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Inhibition of human androgen-independent PC-3 and DU-145 prostate cancers by antagonists of bombesin and growth hormone releasing hormone is linked to PKC, MAPK and *c-jun* intracellular signalling

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Abstract

Bombesin/gastrin-releasing peptide (BN/GRP) antagonists RC-3940-II and RC-3940-Et, and growth hormone-releasing hormone (GHRH) antagonists MZ-J-7-118 and RC-J-29-18 inhibit the growth of human androgen-independent PC-3 and DU-145 prostate cancers in nude mice. Additive inhibitory effects were observed after treatment with both classes of analogs. In the present study, we investigated the effects of these antagonists on intracellular signalling pathways of protein kinase C (PKC), mitogen activated protein kinases (MAPK) and *c-fos* and *c-jun* oncogenes that are involved in tumour cell proliferation. In PC-3 tumours, antagonists of BN/GRP and GHRH decreased significantly the expression of PKC isoforms alpha (α), eta (η) and zeta (ζ) and increased that of delta (δ) PKC protein. MAPK was not detectable. In DU-145 tumours, which constitutively express MAPK, all treatments strongly decreased the levels of p42/44 MAPK. Treatment with the antagonists tended to reduce m-RNA for *c-jun* in both tumour models. In proliferation assays *in vitro*, inhibitors of PKC and MAPK diminished growth of DU-145 and PC-3 cells. These findings suggest that antagonists of BN/GRP and GHRH inhibit the growth of androgen-independent prostate cancer by affecting intracellular signalling mechanisms of PKC, MAPK and *c-jun*.

Keywords: Prostate cancer; PKC isoforms; MAP-kinases; *c-fos*, *c-jun*; PC-3; DU-145

1. Introduction

Prostate cancer is a significant health problem among men in the Western world [1,2]. Whereas many patients with organ confined disease can be cured by radical prostatectomy or radiation therapy, a significant number will develop local recurrence and disseminated, metastatic disease. Androgen deprivation is an established treatment for advanced prostate cancer, but a relapse due to the development of androgen independence is frequently observed. The failure of androgen deprivation therapy could be due to amplification, loss or changes in the specificity of the androgen receptor. It has been shown that growth of advanced prostate cancer can be activated by a wide spectrum of other steroid hormones, non-steroidal antiandrogens and various growth factors [3].

Several growth factors are involved in growth of androgen-independent prostate cancer signal through

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protein kinase C (PKC) pathway [4,5]. The PKC family consists of several isoforms: the conventional (α, βI, βII and γ), the novel PKCs (δ, ε, θ), and the atypical PKCs (ζ, λ and μ) [6]. PKC isoforms are serine/threonine kinases involved in a wide range of physiological and pathological processes including differentiation, proliferation, apoptosis, neoplastic transformation and gene expression. The expression of PKC isoforms has been investigated in different prostate cancer models [7–9] and activation of PKC and its downstream targets is considered to be involved in the development of androgen-independent prostate cancer. Recently, specific functions of each isoform of PKC have been described and the evaluation of isoforms for use as targets of drug action was initiated [10]. ISIS 3521, an antisense oligonucleotide inhibitor of PKC-α, is in clinical trials for the treatment of hormone refractory prostate cancer [11]. The phospholipid signalling cascade includes activation of phospholipase C-β, PKC and finally mitogen activated protein kinase (MAPK), which after phosphorylation is translocated into the nucleus and increases the expression of immediate-early oncogenes such as *c-fos* and *c-jun* [10,12]. MAPK is important in determining the cellular response to several types of stimulation [13] and activated MAPK is frequently found in advanced prostate cancer [14]. The androgen receptor is regulated by phosphorylation and cross-talk with several signalling pathways, including PKC, PKA and MAPK [15]. Recently, it has been demonstrated that MAPK is constitutively activated in DU-145 cells, but not in other cell lines and that the high level of MAPK activity in the DU-145 cell line is linked to the effect of paracrine/autocrine growth factors [16,17].

Peptide growth factors including bombesin/gastrin releasing hormone (BN/GRP), growth hormone-releasing hormone (GHRH) and their receptors have been found in surgical specimens from patients with locally advanced prostate cancer [2,18,19] and in experimental human prostate cancer lines [2,18,20–24]. However, splice variants of GHRH receptors expressed by prostatic and other cancers are different from the pituitary isoform [2].

