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Inhibition of α, βI, δ, η and ζ Protein Kinase C Isoforms by Xanthonolignoids

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The effect of the xanthonolignoids *trans*-(±)-kielcorin C, *cis*-(±)-kielcorin C, *trans*-(±)-kielcorin D, *trans*-(±)-isokielcorin D and *trans*-(±)-kielcorin E on isoforms α, βI, δ, η and ζ of protein kinase C (PKC) was studied using the yeast phenotypic assay.

All the compounds tested revealed an effect compatible with PKC inhibition, similar to that exhibited by the well established PKC inhibitor chelerythrine, and with differences in their potency towards the distinct isoforms tested, being, in general, potent inhibitors of the atypical PKC isoform (PKC-ζ). PKC inhibition caused by these kielcorins was confirmed using an *in vitro* kinase assay.

The present study constitutes the first attempt to unravel the molecular mechanism of kielcorins activity, and shows that xanthonolignoids are a promising group of compounds to investigate for isoform selective PKC inhibitors.

Keywords: Protein kinase C; PKC isoforms; Yeast phenotypic assay; PKC inhibitors; Xanthonolignoids; Kielcorins

INTRODUCTION

The ubiquitous enzyme protein kinase C (PKC) is a family of serine-threonine kinases with pivotal roles in cellular regulation, signal transduction and neoplastic promotion.[1,2] PKC isoforms are grouped into at least three groups: the classical PKCs (cPKCs), which include the isoforms α, βI, βII and γ; the novel PKCs (nPKCs), which include the isoforms δ, ε, θ, and η; and the atypical PKCs (aPKCs), which include the isoforms ζ and λ/ι.[2] More recently, a fourth group of structurally distinct PKCs has been identified, the so-called PKC-related kinases (PRKs).[2] A new isoform, PKCμ/PKD, has also been reported, but its inclusion in the PKC family remains controversial.[2]

Clarification of the role of individual PKC isoforms in the cell has been made difficult by the lack of selective PKC activators and inhibitors,[3] which has been hampered by methodological limitations, caused by the coexistence of several PKC isoforms. These limitations can be circumvented by using the yeast phenotypic assay,[4,5] which uses yeast expressing an individual mammalian PKC isoform. The assay is based on the growth inhibition of transformed yeast (reflecting an increase in the cell doubling time), which is proportional to the degree of PKC activation.[6,7] Because only one mammalian PKC isoform is functionally expressed, separately, and effects non-mediated by that isoform can be easily detected by measuring growth in the absence of expression, it represents a convenient method to screen potential PKC activators and inhibitors and to elucidate their isoform selectivity. In the yeast phenotypic assay, PKC activators cause a concentration-dependent growth inhibition of transformed yeast,[6,7] while PKC inhibitors block that growth inhibition.[8,9] Therefore, the potency of PKC activators can be estimated by the concentration that cause half of the maximal growth inhibition attainable by PKC activators (EC_{50}) and the potency of PKC inhibitors by the increase in the EC_{50} value caused by the PKC inhibitor [EC_{50} ratio = EC_{50}(PKC activator + PKC inhibitor)/EC_{50} (PKC activator)].

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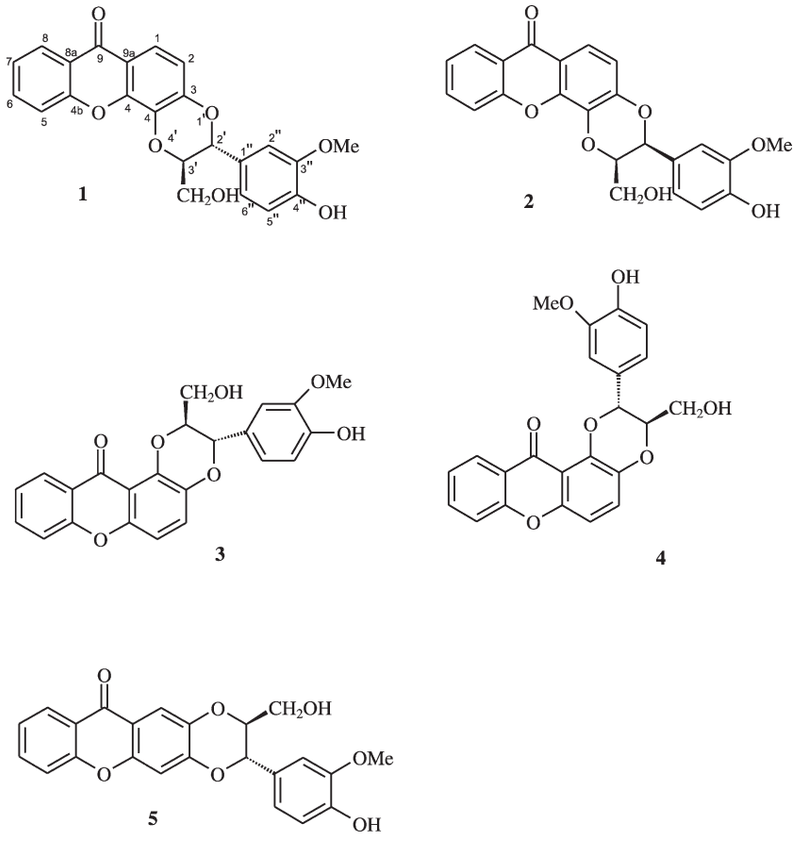

FIGURE 1 Chemical structures of *trans*-(±)-kielcorin C (**1**), *cis*-(±)-kielcorin C (**2**), *trans*-(±)-kielcorin D (**3**), *trans*-(±)-isokielcorin D (**4**) and *trans*-(±)-kielcorin E (**5**).

Xanthone derivatives have been reported to mediate several important biological activities.[10,11,12] Recent studies suggested that these compounds may act, at least in part, by interacting with PKC, causing effects compatible with either PKC activation[4] or PKC inhibition.[5]

Kielcorins are 1,4-benzodioxane derivatives belonging to the xanthonolignoids family, with a skeleton in which a phenylpropane unit is linked to a xanthone nucleus through a dioxane bridge.[10,13,14] Recent studies showed that kielcorins have an hepatoprotective effect[15] and an antiproliferative effect against three human tumor cell lines: MCF-7 (breast), TK-10 (renal) and UACC-62 (melanoma).[13,14] However, to our knowledge, no data is available about the molecular basis of the biological activity of kielcorins. Previously, the biomimetic synthesis of natural kielcorin and kielcorin B was achieved.[16,17] In order to obtain new compounds belonging to this family, synthesis of *trans*-(±)-kielcorin C (**1**), *cis*-(±)-kielcorin C (**2**), *trans*-(±)-kielcorin D (**3**), *trans*-(±)-isokielcorin D (**4**) and *trans*-(±)-kielcorin E (**5**) (Figure 1) was undertaken.[13] The synthetic approach of these constitutional isomers was based on the biomimetic pathway, by oxidative coupling of coniferyl alcohol (**6**) with an appropriate xanthone (**7**)[13] (Figure 2).

In the present study, the effects of *trans*-(±)-kielcorin C (**1**), *cis*-(±)-kielcorin C (**2**), *trans*-(±)-kielcorin D (**3**), *trans*-(±)-isokielcorin D (**4**) and *trans*-(±)-kielcorin E (**5**) on PKC isoforms α and βI (cPKCs), δ and η (nPKCs) and ζ (aPKCs) were

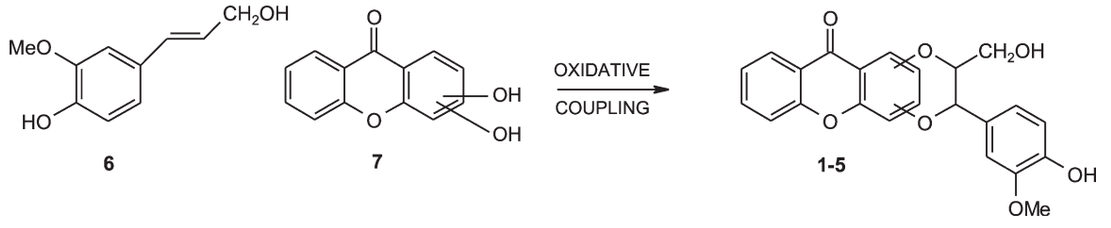

FIGURE 2 General procedure for the biomimetic synthesis of isomeric kielcorins **1**–**5**.

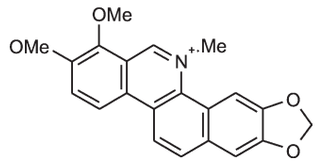

FIGURE 3 Chemical structure of chelerythrine.

characterized in our laboratory using the yeast phenotypic assay. The inhibition of PKC by these five isomeric kielcorin derivatives is reported and their effects and structures were compared to those of the PKC inhibitor chelerythrine[18] (Figure 3). Differences in their potency towards the individual PKC isoforms tested were also detected.

This work represents the first molecular approach for the study of the biological effects of kielcorins and reveals that they act as PKC inhibitors.

