA; 2% for 30 min). After PFA removal, hydrogen peroxide (3% in methanol for 30 min) was used to inhibit endogenous peroxidase activity. Cells were then incubated with normal horse serum (10% for 30 min) to block the non-specific binding sites. Immunocytochemical staining was performed using as the primary antibody a mouse monoclonal immunoglobulin (Ig) G generated against the human C-terminus of ER α (F-10, Santa Cruz Biotechnology; 1:50 overnight at 4 °C). A biotinylated horse-anti-mouse IgG (1:600 for 60 min at room

temperature) was applied as the secondary antibody (Vector Laboratories, Burlingame, CA, USA). Subsequently, the amplification of avidin-biotin-horseradish peroxidase complex (ABC complex/HRP; Vector Laboratories; 1:100 for 30 min at room temperature) was carried out and 3,3'-diaminobenzidine tetrachloride dihydrate (Vector Laboratories) was used as a detection system. Cells were rinsed after each step with Tris-buffered saline (0.05 M Tris-HCl plus 0.15 M NaCl, pH 7.6) containing 0.05% Triton-X100 (TBS-T). In control experiments cells were processed replacing the primary antibody with mouse serum (Dako S.p.A., Milan, Italy) or using a primary antibody pre-absorbed (48 h at 4 °C) with an excess of purified ER α protein (PanVera Corp., Madison, WI, USA).

Ligand binding assay for ERs

The ability of PIC and MYR to compete with [3 H]E2 for binding to ER α and ER β was evaluated and compared with that of E2. Two picomoles of purified recombinant human ER α and ER β proteins (PanVera Corp., Madison, WI, USA), each in 20 mM HEPES, pH 7.4, 1.5 mM EDTA, 0.5 mM dithiothreitol and 10% (v/v) glycerol, were incubated with 1 nM [2,4,6,7- 3 H]E2 (72 Ci/mmol; Amersham) in the presence of serial dilutions of unlabeled E2, PIC or MYR for 20–22 h at 4 °C. Bound and free radioligands were separated on Sephadex G-25 PD-10 columns. The amount of receptor-bound [3 H]E2 was determined by liquid scintillation counting (OptiPhase, HiSafe 3 and 1409; Wallac, Inc., Gaithersburg, MD, USA). Relative counts per minute were plotted against the concentrations of the ligand, and data were evaluated with the use of a non linear, four-parameter logistic model to estimate the median effective concentration (EC $_{50}$) value.

Proliferation assays

For quantitative proliferation assays 1×10^4 MCF7 cells were seeded in 24-well plates in regular growth medium. Cells were washed extensively once they had attached and were incubated in medium without serum for 24 h. On the second day, the medium was changed and supplemented with 5% charcoal-stripped (CS)-FBS. Ligands were added at this point, thereafter the 5% CS-FBS medium was renewed every day together with treatments. On day 6, cells were trypsinized and counted in a haemocytometer using the trypan blue exclusion method.

Statistical analysis

Statistical analysis was performed using ANOVA followed by Newman-Keuls testing to determine differences in means. Data are expressed as means $\pm$ S.D. *P* values <0.05 were considered significant.

Results

Transactivation of the endogenous ER α

We first aimed to evaluate whether a transiently transfected ER reporter gene is able to respond to PIC and MYR. The reporter plasmid XETL carries firefly luciferase sequences under the control of an ERE upstream of the thymidine kinase promoter. As an internal transfection control, we co-transfected a plasmid that expresses renilla luciferase, which is enzymatically distinguishable from firefly luciferase, from the strong cytomegalovirus enhancer/promoter. Using MCF7 cells, which express ER α but no ER β as judged by RT-PCR (data not shown), PIC and MYR were able to induce a substantial XETL expression from the concentration of 10 nM (Fig. 2A). The ER antagonist ICI abolished the activation by E2 and both compounds (Fig. 3A), whereas the antiandrogen OHF, the antiprogesterone and antiglucocorticoid ZK 98299 had no effect (data not shown), suggesting that PIC and MYR transactivate ER α directly. Next, using the natural pS2 ERE-containing promoter (Berry *et al.* 1989) we confirmed that both compounds were able to induce the ER α -mediated transcriptional activity (Fig. 2B), which was no longer noticeable in the presence of ICI (Fig. 3B). For comparison, we performed the same transfection experiments in uterine Ishikawa cancer cells showing that PIC and MYR activated the endogenous ER α (Fig. 2C) in a direct manner since ICI inhibited the transcriptional response also in this cellular context (Fig. 3C).