In previous studies, we showed that antagonists of BN/GRP and GHRH effectively inhibit the growth of various human experimental cancers including prostate cancer and reduce the concentration and receptor levels of tumoural growth factors [2,22,24,25]. The BN/GRP receptors are members of the G-protein coupled receptor super family and the signal transduction pathways involve the activation of phospholipase C, generation of inositol triphosphate, the release of intracellular calcium, and the activation of PKC [26]. Bombesin mediated mitogenesis can be blocked by different BN/GRP antagonists through interrupting the signal transduction process at various post-receptor levels [27]. This mitogenic block is mediated by uncoupling the receptor from its signalling system and is associated with down-regulation of PKC [27]. The PKC pathway could also be an important signalling system involved in the action of GHRH on its pituitary receptors as shown in studies on the control of GHRH secretion from ovine somatotropes [28]. It has also been observed that activation of pituitary receptors for GHRH produces a phosphorylation of MAPK and increases the levels of *c-fos* protein [29].

Nevertheless, the intracellular signalling pathways in prostate cancer involving PKC/MAPK affected by GHRH antagonists have not been studied previously. In H-69 human small-cell lung carcinoma, tumour inhibition by antagonists of BN/GRP and of GHRH is correlated with an inhibition of the PKC-MAPK-*c-fos*/*c-jun* signalling pathway [6]. Recently, we showed that BN/GRP antagonists RC-3940-II and RC-3940-Et; GHRH antagonists MZ-J-7-118 and RC-J-29-18; as well as a combination of BN/GRP antagonist RC-3940-II with GHRH antagonist MZ-J-7-118, strongly inhibited the growth of PC-3 and DU-145 human androgen-independent prostate cancers xenografted into nude mice [25]. In order to elucidate the intracellular signalling mechanisms involved in the antitumour action of these antagonists of BN/GRP and GHRH, we have investigated in the present study whether they affect the protein levels of PKC isoforms and MAPK, as well as the expression of early oncogenes *c-fos* and *c-jun* in PC-3 and DU-145 tumours.

2. Materials and methods

2.1. Peptides and reagents

GHRH antagonists MZ-J-7-118 and RC-J-29-18 were synthesised in our laboratory by methods similar to those described [30]. The chemical structure of MZ-J-7-118 is [CH_3-$(CH_2)_6$-CO-Tyr^1, D-Arg^2, Phe(4-Cl)^6, Ala^8, His^9, Tyr(Et)^{10}, His^{11}, Abu^{15}, Nle^{27}, D-Arg^{28}, Har^{29}]hGHRH(1-29)NH_2, where Phe(4-Cl) is 4-chlorophenylalanine, Abu is α-aminobutyric acid, Nle is norleucine, Har is homoarginine. RC-J-29-18 is the analog of MZ-J-7-118 with a C-terminal ethylamide modification. The BN/GRP antagonist RC-3940-II, originally synthesised in our laboratory [31], was made and provided by Zentaris Gmbh (Frankfurt/Main, Germany) as D-24197. Its chemical structure is [Hca^6, Leu^{13}ψ(CH_2N)-Tac^{14}]BN(6–14), where Hca is desaminophenylalanine, and Tac is thiazolidine-4-carboxylic acid. BN/GRP antagonist RC-3940-Et, which is the analog of RC-3940-II with a C-terminal ethylamide modification, was synthesised in our laboratory as described for RC-3940-II [31]. For daily subcutaneous (s.c.) injection, the compounds were dissolved in 0.1% dimethylsulfoxide (DMSO) in 10% aqueous propylene glycol solution.

2.2. Cell lines and in vivo studies

The PC-3 and DU-145 tumour samples used in the present investigation were obtained from the *in vivo* studies carried out previously [25]. The PC-3 and DU-145 human androgen-independent prostate cancer cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA), maintained in culture and transplanted into male athymic (Ncr nu/nu) nude mice (Frederick Cancer Research and Development Center, Frederick, MD, USA) as described [24]. All experiments were performed in accordance with the institutional ethical guidelines of animal care and were in agreement with guidelines for the welfare of animals in experimental neoplasia.