MATERIALS AND METHODS

Synthesis

Purification of compounds was performed by column chromatography, using Merck sílica gel 60 (0.50–0.20 mm), flash chromatography [Merck silica gel 60 (0.040–0.063 mm)] and preparative thin layer chromatography (tlc), employing Merck sílica gel 60 (GF_{254}). M.p. were determined on a *Köfler microscope* and are uncorrected, IR Spectra on a *Perkin Elmer 257* spectrophotometer with KBr microplates, UV Spectra on a *Varian CARY 1E* spectrophotometer, ^{1}H and ^{13}C-NMR Spectra on a *Bruker AC 200*, *DRX 300*, or *DRX 500* spectrometer at r.t., in $CDCl_3$ or (D_6)DMSO with δ in ppm relative to $SiMe_4$ as an internal reference and coupling constants *J* in Hz. ^{1}H-NMR assignments were made using 2D COSY experiments, while ^{13}C-NMR assignments were made using one-dimensional selective INEPT and 2D HETCOR, HSQC and HMBC experiments (long range C/H coupling constants were optimised to 7 and 1 Hz). NOE experiments were determined by means of NOE difference technique, using an irradiation time of 2 s and a relaxation delay of 4 s. EI-MS Spectra: *Hitachi Perkin-Elmer RMU-6M* spectrometer, in *m/z*. HR-MS Spectra: *VG AutoSpec-Q* spectromer; recorded as EI (electronic impact) mode.

General Procedure for the Synthesis of Kielcorin Derivatives

The appropriate dihydroxyxanthone (**7**, 615 mg, 2.7 mmol) and coniferyl alcohol (**6**, 500 mg, 2.7 mmol) in a mixture of 1:1 toluene:acetone were stirred in the presence of silver oxide (860.6 mg, 3.1 mmol) in the dark for 2–3 days. The suspension was filtered, the filtrate evaporated, and the crude product purified by column chromatography using mixtures of hexane:chloroform (20:80 for compounds **1**, **2**, and **5**) and chloroform:methanol (98:2 for compounds **3** and **4**). The isolation of the components **1**–**2**, **3**–**4** and **5** was then carried out by preparative tlc ($CHCl_3$:MeOH 95:5).

trans-(±)-Kielcorin C (1)

M.p. 243–247° (Me_2CO). UV (EtOH): 309, 283, 239, 204 nm. IR (KBr): 3398, 1642, 1605, 1562, 1449, 1338, 1264, 1025, 861 cm^{-1}. ^{1}H-NMR (300.13 MHz, (D_6)DMSO) δ: 9.25 (*s*, *H*O-C(4″)), 8.20 (*dd*, *J* = 7.7, and 1.6, H-C(8)), 7.87 (*ddd*, *J* = 8.3, 7.4, and 1.6, H-C(6)), 7.70 (*d*, *J* = 8.8, H-C(1)), 7.69 (*d*, *J* = 8.3, H-C(5)), 7.49 (*ddd*, *J* = 7.7, 7.4, and 0.7, H-C(7)), 7.07 (*d*, *J* = 8.8, H-C(2)), 7.07 (*d*, *J* = 1.7, H-C(2″)), 6.92 (*dd*, *J* = 8.1 and 1.7, H-C(6″)), 6.83 (*d*, *J* = 8.1, H-C(5″)), 5.15 (*d*, *J* = 7.9, H-C(2′)), 5.13 (*t*, *J* = 6.1, CH_2OH), 4.36–4.40 (*m*, H-C(3′)), 3.79 (*s*, MeO-C(3″)), 3.71–3.76 (*m*, CH_2OH), 3.40–3.50 (*m*, CH_2OH). ^{13}C-NMR (75.47 MHz, (D_6)DMSO): 175.1 (C(9)), 155.4 (C(4b)), 148.8 (C(3)), 147.7 (C(3″)), 147.2 (C(4″)), 145.9 (C(4a)), 135.2 (C(6)), 131.8 (C(4)), 126.7 (C(1″)), 126.0 (C(8)), 124.5 (C(7)), 121.0 (C(8a)), 120.7 (C(6″)), 118.1 (C(5)), 117.5 (C(1)), 115.7 (C(9a)), 115.4 (C(5″)), 113.9 (C(2)), 111.8 (C(2″)), 78.0 (C(3′)), 76.5 (C(2′)), 59.9 (CH_2OH), 55.7 (*Me*O-C(3″)). EI-MS: 406 (6, M^+), 356 (26), 341 (22), 327 (17), 306 (18), 292 (12), 281 (100), 261 (38), 253 (5), 228 (13), 215 (8), 180 (10), 165 (8), 141 (10), 115 (18), 105 (9), 91 (17), 77 (18), 63 (10), 55 (13). HRMS for $C_{23}H_{19}O_7$: Calc. 407.1131; Found: 407.1130.

cis-(±)-Kielcorin C (2)

M.p. 200–202° (Me_2CO). UV (EtOH): 308, 283, 239, 204 nm. IR (KBr): 3420, 1644, 1607, 1511, 1454, 1379, 1267, 1100, 860 cm^{-1}. ^{1}H-NMR (300.13 MHz, (D_6)DMSO) δ: 9.09 (*s*, *H*O-C(4″)), 8.21 (*dd*, *J* = 7.6 and 1.6, H-C(8)), 7.83 (*ddd*, *J* = 8.0, 7.5, and 1.6, H-C(6)), 7.75 (*d*, *J* = 8.7, H-C(1)), 7.67 (*d*, *J* = 8.0, H-C(5)), 7.46 (*dd*, *J* = 7.6 and 7.5, H-C(7)), 7.05 (*d*, *J* = 8.8, H-C(2)), 6.99 (br. *s*, H-C(2″)), 6.95–6.74 (*m*, H-C(5″) and H-C(6″)), 5.46 (*d*, *J* = 2.8, H-C(2′)), 4.98 (*t*, *J* = 5.4, CH_2OH), 4.63–4.68 (*m*, H-C(3′)), 3.83 (*s*, MeO-C(3″)), 3.42–3.61 (*m*, CH_2OH). ^{13}C-NMR (75.47 MHz, (D_6)DMSO): 175.5 (C(9)), 155.9 (C(4b)), 148.7 (C(3)), 147.8 (C(3″)), 147.1 (C(4″)), 146.7 (C(4a)), 135.2 (C(6)), 131.1 (C(4)), 126.7 (C(1″)), 126.4 (C(8)), 124.6 (C(7)), 121.5 (C(8a)), 119.5 (C(6″)), 118.4 (C(5)), 118.2 (C(1)), 116.4 (C(9a)), 115.9 (C(5″)), 114.1 (C(2)), 111.4 (C(2″)), 77.8 (C(3′)), 76.4 (C(2′)), 58.6 (CH_2OH), 56.0 (*Me*O-C(3″)). EI-MS: 406 (19, M^+), 281 (1.8), 246 (24), 184 (14), 153 (100), 149 (8), 137 (17), 111 (1.3), 93 (60), 83 (22), 72 (8), 65 (38), 59 (14), 57 (41). HRMS for $C_{23}H_{19}O_7$: Calc. 407.1131; Found: 407.1131.

***trans*-(±)-Kielcorin D (3)**

M.p. 258–260° (Me_2CO). UV (EtOH): 372, 278, 259, 239, 237, 212 nm. IR (KBr): 3498, 1635, 1602, 1309, 1270, 1228, 1047, 762 cm^{-1}. ^{1}H-NMR (300.13 MHz, (D_6)DMSO) δ: 9.20 (*s*, *H*O-C(4″)), 8.04 (*dd*, $J = 7.9$ and 1.6, H-C(8)), 7.80 (*ddd*, $J = 8.1$, 7.8, and 1.6, H-C(6)), 7.56 (*d*, $J = 8.1$, H-C(5)), 7.45 (*d*, $J = 9.1$, H-C(3)), 7.40 (*dd*, $J = 7.9$ and 7.8, H-C(7)), 7.14 (*d*, $J = 9.1$, H-C(4)), 7.10 (*d*, $J = 1.7$, H-C(2″)), 6.92 (*dd*, $J = 8.1$ and 1.7, H-C(6″)), 6.83 (*d*, $J = 8.1$, H-C(5″)), 5.05 (*d*, $J = 7.6$, H-C(3′)), 5.05 (*t*, $J = 7.6$, CH_2OH), 4.20–4.23 (*m*, H-C(2′)), 3.78 (*s*, MeO-C(3″)), 3.57–3.62 (*m*, CH_2OH), 3.41 (under H_2O signal, CH_2OH). ^{13}C-NMR (75.47 MHz, (D_6)DMSO): 174.9 (C(9)), 154.6 (C(4b)), 150.9 (C(4a)), 147.6 (C(3″)), 147.0 (C(4″)), 143.1 (C(1)), 138.9 (C(2)), 134.8 (C(6)), 127.1 (C(1″)), 125.9 (C(8)), 124.0 (C(7)), 123.7 (C(3)), 121.7 (C(8a)), 120.5 (C(6″)), 117.6 (C(5)), 115.3 (C(5″)), 111.92 and 111.86 (C(9)a and C(2″)), 109.5 (C(4)), 77.2 (C(2′)), 76.1 (C(3′)), 60.0 (CH_2OH), 55.7 (*Me*O-C(3″)). EI-MS: 406 (6, M^+), 369 (2), 327 (7), 285 (6), 228 (14), 184 (22), 153 (100), 137 (10), 125 (13), 110 (9), 93 (61), 81 (9), 65 (35). HRMS for $C_{23}H_{19}O_7$: Calc. 407.1131; Found: 407.1130.