Activation of ER α and ER β in a heterologous system

To provide mechanistic evidence on the ability of PIC and MYR to transactivate ER α and to examine the response of ER β , we turned to a heterologous system using SKBR3 cells lacking ERs. Chimeric proteins consisting of the DNA-binding domain (DBD) of the yeast transcription factor Gal4 and the ER α or the ER β HBDs containing the ligand-dependent activation function (AF) 2 were both activated by PIC and MYR although with a different potency compared with E2 (Fig. 4A). Hence, the HBD of each ER isoform was sufficient to allow the functional response, while the Gal4-DBD fused to the AF1 domain of ER α did not elicit transcriptional effects upon all treatments (Fig. 4B). Next, we examined the response of diverse ER α HBD mutants in the context of Gal4 proteins. The two-point mutations L525A and G521R that require a high E2 concentration for a substantial transactivation (Ekena *et al.* 1996) elicited very low activity upon PIC and MYR (Fig. 4B and C). The AF2 mutant M543/L544A failed to respond to all treatments (Fig. 4C), while the point mutation G400 V and the mutant lacking the F-domain at the C-terminal HBD core exhibited a strong response

to E2, PIC and MYR (Fig. 4D). Taken together, our results argue that mutations of ER α in the hormone binding pocket are able to alter the transcriptional activity induced by E2 as well as that of the compounds tested.

Nuclear immunolocalization of ER α

Upon ligand binding, ER α undergoes conformational changes allowing the formation of receptor dimers and the interaction with specific EREs contained within the regulatory region of target genes (Tsai & O'Malley 1994, Beato *et al.* 1996, Pratt & Toft 1997). Performing an immunocytochemical assay we aimed to evaluate the ability of short PIC and MYR treatments (1 h) to elicit a nuclear localization of the endogenous ER α in MCF7 cells. No detectable ER α immunoreactivity was observed in basal experimental conditions obtained by maintaining cells in medium lacking phenol red as well as serum for 3 days (Fig. 5). Only in the nuclear compartment did E2, PIC and MYR produce strong staining signals and these were abrogated in the presence of the ER antagonist ICI 182 780 (Fig. 5). No signals were observed after replacing the anti-ER α antibody by irrelevant rabbit IgG or by using the primary antibody pre-absorbed with an excess of receptor protein (data not shown). These findings further supported the functional activity elicited by compounds tested in transfection experiments.

Down-regulation of ER α mRNA and protein levels

Levels of ER α in breast cancer cells are autoregulated by E2 through different mechanisms which include a reduced transcription rate of the ER α gene together with an increased turnover of the ligand-activated protein (Santagati *et al.* 1997). Hence, to evaluate whether PIC and MYR are also able to modulate the mRNA of ER α in MCF7 cells we performed a semiquantitative RT-PCR standardizing its expression by the housekeeping gene 36B4. Interestingly, both compounds induced a receptor down-regulation (Fig. 6A and B) which was observed even at the protein level depending on new synthesis (Fig. 6C and D) (El Khissiini & Leclercq 1999, Nonclercq *et al.* 2004).

Figure 2 PIC and MYR activate the endogenous ER α . MCF7 breast cancer cells were transfected with the luciferase reporter plasmid XETL(A) or TFF1/pS2 (B) and treated with increasing concentrations of E2, PIC and MYR. (C) Uterine Ishikawa cancer cells were transfected as described for panel A. Luciferase activities were standardized to the internal transfection control and expressed as the ratio of induced activity to the activity in the absence of ligand. Each data point represents the mean of triplicate samples of three separate experiments.

Up-regulation of estrogen target genes

As aforementioned, the E2/ER α -activated complex regulates the expression of genes that contain ERE sequences within the promoter regions (Truss & Beato 1993). Following the same experimental procedure described above, we documented the ability of PIC and MYR to up-regulate, like E₂ the mRNA expression of target genes – such as pS2/TIFF1, cathepsin D and EFP E2, (Fig. 6A and B).

Competition for ER binding

The results obtained strongly suggested that PIC and MYR are ERs for ligands. This issue was examined directly by a competition binding experiment with purified recombinant human ER α and ER β proteins. Our data demonstrated that both compounds compete with the radiolabeled E2 tracer for binding to the ERs in a concentration-dependent manner, however they are poor binders particularly for ER α in comparison to ER β (Fig. 7). The latter phenomenon is in agreement with a previous report on ER isoform-specific preferences in binding to environmental estrogens (Kuiper *et al.* 1997).

Proliferative effects on hormone-sensitive breast cancer cells

Having clearly demonstrated that PIC and MYR are functional activators of ER α , we evaluated in a time course study the proliferative response of MCF7 cells to increasing concentrations of both ligands. Cells were lysed on the indicated days, counted in a hemocytometer, and results obtained upon treatments were compared with those of untreated cells. Notably, both compounds were able to induce progressive proliferative effects (Fig. 8A) which were no longer noticeable in presence of ICI (Fig. 8B), suggesting that even the growth stimulation is mediated by an ER α -dependent mechanism.