2.3. Experiment 1

PC-3 tumour tissue was harvested aseptically from donor animals after 10 weeks of s.c. growth. Male nude mice were xenografted s.c. with 3 mm^3 pieces of PC-3 tumour tissue using a trocar needle. When tumours had grown to a volume of approximately 70 mm^3, the animals were randomly assigned to six experimental groups, each containing eight animals. Antagonists of BN/GRP and GHRH were injected s.c. at the doses of 10 and 5 μg/day, respectively, and one group received combination of RC-3940-II (10 μg/day) and MZ-J-7-118 (5 μg/day). Controls received vehicle solution. The experiment was ended on day 28.

2.4. Experiment 2

DU-145 tumour tissue was harvested aseptically from donor animals after 15 weeks of s.c. growth. Male nude mice were xenografted s.c. with 3 mm^3 tumour pieces using a trocar needle. When tumours had grown to a mean volume of 45 mm^3, the animals were divided into four groups each containing 6–7 animals. Antagonists of BN/GRP and GHRH were administered by s.c. injection as follows: RC-3940-II (10 μg/day), MZ-J-7-118 (5 μg/day), and the combination of RC-3940-II (10 μg/day) and MZ-J-7-118 (5 μg/day). Controls received vehicle solution. The experiment was ended on day 56. At the end of experiments 1 and 2, mice were sacrificed, tumours were carefully excised, weighed, snap-frozen and stored at −70 °C for further investigations.

2.5. RNA extraction and reverse transcription (RT)-PCR

Total RNA was extracted from tumours using the Tri-Reagent (Sigma Chemical Co., St. Louis, MO, USA) following manufacturer's instructions. For amplification of cDNA transcripts, gene-specific primers for human *c-fos*, *c-jun* and β-*actin* were used as described in detail [12]. PCR products were subjected to electrophoresis on a 1.8% agarose gel, stained with ethidium bromide and analysed.

Semi-quantitatively by using the Kodak imaging system with Kodak Image Analysis Software. The mRNA levels for each gene were normalised *vs.* the corresponding levels of mRNA for β-actin. Triplicate RNA samples from each group were also subjected to cDNA synthesis using the Quantitect Reverse Transcription Kit (Qiagen, Valencia, CA) with a step to remove genomic DNA. This cDNA was then used as template in a real-time PCR reaction using the pre-validated Quantitect Gene Expression Assay Kit (Qiagen), where beta actin, *c-fos*, and *c-jun* expression levels were quantified. Samples were done in triplicate yielding a sample size of 9 for each group and the data were quantified according to methods established by Pfaffl [32].

2.6. Western blotting assays for PKC and MAP kinases

Protein-matched samples were subjected to electrophoresis on 8% SDS-PAGE and were then transferred to nitrocellulose membranes. The membranes were incubated in 5% (w/v) non-fat dry milk in PBS-Tween for 1 h and then incubated in the primary antibody solutions at 4 °C overnight. Experiments were performed with monoclonal antibodies for βI and βII PKC (1:2000), and polyclonal antibodies to α-, β-, γ-, δ-, ε-, η- and ζ-PKC isoforms (1:1000), (Sigma), p42/44 MAPK (1:1000); (Cell Signalling, Beverly, MA, USA) or polyclonal antibody to β-actin (1:5000) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The specificity of the immunoreactive bands was confirmed by the disappearance of the bands after incubation of the membranes with the specific peptide fragments corresponding to each PKC isoform. The nitrocellulose membranes were washed 3×15 min in 5% (w/v) non-fat dry milk in TBS-Tween and then incubated in horseradish peroxidase-conjugated secondary antibody solution (1:1000) for 1.5 h (Santa Cruz Biotechnology). The blots were washed 3×10 min in 5% (w/v) non-fat dry milk in TBS-Tween, 2×10 min in TBS-Tween and visualised with the enhanced chemiluminescence detection system (Amersham, Arlington Heights, IL, USA). Experiments were performed at least twice and the reactive bands corresponding to PKC-isoforms and MAPK were quantitated using the Kodak EDAS 290 imaging system with Kodak 1D Image Analysis Software. The relative protein levels were normalised *vs.* the corresponding levels of human β-actin.