***trans*-(±)-Isokielcorin D (4)**

M.p. 136–139° (Me_2CO). UV (EtOH): 374, 280, 260, 240, 209 nm. IR (KBr): 3436, 1626, 1520, 1459, 1383, 1319, 1269, 1110, 1060, 1028, 809 cm^{-1}. ^{1}H-NMR (500.13 MHz, (D_6)DMSO) δ: 9.20 (*s*, *H*O-C(4″)), 8.12 (*dd*, $J = 7.8$ and 1.4, H-C(8)), 7.81 (*ddd*, $J = 8.1$, 7.4, and 1.4, H-C(6)), 7.57 (*d*, $J = 8.1$, H-C(5)), 7.43 (*d*, $J = 9.1$, H-C(3)), 7.43 (*dd*, $J = 7.8$ and 7.4, H-C(7)), 7.12 (*d*, $J = 9.1$, H-C(4)), 7.04 (*d*, $J = 1.4$, H-C(2″)), 6.89 (*d*, $J = 8.1$ and 1.4, H-C(6″)), 6.80 (*d*, $J = 8.1$, H-C(5″)), 5.00 (*d*, $J = 7.8$, H-C(2′)), 4.99 (*t*, $J = 7.6$, CH_2OH), 4.31–4.34 (*m*, H-C(3′)), 3.77 (*s*, MeO-C(3″)), 3.71–3.73 (*m*, CH_2OH), 3.41 (under H_2O signal, CH_2OH). ^{13}C NMR (75.47 MHz, (D_6)DMSO): 174.6 (C(9)), 154.5 (C(4b)), 150.0 (C(4a)), 147.7 (C(3″)), 147.3 (C(4″)), 143.2 (C(1)), 139.2 (C(2)), 135.0 (C(6)), 127.3 (C(1″)), 126.2 (C(8)), 123.8 (C(3)), 123.7 (C(7)), 122.3 (C(8a)), 120.8 (C(6″)), 117.7 (C(5)), 115.1 (C(5″)), 111.7 (C(9a) and C(2″)), 107.9 (C(4)), 78.0 (C(3′)), 75.2 (C(2′)), 59.4 (CH_2OH), 55.4 (*Me*O-C(3″)). EI-MS: 406 (50, M^+), 239 (7), 228 (100), 199 (25), 180 (73), 170 (11), 153 (18), 137 (64), 124 (44), 103 (5), 91 (16), 77 (14), 65 (10). HRMS for $C_{23}H_{19}O_7$: Calc. 407.1131; Found: 407.1131.

***trans*-(±)-Kielcorin E (5)**

M.p. 247–249° (Me_2CO). UV (EtOH): 353, 307, 277, 241 nm. IR (KBr): 3498, 1635, 1602, 1309, 1270, 1228, 1047, 762 cm^{-1}. ^{1}H-NMR (300.13 MHz, (D_6)DMSO) δ: 9.25 (*s*, *H*O-C(4″)), 8.17 (*dd*, $J = 7.7$ and 1.2, H-C(8)), 7.84 (*ddd*, $J = 8.0$, 7.6, and 1.2, H-C(6)), 7.62 (*d*, $J = 8.0$, H-C(5)), 7.61 (*s*, H-C(1)), 7.46 (*ddd*, $J = 7.7$ and 7.5, H-C(7)), 7.23 (*s*, H-C(4)), 7.06 (*d*, $J = 1.3$, H-C(2″)), 6.90 (*dd*, $J = 8.1$ and 1.3, H-C(6″)), 6.82 (*d*, $J = 8.1$, H-C(5″)), 5.13 (*d*, $J = 8.1$, H-C(3′)), 5.06 (br. *s*., CH_2OH), 4.33–4.35 (*m*, H-C(2′)), 3.78 (*s*, MeO-C(3″)), 3.58 (br. *d*, $J = 11.4$, CH_2OH), 3.38 (under H_2O signal, CH_2OH). ^{13}C-NMR (75.47 MHz, (D_6)DMSO): 174.8 (C(9)), 155.7 (C(4b)), 151.1 (C(3)), 150.6 (C(4a)), 147.7 (C(3″)), 147.3 (C(4″)), 141.3 (C(2)), 135.0 (C(6)), 126.5 (C(1″)), 125.8 (C(8)), 124.1 (C(7)), 120.8 (C(6″)), 120.6 (C(8a)), 118.0 (C(5)), 115.4 (C(5″) and C(9a)), 111.9 (C(2″)), 111.2 (C(1)), 104.8 (C(4)), 77.8 (C(2′)), 77.0 (C(3′)), 60.0 (CH_2OH), 55.7 (*Me*O-C(3″)). EI-MS: 406 (0.02, M^+), 369 (0.8), 327 (6), 310 (0.8), 285 (6), 267 (0.6), 260 (1.4), 246 (1), 215 (2), 208 (0.6), 184 (21), 153 (100), 137 (3), 125 (12), 110 (9), 93 (62), 81 (8), 65 (35). HRMS for $C_{23}H_{19}O_7$: Calc. 407.1131; Found: 407.1131.

Molecular Calculations

All calculations were performed with *Gaussian 98*.[19] The geometry optimisation was performed using the AM1 semi-empirical method. The optimised conformations were submitted to a single point calculation, using the density functional theory, with the B3LYP functional at 3-21G basis set. The potential isosurfaces were obtained using the cubegen utility present in the *Gaussian* package. All visualizations were obtained with *Molekel 4.2*.[20]

General Methods for the Yeast Phenotypic Assay

Yeast nitrogen base was from DIFCO (Merck Portugal, Lisboa, Portugal). The kit for protein quantification was from Pierce (Biocontec, Lisboa, Portugal). The secondary alkaline phosphatase-conjugated anti-rabbit IgG detection kit (AP-10) and recombinant proteins, PKC-α (PK11), PKC-βI (PK16), PKC-δ (PK31), PKC-η (PK46) and PKC-ζ (PK41), were from Oxford Biomedical Research (LabClinics, Barcelona, Spain). Nitrocellulose membranes and all reagents for sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and immunoblots were from BioRad (PACI, Lisboa, Portugal). Acid-washed glass beads, antibodies to PKC-α, PKC-βI, PKC-δ, PKC-η and PKC-ζ, aprotinin, arachidonic acid sodium salt, chelerythrine cloride, leupeptin, pepstatin A, phenylmethylsulfonyl fluoride, phorbol 12-myristate 13-acetate (PMA) were from Sigma Aldrich (Sintra, Portugal). All other chemicals used were of analytical grade.

Yeast Transformation and Cell Cultures

Constructed yeast expression plasmids YEp52 and YEp51, encoding the cDNA for bovine PKC-α and for rat PKC-βI, respectively (kindly provided by Dr. Heimo Riedel, Wayne University, Detroit, USA) and YEplac181, encoding the cDNA for the rat

PKC-δ, mouse PKC-η or PKC-ζ (kindly provided by Dr. Nigel Goode, Royal Veterinary College, London, UK) were amplified in *Escherichia coli* DH5α and confirmed by restriction analysis. The plasmids used contain galactose-inducible transcriptional elements and the leu2 gene for selection. *Saccharomyces cerevisiae* (*S. cerevisiae*; strain CG379; α *ade5 his7-2 leu2-112 trp1-289α ura3-52 [Kil-O]*; Yeast Genetic Stock Center, University of California, Berkeley, USA) was transformed using the lithium acetate method.[21] To ensure the selection of transformed yeast, cells were grown in leucine-free medium, in 1.5% agar plates, at 30°C.

For the yeast phenotypic assay, transformed cells were incubated in leucine free-medium, with slow shaking, at 30°C. The leucine free-medium contained 0.7% yeast nitrogen base, 2% glucose (w/v) or the indicated carbon source, amino acids, purines and pyrimidines, according to the transformed yeast requirements. Galactose (2%; w/v), instead of glucose, was included to induce transcription of the mammalian PKC gene.

Cell Lysis and Immunoblotting

Cell lysis was performed basically as described.[4] The protein concentration was determined using a kit for protein quantification (*Coomassie® Protein Assay Reagent Kit*, Pierce, Biocontec, Lisboa, Portugal). Similar amounts of protein (~40 μg) from protein extracts were then separated on 10% SDS-polyacrylamide gels (Mini-Protean II, BioRad, Hercules, CA, USA). Positive controls (4 μg) were obtained using recombinant proteins PKC-α (M_r 76,799 Da), PKC-βI (M_r 76,790 Da), PKC-δ (M_r 77,517 Da), PKC-η (M_r 77,600 Da) and PKC-ζ (M_r 67,740 Da). Proteins were electrophoretically transferred to nitrocellulose membranes and probed on immunoblots with specific rabbit antibodies to the individual mammalian PKC isoforms and revealed with a secondary alkaline phosphatase-conjugated anti-rabbit IgG (AP-10, Oxford Biomedical Research, LabClinics, Barcelona, Spain).