Discussion

Our findings provide new insights into the activity exerted by the wine-derived phenolics PIC and MYR which can be considered ER agonists on the basis of their ability: (1) to activate endogenous ER α in hormone-dependent MCF7 and Ishikawa breast and uterine cancer cells respectively; (2) to activate the ligand-dependent AF2 domain of ER α and ER β in the

Figure 3 The ER antagonist ICI inhibits the ER α transactivation by PIC and MYR. Cells were transfected with XETL (A and C) or with TFF1/pS2 ERE (B) and treated as indicated. Each data point represents the mean of triplicate samples of three separate experiments. ●, ■, ▲, $P < 0.05$.

Figure 4 PIC and MYR are ER α and ER β agonists in a heterologous system. Dose-response to E2, PIC and MYR of fusion proteins consisting of the Gal4-DBD and the ER α or ER β HBD (A), the Gal4-DBD fused to the ER α AF1 domain and the Gal4-ER α HBD mutant G521R (B), the Gal4-ER α HBD mutants M543A-L544A and L525A (C), the Gal4-ER α HBD mutant G400 V and the Gal4-DBD fused to the ER α HBD lacking the F-domain (D) (see Materials and methods section for other details). Each data point represents the mean of triplicate samples of three separate experiments.

context of chimeric proteins with the Gal4 DBD in ER-negative SKBR3 breast cancer cells; (3) to elicit responses similar to E2 using different expression vectors for ER α mutated in the AF2 domain; (4) to rapidly induce a nuclear immunodetection of ER α ; (5) to regulate ER α mRNA and protein levels as well as the expression of estrogen target genes such as TFF1/pS2, cathepsin D and EFP; (6) to compete with E2 for binding to purified ER α and ER β proteins; (7) to stimulate the proliferation of MCF7 cells at concentrations that may be physiologically achievable through a moderate wine intake.

In recent years, the biological properties of natural estrogen-like compounds have attracted increasing interest for the potential beneficial effects on human health (Cotton 1994, Safe 1995, Lopez-Otin & Diamandis 1998). In this regard, a reduced incidence and mortality from a variety of diseases has been associated with moderate wine consumption (Fuhrman *et al.* 1995, Goldberg *et al.* 1995, Whitehead *et al.* 1995, German & Walzem 2000, Gronbaek *et al.* 2000), which is considered a relevant source of estrogenic compounds like the well-characterized resveratrol (Kopp 1998). In order to ascertain the mechanisms involved in the

Figure 6 PIC and MYR regulate the expression of estrogen target genes. (A) Semiquantitative RT-PCR of ER α , TFF1/pS2, cathepsin D and EFP mRNA. MCF7 cells were stimulated for 24 h with 1 μ M E2, PIC and MYR; 36B4 mRNA levels served as a control. (B) Quantitative representation of data of two independent experiments including that of panel A after densitometry and correction for 36B4 expression. (C) Immunoblot of ER α from MCF7 cells treated for 24 h with 1 μ M E2, PIC and MYR and 50 μ M Cx where applicable. (D) Quantitative representation of data of two independent experiments including that of panel C after densitometry. β -Actin served as a loading control. $\circ$, $\square$, $\bullet$, $\blacksquare$, $P < 0.05$.

potential protective role exerted by resveratrol, many studies have been performed demonstrating its ability to regulate lipid metabolism and platelet function as well as to trigger apoptotic events in different cancer cells (Bagchi *et al.* 2001, Bianchini & Vainio 2003, Pervaiz 2003). However, other investigations evaluating the potential estrogen-like properties of resveratrol have documented an agonistic activity for ER α including the ability to stimulate the proliferation of breast tumor cells (Gehm *et al.* 1997, Basly *et al.* 2000, Nakagawa *et al.* 2001, Levenson *et al.* 2003, Gehm *et al.* 2004). These findings are reminiscent of those reported for other abundant dietary flavonoids (Hsieh *et al.* 1998, Maggiolini *et al.* 2001 and references therein), and may suggest that the biological actions of resveratrol are either mediated by ER α or involve ER-independent mechanisms mainly in relationship to the cellular

environment and levels of exposure (Gehm *et al.* 1997, Mgbonyebi *et al.* 1998, Ashby *et al.* 1999, Lu & Serrero 1999, Bowers *et al.* 2000, Damianaki *et al.* 2000, Bhat *et al.* 2001). Hence, phytoestrogens and other natural compounds are potentially responsible for diverse pharmacological effects, which depend on bioavailability as well as metabolic clearance rates influencing consequently ER agonism/antagonism and even anti-cancer activity. In this respect, it is worth noting that resveratrol can undergo hydroxylation to yield PIC via different cytochrome P450 enzymes like CYP1B1 which has been found overexpressed in a wide variety of human tumors (Potter *et al.* 2002, Piver *et al.* 2004). Taking into account that PIC also derives directly from wine intake, we performed a panel of *in vitro* assays designed to better define its estrogen-like properties together with those of MYR, an additional phytochemical