2.7. Cell proliferation assay

PC-3 and DU-145 cells were seeded in F12k Nutrient Mixture (Kaighn's modification), 10% FBS and Minimum Essential Medium (MEM), 1 mM pyruvate, 10% FBS, respectively, into 96-well plates at low density, corresponding to a confluence of 2–5%. After a recovery

period of 24–48 h, the cell culture medium was removed and replaced with fresh media containing the test compounds dissolved in 0.1% (v/v) DMSO. According to our previous experiments, DMSO added to the media at the concentration of 0.1% (v/v) did not produce any significant effects on proliferation of PC-3 or DU-145 cells compared to the medium alone. The test compounds included PKC inhibitors BIM and PKC(19-31) at 10 μM concentrations, PKC activators PMA and PDBu (0.10 μM) and a MAPK inhibitor PD98059 (Sigma) (50 μM) in octuplicate wells each. Controls received only culture medium containing 0.1% (v/v) DMSO. After another 85 h, when PC-3 or DU-145 cells reached a maximum confluence of 80–90%, the plates were fixed with glutaraldehyde and *in vitro* cell growth was estimated by the crystal violet method as described [33]. Results were calculated as $\%T/C$, were $T =$ optical density of treated cultures and $C =$ optical density of untreated cultures. Each experiment was repeated 3–5 times and similar results were obtained.

2.8. Statistical analysis

Data are expressed as mean ± SE. Differences between the values were evaluated with one way ANOVA followed by the Fisher LSD test, $P < 0.05$ being considered as statistically significant.

3. Results

3.1. Effects of antagonists of BN/GRP and GHRH and their combination on growth of PC-3 and DU-145 tumours

Antagonists of BN/GRP and GHRH and their combination significantly inhibited growth of androgen-independent PC-3 and DU-145 prostate cancers xenografted into nude mice as reflected by final tumour weights (Figs. 1 and 2). The effect of BN/GRP antagonists was stronger than that of GHRH antagonists in both models. The combination treatment with the two types of antagonists had the greatest inhibitory effect on tumour growth (Figs. 1 and 2). These results were previously reported in detail [25].

3.2. Effects of antagonists of BN/GRP and GHRH on the expression of PKC isoforms in PC-3 androgen-independent prostate cancers

PKC isoenzymes were investigated by immunoblotting of s.c. grown PC-3 tumours from animals treated with antagonists of BN/GRP, GHRH or a combination of both compounds. Monoclonal antibodies to βI- and βII-PKC isoforms and polyclonal antibodies to α-, β-, γ-, δ-, ε-, η-, and ζ-PKC isoforms were used. Significant

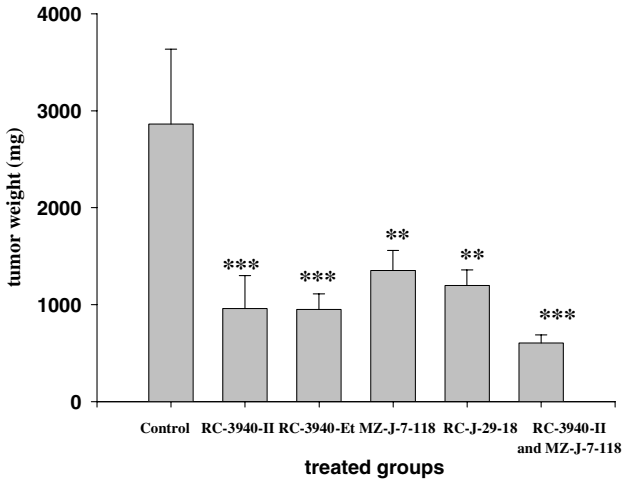

Fig. 1. Final average tumour weights of mice bearing s.c. PC-3 human androgen-independent prostate cancers treated with BN/GRP antagonists RC-3940-II and RC-3940-Et, GHRH antagonists MZ-J-7-118 and RC-J-29-18, or a combination of RC-3940-II and MZ-J-7-118. Vertical bars, SE, $^{**}P < 0.01$ *vs.* controls, $^{***}P < 0.001$ *vs.* controls.