Yeast Phenotypic Assay

Transformed yeast cultures were incubated in leucine-free medium. Optical density values, measured at 620 nm (OD_{620}; Cary 1E Varian spectrophotometer, Palo Alto, CA, USA), were used as an indicator of growth. Transformed yeast were grown to an OD_{620} of approximately 1, collected by centrifugation and diluted to an OD_{620} of 0.05, in medium containing 2% (w/v) galactose (gene transcription-inducer) and 3% (v/v) glycerol (alternative carbon source). Diluted cultures (200 μl) were transferred to 96-wells microtitre plates and incubated for up to 100 h, with slow shaking at 30°C, either in the presence of drugs or solvent (DMSO 0.1%; final concentration). Growth was monitored by determining the OD_{620} using a plate reader (BioRad Benchmark Microplate Reader; Hercules, CA, USA). In preliminary experiments, growth curves for individual isoforms were determined and the duration of the logarithmic and stationary phases identified. Estimation of drug effects was based on OD_{620} measurements at fixed time points (at 65 h for cPKC isoforms or at 48 h incubation for nPKC and aPKC), times occurring during the respective logarithmic phase and where a "steady-state growth inhibition" (period of time during which maximal inhibition of growth was reached and remained constant or changed only slightly) was reached. In individual experiments, OD_{620} was routinely monitored for up to 100 h to confirm whether the "fixed time points" chosen were appropriate for all the compounds studied (PMA or arachidonic acid). The difference between the maximal OD_{620} reached and that measured at the beginning of incubation was used as an index of yeast growth. Drugs or solvent were added at the beginning and kept throughout the incubation. Yeast growth in the presence of drugs was expressed as percentage of growth observed in parallel experiments in the presence of solvent; it was further transformed into growth inhibition by subtracting that value from 100. Because growth inhibition caused by a maximal concentration of the standard PKC activator varied between isoforms, 100% growth inhibition was assumed to be that caused by 10^{-5} M PMA (or arachidonic acid for PKC-ζ), in order to standardise the maximal inhibition attainable; 0% growth inhibition would occur when growth in the presence of drugs was identical to that in the presence of solvent. Effects of PKC activators were expressed as a percentage of that effect.

For interaction experiments, a single concentration of PKC inhibitor chelerythrine or kielcorins **1**–**5** was added to PMA (10^{-8}–10^{-5} M; arachidonic acid for PKC-ζ). Concentration-response curves for the PKC activator in the absence or in the presence of chelerythrine or kielcorins **1**–**5**, were obtained and the concentration of PKC activator that caused 50% growth inhibition (EC_{50}) calculated. For each compound the EC_{50} ratios [EC_{50} (PKC activator + compound)/EC_{50} (PKC activator)] were calculated on each PKC isoform tested.

In Vitro Kinase Assay

The direct interaction between the compounds tested (PKC inhibitor chelerythrine and kielcorins **1**–**5**) and PKC was studied *in vitro* using the IQ™ PKC Assay Kit—Pseudosubstrate Peptide Substrate from Pierce Biotechnology (PACI, Lisboa, Portugal).

The assay was performed according to the manufacturer instructions for the 96-well plate format. Kielcorins **1**–**5** and the standard PKC inhibitor chelerythrine were tested at the concentration of 10^{-8} M. Purified PKC enzyme standard from rat brain (containing a mixture of α, β and γ isoforms) was from Pierce Biotechnology (PACI, Lisboa, Portugal); it was used at a final concentration of 0.01 U. Reaction mixtures containing the endogenous PKC activator phosphatidyl-L-serine, were incubated for 90 min and fluorescence intensity determined using a microplate spectrofluorometer SPECTRAmax®GEMINI XS (Molecular Devices Corporation, Sunnyvale, CA, USA). In the presence of PKC, there was a decrease in relative fluorescence units (RFU), reflecting phosphorylation of the fluorescent substrate. In the presence of PKC inhibitors, substrate phosphorylation is reduced causing an increase in RFU. RFU in samples not containing PKC were considered as 100%. Effects of drugs were expressed as a percentage of that value.

Statistical Analysis

Results are given as arithmetic means ± SEM and *n* represents the number of determinations. Differences between means were tested for significance using either paired Student's *t* test and unpaired Student's *t* test. A *P* value less than 0.05 was taken to be statistically significant.

RESULTS

Expression of mammalian PKC-α, -βI, -δ, -η and -ζ was confirmed by immunoblotting, using protein extracts of yeast cells, transformed with the plasmid containing the gene for a single mammalian PKC isoform, grown in the presence of the transcription inducer (2% galactose). Expression of each of these PKC isoforms resulted in a single antigenic band, which co-migrated with the respective recombinant protein. Protein extracts of transformed yeast cells grown in the absence of galactose did not present antigenic bands (Figure 4).

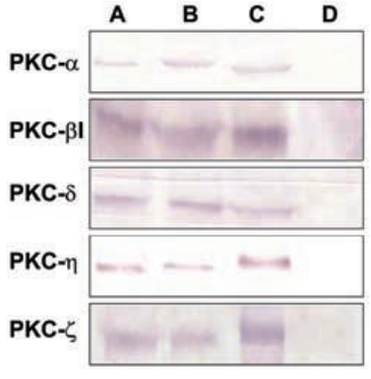

FIGURE 4 Immunodetection of PKC-α, -βI, -δ, -η and -ζ isoforms expressed in transformed *Saccharomyces cerevisiae* (CG379). Individual immunoblots are presented in a horizontal arrangement and were obtained from proteins extracts (~40 μg protein/lane) from cultures grown in selective medium in the presence of 2% galactose (lanes A and B; duplicate samples) or in the absence of 2% galactose (lane D). Positive controls (lane C; 4 μg) were obtained using recombinant proteins PKC-α (M_r 76,799 Da), PKC-βI (M_r 76,790 Da), PKC-δ (M_r 77,517 Da), PKC-η (M_r 77,600 Da) and PKC-ζ (M_r 67,740 Da).

Yeast Phenotypic Assay

PMA (considered a standard activator for the classical and novel PKC isoforms) was tested in yeast expressing PKC-α, -βI, -δ or -η, in concentrations up to 10^{-5} M (higher concentrations were not possible to test due to its low solubility in the culture medium). Since atypical PKC isoforms are not activated by phorbol esters,[22] arachidonic acid (up to 10^{-5} M) was used as the standard activator for PKC-ζ.[23] Maximal values of growth inhibition caused by 10^{-5} M PMA (or arachidonic acid for PKC-ζ), on the PKC isoforms tested are presented in Table I and were assumed to correspond to 100% growth inhibition. Effects of drugs were expressed as percentage of that maximal growth inhibition.

In the absence of galactose, neither PMA nor arachidonic acid altered yeast growth. The solvent used (DMSO; final concentration 0.1%) did not change yeast growth either in the presence or in the absence of galactose (not shown).

The influence of the kielcorin derivatives: *trans*-(±)-kielcorin C (**1**), *cis*-(±)-kielcorin C (**2**), *trans*-(±)-kielcorin D (**3**), *trans*-(±)-isokielcorin D (**4**) and *trans*-(±)-kielcorin E (**5**) (Figure 1) and of the established PKC inhibitor chelerythrine (Figure 3) on the growth of yeast expressing individual PKC isoforms was studied. In the absence of galactose, chelerythrine and kielcorins **1**–**5**, all tested at a concentration of 10^{-5} M, did not influence yeast growth.

In the presence of galactose, PMA caused a concentration-dependent inhibition of growth of yeast expressing cPKCs or nPKCs isoforms.

TABLE I Yeast growth inhibition caused by 10^{-5} M PMA on the PKC isoforms studied

PKC isoforms	Growth inhibition caused by 10^{-5} M PMA (% of control)
α	40.6 ± 1.9 ($n = 36$)
βI	36.1 ± 1.1 ($n = 56$)
δ	26.6 ± 0.6 ($n = 52$)
η	21.3 ± 0.7 ($n = 52$)
ζ	0.2 ± 1.4 ($n = 36$)[a]

[a] Growth inhibition caused by 10^{-5} M arachidonic acid was 23.9 ± 0.7 ($n = 56$). Growth in the presence of solvent was considered to be 0% growth inhibition (100% growth; see Experimental for details). Each value represents means ± SEM of the indicated *n* determinations.