Figure 5 Nuclear immunodetection of ER α . MCF7 cells were serum deprived for 3 days and then treated for 1 h with vehicle (–), 1 μ M E2, PIC, MYR and 1 μ M ER antagonist ICI where applicable. Each panel shown is representative of at least ten experiments. Scale bars, 5 μ m.

Figure 7 PIC and MYR compete with [3 H]E2 for ER α and ER β binding. Competitive binding of increasing concentrations of PIC and MYR to purified recombinant human ER α (A) and ER β (B) protein. Each data point represents the mean of triplicate samples of three separate experiments.

occurring abundantly in red wine. At a low concentration range physiologically achievable through wine consumption, both compounds stimulated the growth of hormone-sensitive breast tumor cells as ER α activators. Moreover, the results obtained in transfection experiments with Gal4 chimeras suggested that PIC and MYR

Figure 8 PIC and MYR stimulate the proliferation of breast cancer cells. (A) Time- and dose-response study to PIC and MYR in MCF7 cells. Data obtained in cells after treatments were compared with those of vehicle-treated cells set as 100%. (B) MCF7 cells were treated as indicated, lysed and counted on day 6. Each data point represents the mean of triplicate samples of three separate experiments. □, $P < 0.05$.

are AF2 agonists alongside other selective estrogen receptor modulators (SERMs) grouped as class I estrogens, according to the molecular classification previously proposed (Jordan *et al.* 2001). The ability of ligands to modulate intracellular levels of ER α is important for ER α pharmacology. The human ER α is a substrate for ubiquitination in the presence of E2 and the exposure to different ligands has been shown to lower ER α levels by a proteasome-mediated proteolysis (Nawaz *et al.* 1999). The down-regulation of ER α following treatments with PIC and MYR in MCF7 cells further supported their agonistic activity observed also through a ligand-dependent receptor degradation process. Moreover, despite a low binding affinity for ER α both compounds up-regulated the expression profiles of diverse estrogen target genes providing a reliable assessment of the transcriptional efficacy exhibited in the context of natural DNA response elements.

A large body of evidence has suggested that ER α -mediated estrogen signaling plays a major role in the etiology and progression of breast carcinoma (Ciocca & Fanelli 1997, Lopez-Otin & Diamandis 1998). Consequently, ER α antagonists have been extensively utilized as adjuvant endocrine treatment to prevent such malignancy and/or its recurrence (Early Breast Cancer Trialists Collaborative Group 1998, Cosman & Lindsay 1999). However, about half of ER α -positive tumors fail to respond to antiestrogen therapy from the beginning and most of the remaining ones eventually acquire pharmacological resistance. In this context, it should be noted that E2 and diverse phytoestrogens irrespective of ER expression trigger rapid genomic and non-genomic events (Brownson *et al.* 2002, Maggiolini *et al.* 2004) which, at least in part, contribute to the failure of antiestrogen treatment as well as to breast tumor progression. On the basis of the aforementioned observations and the present findings, the estrogenic properties of phytochemicals studied might be considered as a risk factor in postmenopausal women who habitually consume red wine (van den Brandt *et al.* 1995, Ferraroni *et al.* 1998, Feigelson *et al.* 2001, Poschl & Seitz 2004). However, taking into account that PIC and MYR exert inhibitory effects in different tumor cells (Markovits *et al.* 1989, Azuma *et al.* 1995, Chang *et al.* 1995, Wieder *et al.* 2001, Wolter *et al.* 2002, Larrosa *et al.* 2004), their concentrations as well as the cellular context may direct various biological responses as has been previously reported for resveratrol and other phytoestrogens (Gehm *et al.* 1997, Hsieh *et al.* 1998, Basly *et al.* 2000, Maggiolini *et al.* 2001, Nakagawa *et al.* 2001, Bianchini & Vainio 2003, Pervaiz 2003). Herein, we have provided mechanistic insight into the estrogenic potential of PIC and MYR that may be involved in the beneficial as well as the adverse effects associated with wine consumption. In this vein, further investigations are needed to

elucidate the level of absorption and exposure to these phytochemicals, and the dose-dependent properties as well as the activity exerted *in vivo* in estrogen target tissues such as breast, uterus and bone.

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