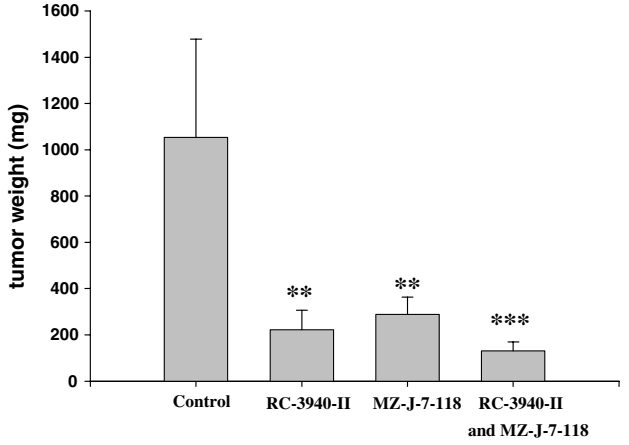

Fig. 2. Final average tumour weights of mice bearing s.c. DU-145 human androgen-independent prostate cancers treated with BN/GRP antagonist RC-3940-II, GHRH antagonist MZ-J-7-118 and a combination of RC-3940-II and MZ-J-7-118. Vertical bars, SE, $^{**}P < 0.01$ *vs.* controls, $^{***}P < 0.001$ *vs.* controls.

immunoreactive bands were detected in PC-3 tumours with specific antisera to α, δ-, η- and ζ-isoform of PKC (Fig. 3). No reactive signal was observed with antisera to β-, γ- and ε-isoforms. The bands were submitted to densitometric analyses and normalised to β-actin levels (Table 1). Fig. 3 shows the level of each specific PKC isoform as the percentage of control. BN/GRP antagonists alone or in combination with GHRH antagonist strongly decreased the levels of α- and η-PKC isoforms (Fig. 3, Table 1). GHRH antagonists alone also reduced the levels of α- and η-PKC, but only the effect of RC-J-29-18 was statistically significant. ζ-PKC was significantly decreased by antagonists of BN/GRP and GHRH, as well as by the combination of both compounds (Fig. 3, Table 1). In contrast to the other three

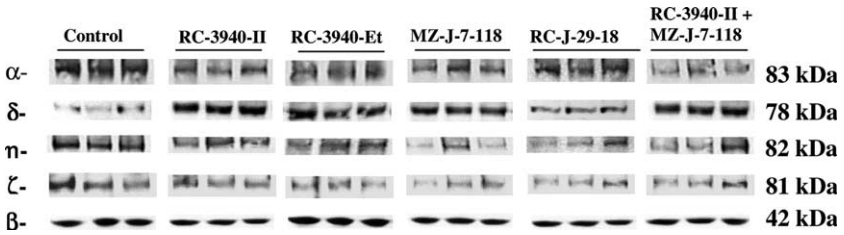

Fig. 3. Expression of PKC isoforms in PC-3 tumour samples from animals untreated or treated with BN/GRP antagonists RC-3940-II and RC-3940-Et, GHRH antagonists MZ-J-7-118 and RC-J-29-18, or a combination of RC-3940-II and MZ-J-7-118. Western blot analysis was performed on the whole tissue homogenates of tumours using antibodies for α-, δ-, η-, and ζ-PKC isoforms. The molecular masses of PKC isoforms and β-actin (internal control) are shown.

Table 1
Protein levels of PKC α, δ, η and ζ in PC-3 tumours after treatment with BN/GRP antagonists RC-3940-II and RC-3940-Et, GHRH antagonists MZ-J-7-118 and RC-J-29-18 and the combination of MZ-J-7-118 with RC-3940-II

	Protein (% of Control)					
	Control	RC-3940-II	RC-3940-Et	MZ-J-7-118	RC-J-29-18	MZ-J-7-118 + RC-3940-II
α-PKC	100 ± 9	49.6 ± 4.0**	38.5 ± 2.4**	94.1 ± 4.5	73.2 ± 12.0*	37.8 ± 4.7**
δ-PKC	100 ± 21	192.6 ± 14.7*	226.9 ± 25.6**	206.5 ± 16.2*	117.2 ± 15.9	232.8 ± 22.3**
η-PKC	100 ± 16	51.3 ± 3.1**	51.6 ± 7.4**	82.6 ± 11.5	60.7 ± 9.5*	40.9 ± 5.7**
ζ-PKC	100 ± 4	63.9 ± 9.2*	66.3 ± 5.0**	39.7 ± 8.1**	57.9 ± 17.6**	45.4 ± 9.5**