EC_{50} values (nM) were, for the isoform indicated, 99.4 ± 10.1 (PKC-α), 214.7 ± 48.0 (PKC-βI), 453.1 ± 32.8 (PKC-δ) and 7.6 ± 0.6 (PKC-η) ($n = 64$). In yeast expressing PKC-ζ, arachidonic acid, but not PMA, caused a concentration-dependent growth inhibition of yeast expressing this isoform, with an EC_{50} of 208.2 ± 30.3 nM ($n = 64$).

In the yeast phenotypic assay, a PKC inhibitor blocks the growth inhibition caused by a PKC activator.[8,9] Therefore, the effects of chelerythrine and kielcorins **1**–**5** on growth inhibition caused by the appropriate PKC activator (PMA for PKC-α, βI, -δ and -η; arachidonic acid for PKC-ζ) were investigated.

In a first approach, chelerythrine and kielcorins **1**–**5** were tested alone. At the maximal concentration feasible under these experimental conditions (10^{-5} M), chelerythrine and kielcorins **1**–**5** caused, *per se*, stimulation of growth of yeast expressing individual mammalian PKC isoforms. A decrease in the concentration reduced this growth stimulation and, at a concentration of 10^{-8} M, chelerythrine and kielcorins **1**–**5** failed to cause a significant stimulation of yeast growth (data not shown).

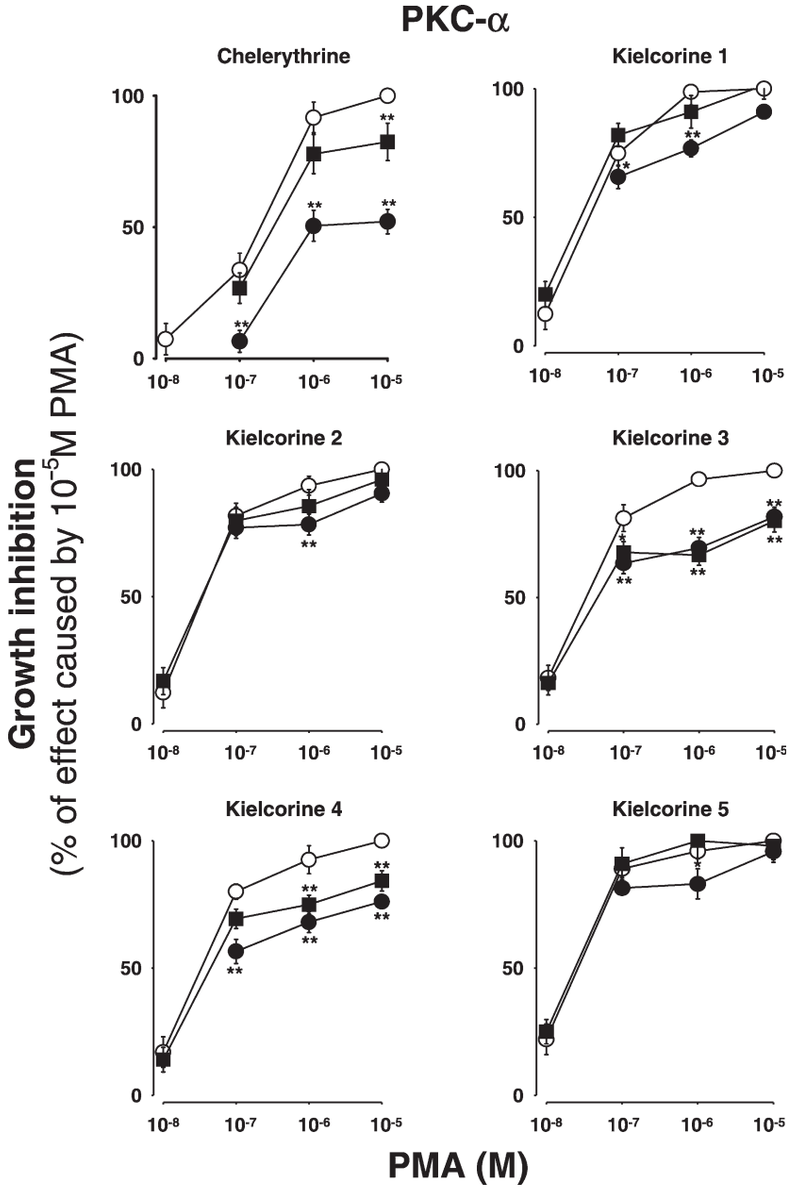

FIGURE 5 Interaction experiments on PKC-α: concentration-response curves for PMA alone (open circles) and in the presence of 10^{-5} M (filled circles) or 10^{-8} M (filled squares) of chelerythrine or kielcorins **1**–**5**. Results are expressed as % of the maximal effect caused by 10^{-5} M PMA. Shown are means ± SEM of 16 – 20 determinations. Significantly different from growth inhibition caused by PMA alone: $^{*}P < 0.05$, $^{**}P < 0.001$ (unpaired Student's *t* test).

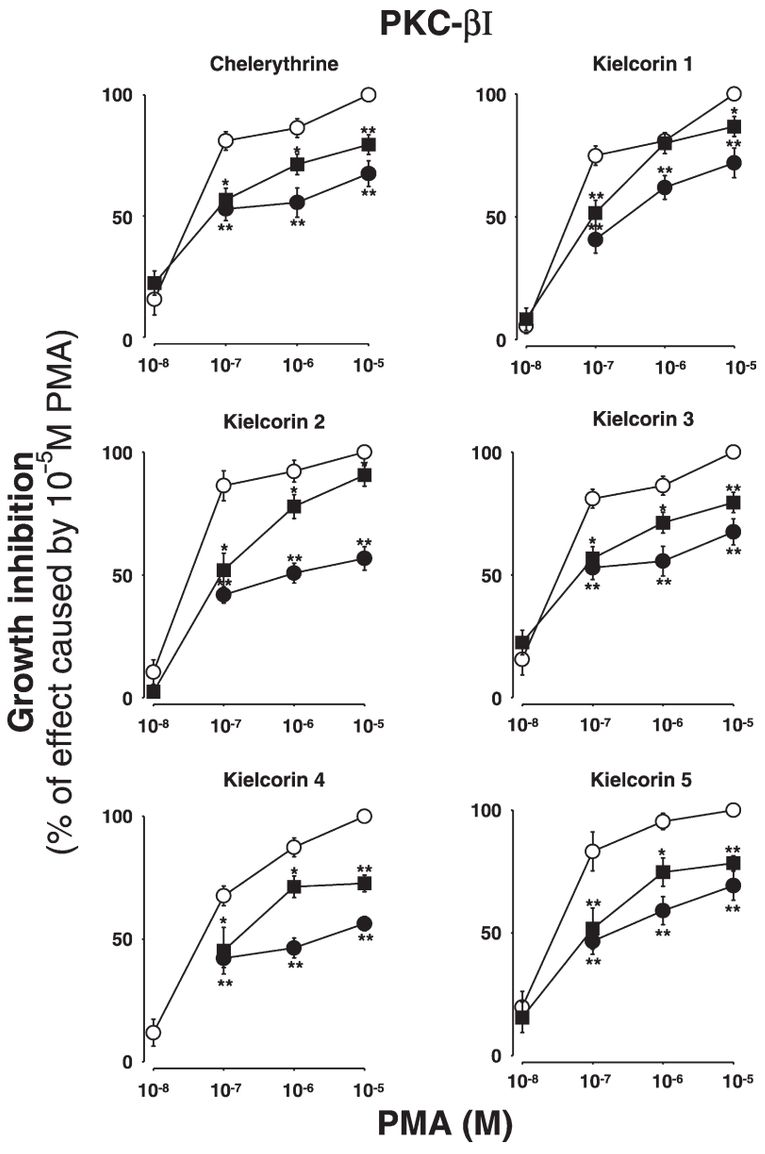

FIGURE 6 Interaction experiments on PKC-βI: concentration-response curves for PMA alone (open circles) and in the presence of 10^{-5} M (filled circles) or 10^{-8} M (filled squares) of chelerythrine or kielcorins **1**–**5**. Results are expressed as % of the maximal effect caused by 10^{-5} M PMA. Shown are means ± SEM of 16–20 determinations. Significantly different from growth inhibition caused by PMA alone: $^{*}P < 0.05$, $^{**}P < 0.001$ (unpaired Student's *t* test).

In a subsequent series of experiments, the ability of chelerythrine and kielcorins **1**–**5** to block growth inhibition caused by the appropriate PKC activator, was investigated. Chelerythrine and kielcorins **1**–**5** were tested at the maximal concentration feasible (concentration at which a stimulation of yeast growth was observed) and at 10^{-8} M (concentration at which no stimulation of yeast growth was observed). Effects of chelerythrine and kielcorins **1**–**5** on the growth inhibition caused by the PKC activator PMA on yeast expressing PKC-α, -βI, -δ or -η are shown in Figures 5,6,7 and 8, respectively. Figure 9 shows effects of chelerythrine and kielcorins **1**–**5** on the growth inhibition caused by arachidonic acid on yeast expressing PKC-ζ.