* $P < 0.05$.
** $P < 0.01$ *vs.* control.

isoforms, the level of δ-PKC was up-regulated by all compounds and this effect was significant in all treatment groups except for RC-J-29-18 (Fig. 3, Table 1). The inhibition of α-, η- and ζ-isoforms, as well as the increase in the levels of δ-PKC expression was not more pronounced in the group that received the combination of RC-3940-II and MZ-J-7-118 as compared to the single agents (Fig. 3, Table 1).

3.3. Effects of antagonists of BN/GRP and GHRH on the expression of p42/44 MAPK in PC-3 and DU-145 tumours

We used a site specific antibody recognising phosphorylated forms of p42/p44 MAPK to estimate changes in protein expression after treatment of tumours with antagonists of BN/GRP and GHRH. To examine the basal expression of MAPK in DU-145 and PC-3 models, Western blots were performed on control tumours and on cells cultured *in vitro*.

The expression of phosphorylated MAPK in PC-3 tumour cells was very low, being more than 10-fold lower than in DU-145 tumours (data not shown). Therefore, the effects of treatments on the MAPK levels in PC-3 tumours could not be investigated. However, high basal levels of MAPK were found in the DU-145 model, and the effects of treatments on the MAPK expression in this tumour model were evaluated (Fig. 4). BN/GRP antagonist RC-3940-II, GHRH antagonist MZ-J-7-118 and the combination of both compounds very potently decreased the expression of MAPK in DU-145 tumours (Fig. 4).

3.4. Effects of PKC inhibitors and activators, and MAPK inhibitor on the proliferation of PC-3 and DU-145 cells

Cell proliferation assays *in vitro* were carried out to investigate the effects of PKC inhibitors BIM and PKC(19-31); PKC activators PMA and PDBU; and the MAPK inhibitor PD98059 on PC-3 and DU-145

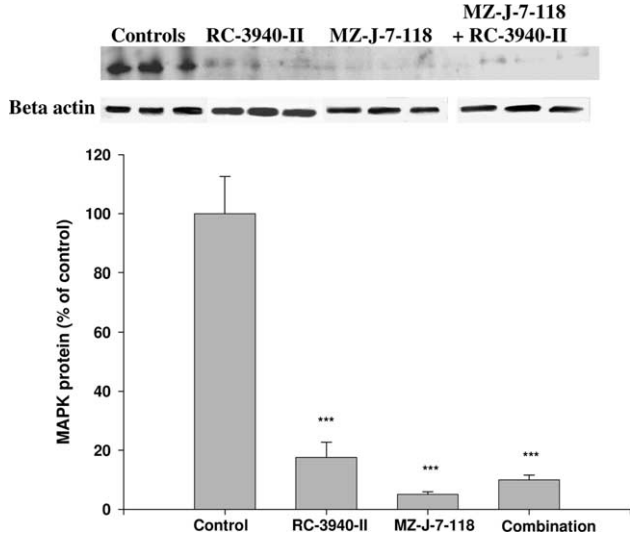

Fig. 4. Expression of MAPK isoforms in DU-145 tumours untreated or treated with BN/GRP antagonists RC-3940-II, GHRH antagonists MZ-J-7-118, or a combination of RC-3940-II and MZ-J-7-118. Western blot analysis was performed on the whole tissue homogenates using antibodies for p42/44MAPK. The molecular masses of p42/44MAPK isoforms and β-actin (internal control) are shown. Vertical bars, SE, *** $P < 0.001$ *vs.* controls.

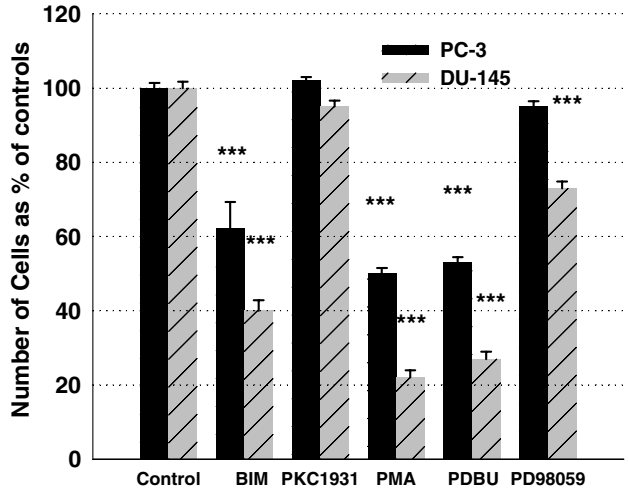

Fig. 5. Effects of PKC inhibitors BIM and PKC (19-31) (10 μM), PKC activators PMA and PDBu (0.1 μM) and MAPK inhibitor PD98059 (50 μM) on the cellular proliferation of PC-3 and DU-145 cells *in vitro*. Cultured cells were exposed to the vehicle or the test compounds, in octuplicate wells each and the cell growth was determined by Crystal Violet assay. Data are expressed as percentage *T*/*C* values from three experiments where *T* represents absorbance of treated cultures and *C* represents absorbance of control cultures. The measured absorbance is proportional to the number of living cells in the assay performed. Vertical bars, SE, $^{***}P < 0.001$ *vs.* controls.

cells. Significant cell growth inhibition was found in both models after 85 h exposure to 10 μM BIM, but not to10 μM PKC(19-31). PKC activators PMA and PDBu at 0.1 μM each, which are known to down-regulate the expression of PKC after long-time exposure such as that used in our experiments, also significantly diminished the growth of PC-3 and DU-145 cells as compared to controls. Inhibition by PD98059 at 50 μM was only significant in DU-145 cells (Fig. 5).

3.5. Effects of antagonists of BN/GRP and GHRH on the expression of c-fos and c-jun mRNA levels in PC-3 and DU 145 tumours

We evaluated the effect of BN/GRP antagonists RC-3940-II and RC-3940-Et; the GHRH antagonists MZ-J-7-118 and RC-J-29-18; and the combination of RC-3940-II and MZ-J-7-118 on the mRNA expression of the oncogenes *c-fos* and *c-jun* in PC-3 and DU-145 tumours. PCR products of 612bp and 409bp, corresponding respectively to the *c-fos* and *c-jun*, were detected in all tumours. Fig. 6 shows RT- PCR products in controls and treated tumours, and Table 2 presents the expression levels of mRNA for *c-fos* and *c-jun* after normalisation to the corresponding β-actin levels (459 bp internal control). The expression of mRNA for *c-fos* was not affected, but there was a tendency for the inhibition of *c-jun* mRNA expression in both tumour models after treatment with the antagonists. However, none of the inhibitory effects was significant due to the high variation of the samples (Fig. 6(a)–(c), Table 2). In the experiment with DU-145 tumours, mRNA levels for *c-fos* and *c-jun* were also measured by real-time PCR. The results are shown in Table 3. Real time PCR revealed a down-regulation in *c-jun* after all the treatments. A single treatment with RC-3940-II or MZ-J-7-118 produced a 3.3-fold and 1.4-fold decrease in *c-jun* expression, respectively, and the combination of both these analogs showed a 1.9-fold down-regulation of *c-jun* expression. The expression of *c-fos* was only slightly down-regulated by the combination of both analogs (1.2-fold). The