Chelerythrine reduced growth inhibition caused by the PKC activator (PMA or arachidonic acid), causing a shift to the right for the concentration-response curve of the PKC activator on all isoforms tested (compare open and filled symbols in left upper panel of Figures 5,6,7,8 and 9). This shift to the right was concentration-dependent, being the displacement caused by 10^{-8} M chelerythrine which was less pronounced than that caused by 10^{-5} M (compare filled squares and circles in left upper panel of Figures 5,6,7,8 and 9). In general, kielcorins also caused a shift to the right of the concentration-response curve for the PKC activator (compare open and filled symbols of Figures 5,6,7,8 and 9). The main exception occurred with PKC-α, where kielcorins **1**, **2**

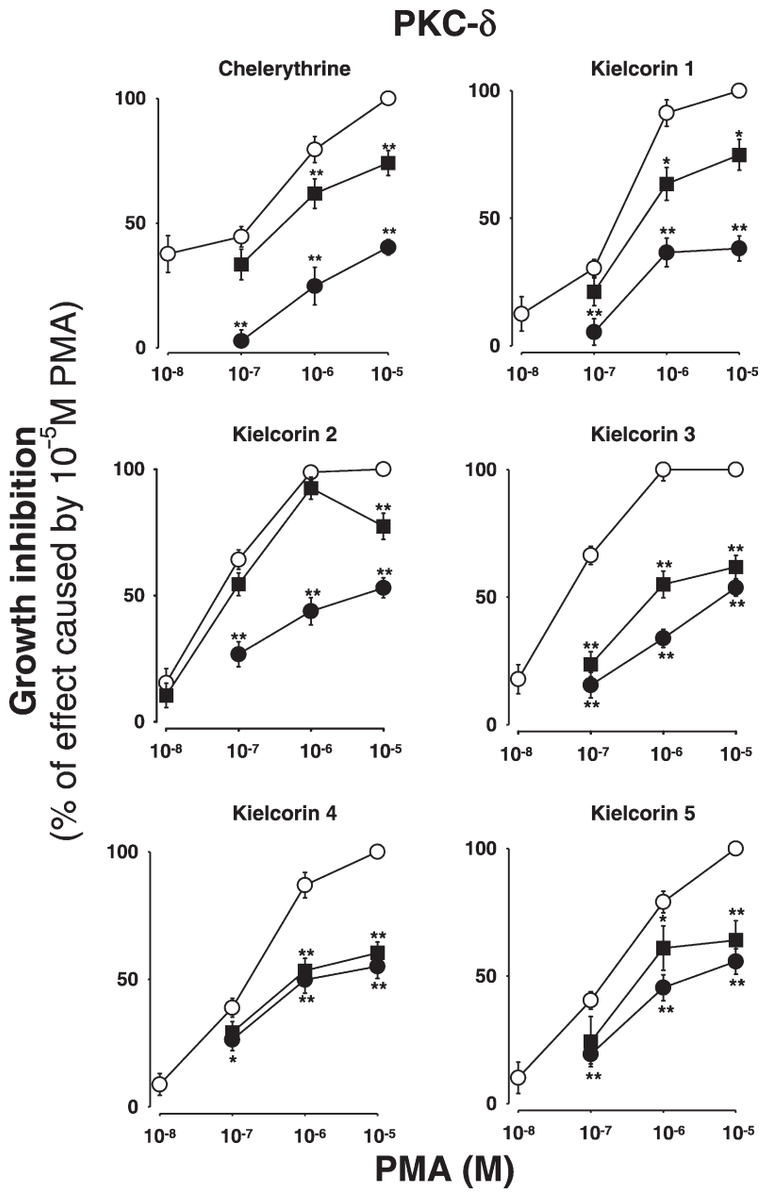

FIGURE 7 Interaction experiments on PKC-δ: concentration-response curves for PMA alone (open circles) and in the presence of 10^{-5} M (filled circles) or 10^{-8} M (filled squares) of chelerythrine or kielcorins **1**–**5**. Results are expressed as % of the maximal effect caused by 10^{-5} M PMA. Shown are means ± SEM of 16–20 determinations. Significantly different from growth inhibition caused by PMA alone: *$P < 0.05$, **$P < 0.001$ (unpaired Student's t test).

and **5**, at 10^{-8} M, failed to significantly influence the concentration-response curve for PMA (compare open and filled symbols of Figure 5). The shift to the right caused by kielcorins was, in general, concentration-dependent, being the displacement caused by 10^{-8} M which was less pronounced than that caused by 10^{-5} M kielcorins **1**–**5** (compare filled squares and circles of Figures 5,6,7,8 and 9). Exceptions occurred for kielcorin **5** on PKC-βI, kielcorins **4** and **5** on PKC-δ and kielcorin **3** on PKC-ζ, where no concentration-dependence was detected; in the presence of the highest and lowest concentration of these kielcorins the responses to the PKC activator were similar ($P > 0.05$; unpaired Student's t test).

In order to compare the potencies of chelerythrine and kielcorins **1**–**5** to block the growth inhibition caused by the appropriate PKC activator, EC_{50} values (*i.e.* the concentration of the PKC activator that caused half of the growth inhibition caused by 10^{-5} M PMA; arachidonic acid for PKC-ζ), obtained in the absence and in the presence of 10^{-8} M chelerythrine or kielcorins **1**–**5**, were calculated. When EC_{50} values, obtained in the presence of 10^{-8} M chelerythrine or one of the kielcorins **1**–**5**, were different from the EC_{50} values obtained in their

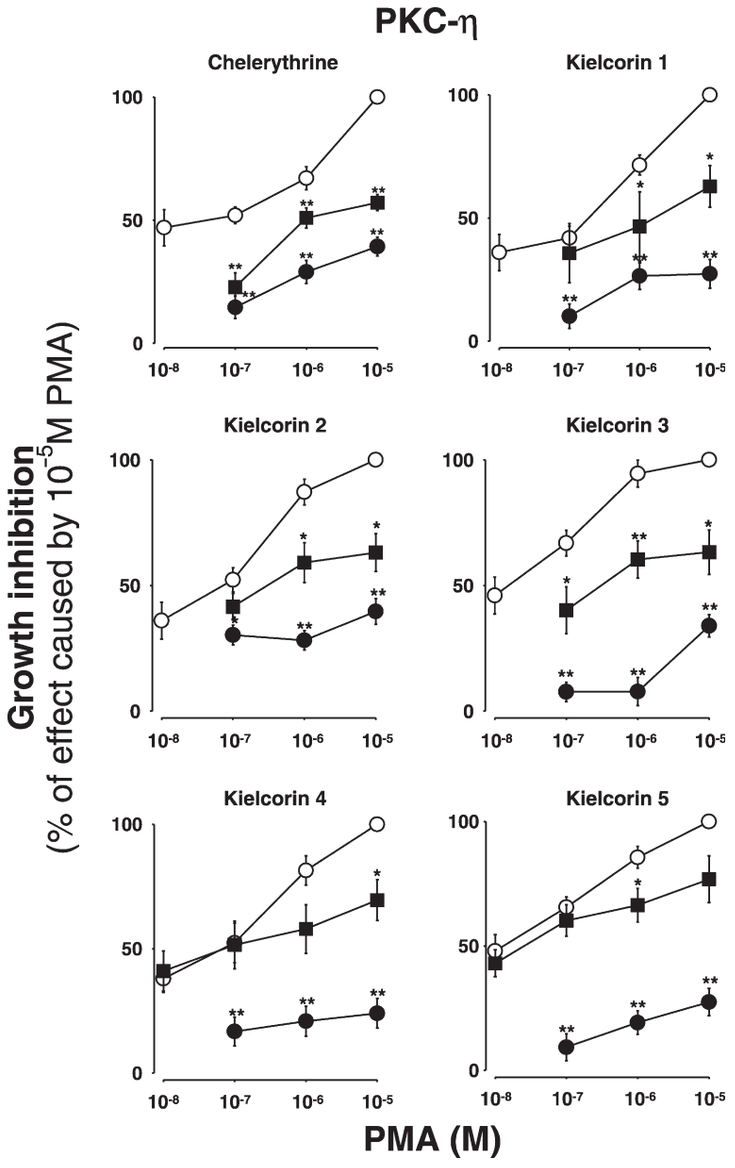

FIGURE 8 Interaction experiments on PKC-η: concentration-response curves for PMA alone (open circles) and in the presence of 10^{-5} M (filled circles) or 10^{-8} M (filled squares) of chelerythrine or kielcorins **1**–**5**. Results are expressed as % of the maximal effect caused by 10^{-5} M PMA. Shown are means ± SEM of 16–20 determinations. Significantly different from growth inhibition caused by PMA alone: $^{*}P < 0.05$, $^{**}P < 0.001$ (unpaired Student's *t* test).

absence, EC_{50} ratios were calculated. EC_{50} ratios were used to compare: *i*) the potency of kielcorins with that of chelerythrine and *ii*) the potencies of chelerythrine and kielcorins **1**–**5** on the different mammalian PKC isoforms individually expressed. Table II presents the EC_{50} ratios obtained with 10^{-8} M chelerythrine and kielcorins **1**–**5** (EC_{50} ratios obtained with 10^{-5} M were not calculated, because of the growth stimulation caused by chelerythrine and kielcorins **1**–**5**, *per se*).

Chelerythrine and kielcorins **1**–**5** presented differences in potencies towards the distinct PKC isoforms tested, as estimated by comparison of the respective EC_{50} ratios. The following order of potencies for the compounds studied have been established: chelerythrine, $PKC-\eta > -\beta I > -\alpha > -\zeta = -\delta$; kielcorin **1**, $PKC-\zeta > -\eta > -\delta = -\beta I$; kielcorin **2**, $PKC-\zeta > -\eta > -\beta I = -\delta$; kielcorin **3**, $PKC-\zeta = -\eta = -\delta$; kielcorin **4**, $PKC-\zeta > -\delta = -\beta I$; kielcorin **5**, $PKC-\zeta = -\beta I = -\delta$ (for definition of the rank order of potency, the $>$ was applied only when the EC_{50} ratio of the isoform placed at the left was significantly higher than that of the EC_{50} ratio placed at the right of the sign; otherwise $=$ was applied; $P < 0.05$; unpaired Student's *t* test).

When compared to chelerythrine, kielcorins **1**–**5** were shown to be less potent on PKC-α, failing, at the concentration tested, to change the PMA EC_{50}

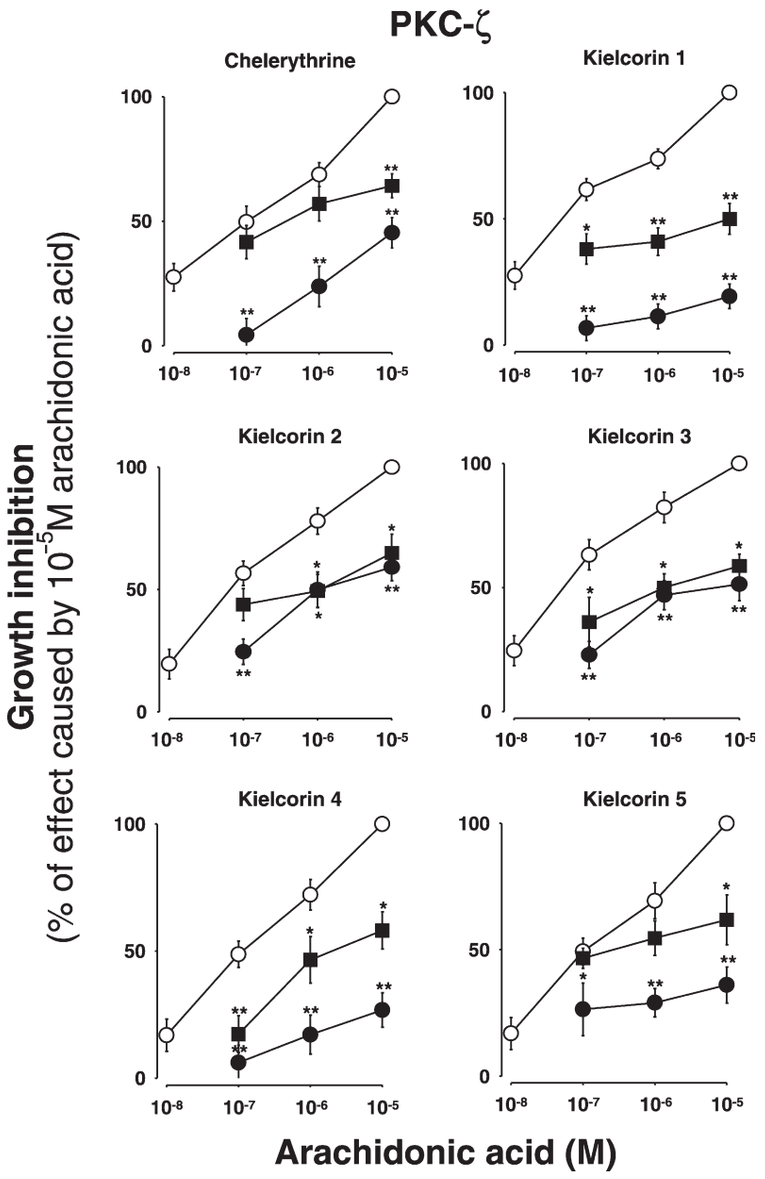

FIGURE 9 Interaction experiments on PKC-ζ: concentration-response curves obtained for arachidonic acid alone (open circles) and in the presence of 10^{-5}M (filled circles) or 10^{-8}M (filled squares) of chelerythrine or kielcorins **1–5**. Results are expressed as % of the maximal effect caused by 10^{-5}M of arachidonic acid. Shown are means ± SEM of 16–20 determinations. Significantly different from growth inhibition caused by arachidonic acid alone: $^{*}P < 0.05$, $^{**}P < 0.001$ (unpaired Student's *t* test).

TABLE II EC_{50} ratios for chelerythrine and kielcorin derivatives on the individual PKC isoforms tested

	EC_{50} Ratio[a]				
Compound	PKC-α	PKC-βI	PKC-δ	PKC-η	PKC-ζ
Chelerythrine	1.9 ± 0.3	2.9 ± 0.3*	1.3 ± 0.1	63.2 ± 1.7*	1.4 ± 0.2
Kielcorin **1**	*ND*	1.8 ± 0.5*	2.2 ± 0.6*	9.8 ± 1.7*†	213.0 ± 20.8†
Kielcorin **2**	*ND*	2.8 ± 0.7*	1.6 ± 0.3*	4.9 ± 0.6*†	17.9 ± 6.4†
Kielcorin **3**	*ND*	*ND*	12.6 ± 4.1†	24.7 ± 6.2†	25.1 ± 6.6†
Kielcorin **4**	*ND*	3.2 ± 0.9*	5.2 ± 0.9*†	*ND*	16.7 ± 5.2†
Kielcorin **5**	*ND*	2.6 ± 0.6	2.0 ± 0.8	*ND*	3.4 ± 0.8†

[a] EC_{50} ratio = EC_{50} (PKC activator + 10^{-8}M compound)/EC_{50} (PKC activator). The EC_{50} values were considered the concentration of PKC activator that caused half of the growth inhibition caused by 10^{-5}M of PMA (arachidonic acid for PKC-ζ). Shown are means ± SEM of 16–20 determinations. *ND*: Not determined (identical EC_{50} values in the absence or in the presence of the compound). Significant differences: from PKC-ζ, $^{*}P < 0.05$; from chelerythrine, $^{\dagger}P < 0.05$ (unpaired Student's *t* test).

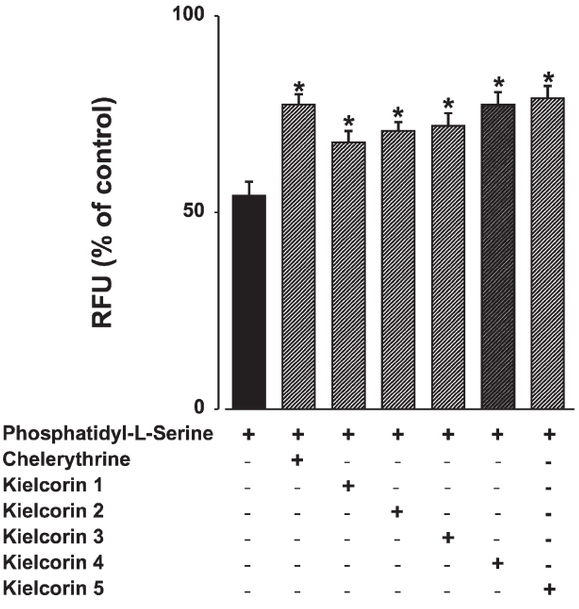

FIGURE 10 *In vitro* kinase assay using a purified rat brain PKC enzyme: effects of chelerythrine, and kielcorins **1**–**5** (all tested at the concentration of 10^{-8} M). Relative fluorescence units (RFU) were measured in samples without enzyme (100%; control) or with PKC, which caused a decrease in RFU (see Experimental for details). Phosphatidyl-L-serine, the recommended PKC activator, was present in all samples; drugs or solvent, were tested in the absence or in the presence of PKC. Effects of drugs were expressed as % of RFU obtained in the absence of PKC. Shown are means ± SEM of 4 assays (8 assays for phosphatidyl-L-serine alone). Significantly different from solvent (phosphatidyl-L-serine alone): $^*P < 0.05$ (one way ANOVA, followed by Tukey's *post-hoc* test).

value. On PKC-βI, kielcorin **3** also did not change the PMA EC_{50}, but kielcorins **1**, **2**, **4** and **5** were shown to be as potent as chelerythrine. On PKC-δ, kielcorins **1**, **2** and **5** were shown to be as potent as chelerythrine, whereas kielcorins **3** and **4** were more potent than chelerythrine, kielcorin **3** having a much higher potency. On PKC-η, kielcorins **4** and **5** failed to alter the PMA EC_{50}, whereas kielcorins **1**, **2** and **3** markedly inhibited PMA effects, although less than chelerythrine, which on this PKC isoform showed its highest potency. On PKC-ζ, all kielcorins were more potent than chelerythrine, kielcorin **1** having an extraordinary high potency when compared with those of other kielcorins or chelerythrine.

In Vitro Kinase Assay

To test whether kielcorins **1**–**5** were interacting directly with PKC, their effects were studied *in vitro*, using purified rat brain PKC. The assay used is based on the ability of PKC inhibitors to reverse the fluorescence intensity quenching (a decrease on the relative fluorescence units, RFU) caused by the PKC activator (phosphatidyl-L-serine as recommended by the method). Reversing of fluorescence quenching (which reflects a reduction of substrate phosphorylation) is assumed to be an indicator of inhibition of PKC catalytic activity. Kielcorins **1**–**5** and chelerythrine were used at the lowest concentration tested (10^{-8} M), the same used to obtain the EC_{50} ratios presented in Table II. Kielcorins **1**–**5** were able to reverse the effect of the endogenous PKC activator. The effects elicited by these compounds were similar to that caused by the PKC inhibitor chelerythrine (Figure 10).

Computational Studies

The optimised geometries of kielcorins **1**–**5** and chelerythrine were calculated and their potential isosurfaces obtained and these are shown in Figure 11. All kielcorins tested (**1**–**5**) contain three main negative potential points, namely, one over the carbonyl of the xanthonic nucleus, another near the hydroxymethyl group and the third located over

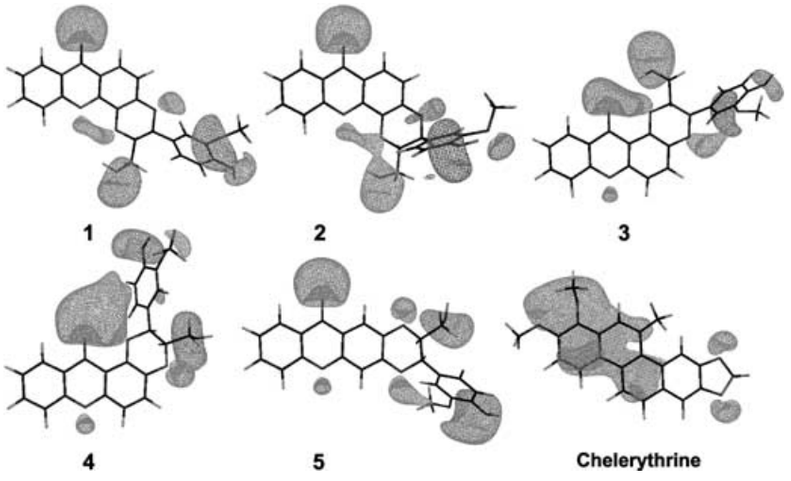

FIGURE 11 Potential isosurfaces at -20 kcal mol^{-1} for kielcorins **1**–**5** and chelerythrine.

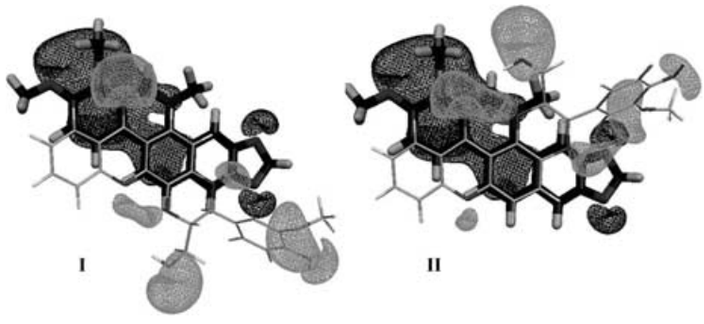

FIGURE 12 Two examples of correspondence of negative potential surfaces: (I) chelerythrine and kielcorin **1**; (II) chelerythrine and kielcorin **3**.

the oxygenated substituents of the aromatic ring in the coniferyl unit. For kielcorins **3** and **4**, the negative potential point over the carbonyl is reinforced by the proximity of the other two regions. Concerning chelerythrine, a strong negative potential over the aromatic area and two others over the methylenedioxy group can be noted. A correspondence of the potential points referred to above for the kielcorins **1**–**5** with chelerythrine was achieved (Figure 12). For kielcorins **3**–**5** the correspondence of the potential bulk of the carbonyl group with the aromatic region of chelerythrine, allows a further correspondence among the other potential points. Figure 12 shows the common features of the negative potential surfaces with chelerythrine, for kielcorin **1** (with a very high potency on PKC-ζ) and also for kielcorin **3** (the most potent on PKC-δ and, like chelerythrine, with a high potency on PKC-η).

DISCUSSION

The yeast phenotypic assay, which uses transformed yeast expressing an individual mammalian PKC isoforms, has been proposed as a rapid and simple assay for the screening of potential PKC modulators. In the present study, either mammalian PKC-α, -βI, -δ, -η or -ζ isoforms were expressed using the same yeast strain. According to previous studies,[6,7] exposure of transformed yeast expressing a mammalian PKC isoform to a PKC activator, led to a concentration-dependent inhibition of yeast growth (reflecting an increase in the cell doubling time). Inhibition of transformed yeast growth caused by the PKC activator was assumed to be due to its interaction with the mammalian PKC isoform expressed because it did not occur when transformed yeast cells were incubated in medium lacking galactose, the expression inducer. Confirmation that mammalian PKC isoforms were expressed in the presence of galactose (and not on its absence) was obtained by immunobloting.

Absolute values of growth inhibition caused by the PKC activators used (PMA or arachidonic acid) differed among the expressed PKC isoforms. These differences can be ascribed to different levels of expression of the mammalian PKC isoform or to differences in the catalytic potential of the expressed isoform. The latter possibility is supported by results obtained *in vitro* that showed that identical amounts of PKC isoforms, in the presence of the same concentration of a PKC activator, led to different degrees of phosphorylation of a given substract.[24] In order to avoid the influence of different absolute values on data interpretation, growth inhibition caused by PKC activators was standardised. For each isoform, growth inhibition caused by a maximal concentration of PKC activator was assumed to be the 100% of growth inhibition attainable by PKC activators under the present experimental conditions and EC_{50} values estimated from the concentration-response curves obtained.

Chelerythrine and kielcorins **1**–**5**, when tested in the micromolar range, cause a slight stimulation of growth of transformed yeast. This effect was observed only when a mammalian PKC isoform was expressed and, therefore seems to result from an interaction with the expressed mammalian PKC isoform. A direct stimulation of growth of transformed yeast has been reported previously[8] and may result from blockade of an endogenous activation of PKC.

Blockade of PKC activator-induced growth inhibition by established PKC inhibitors has been reported to reflect inhibition of the mammalian PKC isoform expressed.[8,9] Under these conditions, PKC inhibitors caused a shift to the right of the concentration-response curve of the PKC activator. When this occurred, the ratio between the PKC activator EC_{50} values (obtained in the presence and in the absence of the PKC inhibitor) was used as an index of the PKC inhibitor potency on the mammalian PKC isoform expressed. To avoid putative influences on the shift to the right caused by the growth stimulation observed with the highest

concentration of chelerythrine and kielcorins, their potencies were compared by the shift caused by 10^{-8} M (same concentration for all the compounds tested). As expected, chelerythrine inhibited effects of the PKC activators on growth of transformed yeast, being active on all isoforms tested. Kielcorins also inhibited effects of PKC activators. Kielcorins were particularly potent on PKC-ζ and showed with PKC-α their lowest potency. Kielcorins were less potent than chelerythrine on PKC-η, and were as potent as chelerythrine on PKC-βI. However, on PKC-δ, kielcorins **3** and **4** were more potent than chelerythrine, and on PKC-ζ, all kielcorins **1**–**5** were more potent than chelerythrine. Among the kielcorins tested, kielcorin **1** presented the most marked difference on potency, being particularly potent on PKC-ζ; the less marked differences were observed with kielcorin **5**. Kielcorin **3** presented a similar high potency on several isoforms (PKC-δ, -η and -ζ).

Confirmation that kielcorins blocked the effect of PKC activators on yeast growth by interacting with PKC was obtained using an *in vitro* kinase assay. At the lowest concentration used on the yeast phenotypic assay, kielcorins **1**–**5** inhibited PKC catalytic activity, an effect similar to that caused by the established PKC inhibitor chelerythrine.

The similarity of results obtained for chelerythrine and kielcorins, in the yeast phenotypic assay and in *in vitro* kinase assay, raised the possibility that they could have common electronic properties. Although PKC inhibitors are known for their large structural diversity,[25] common structural and electronic features were found among kielcorins **1**–**5** and chelerythrine (preliminary computational studies described in Results) which can explain the functional similarities detected between these two groups of compounds as PKC inhibitors.

This study is, to our knowledge, the first attempt to unravel the molecular basis for the biological activity of kielcorins, and suggests that kielcorin derivatives constitute a promising group from which to obtain new isoform selective PKC inhibitors.

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