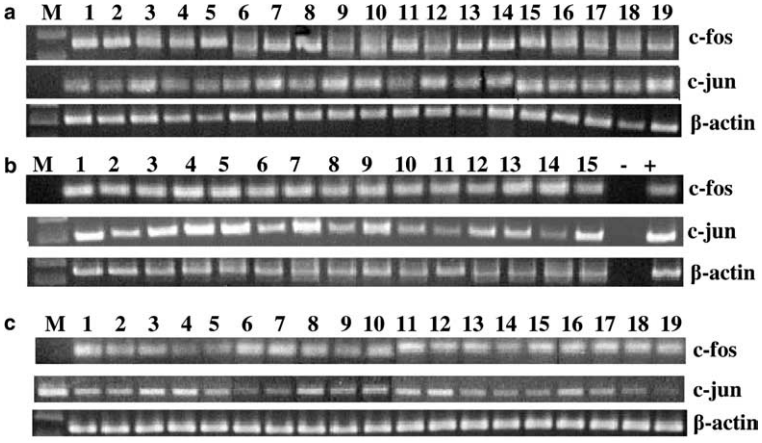

Fig. 6. Expression of mRNA for *c-fos* and *c-jun* oncogenes in PC-3 (a,b) and DU-145 (c) tumours grown in nude mice after treatment with BN/GRP antagonists RC-3940-II and RC-3940-Et, GHRH antagonists MZ-J-7-118 and RC-J-29-18 or the combination of RC-3940-II and MZ-J-7-118. Total RNA was reverse transcribed, and cDNA was amplified with specific primers. The PCR products were resolved on a 1.8% agarose gel and stained with ethidium bromide. (a) M, molecular marker; lanes 1–5, control; lanes 6–10, RC-3940-II; lanes 11–14, RC-3940-Et; lane 15–19, MZ-J-7-118. (b) Lanes 1–5, control samples; lanes 6–10, RC-J-29-18; lanes 11–15, combination of RC-3940-II and MZ-J-7-118. H-69 small cell lung cancer cell line was used as a positive control for β-actin,*c-fos*, and *c-jun*. (c) Lanes 1–5, control samples; lanes 6–10, RC-3940-II, lanes 11–15, MZ-J-7-118 and lanes 16–19, combination of RC-3940-II and MZ-J-7-118.

Table 2
Levels of *c-fos* and *c-jun* MRNA in PC-3 and DU-145 prostate tumours grown in nude mice treated with antagonists of bombesin/GRP RC-3940-II and RC-3940-ET, antagonists of GHRH MZ-J-7-118 and RC-J-29-18 and the combination of RC-3940-II and MZ-J-7-118 (values are % of controls)

Treatment	*c-fos* (PC-3)	*c-fos* (DU-145)	*c-jun* (PC-3)	*c-jun* (DU-145)
Control	100 ± 10.1	100 ± 4.2	100 ± 8.0	100 ± 10.3
RC-3940-II	107.0 ± 5.1	97.4 ± 0.8	84.7 ± 11.0	65.7 ± 5.8
RC-3940-Et	92.2 ± 10.4	n.i.	75.2 ± 16.0	n.i.
MZ-J-7-118	106.9 ± 3.6	92.9 ± 12.0	92.0 ± 4.3	83.8 ± 16.0
RC-J-29-18	93.9 ± 9.5	n.i.	89.5 ± 13.3	n.i.
RC-3940-II and MZ-J-7-118	98.9 ± 8.6	97.1 ± 5.1	74.0 ± 16.4	45.3 ± 36.1

Table 3
Real time PCR values of the relative expression and ratio of mRNA for *c-jun* and *c-fos* in DU-145 tumour tissue after treatment with bombesin/GRP antagonists RC-3940-II, GHRH antagonist MZ-J-7-118, or the combination of the two analogs

	Control	RC-3940-II		MZ-J-7-118		Combination	
	ΔC_t	ΔC_t	Ratio	ΔC_t	Ratio	ΔC_t	Ratio
c-jun	7.94 ± 4.20	9.47 ± 0.16	0.32	8.45 ± 2.00	0.70	8.89 ± 4.58	0.52
c-fos	7.39 ± 3.04	7.33 ± 0.79	1.17	6.08 ± 1.52	2.57	7.71 ± 3.29	0.83

Samples were normalised to beta actin, where the ΔC_t is the difference in the cycle threshold of beta actin and each target gene. The ratio represents the gene expression level in the treatment group as compared to the control group. Data is presented as the means ± SE of three samples each run in triplicate.

treatment with RC-3940-II and MZ-J-7-118 yielded a 1.2 and 2.6-fold up-regulation of *c-fos*, respectively. However none of these findings were statistically significant because of large standard errors.

4. Discussion

In the present study, we investigated whether PKC and MAPK signalling transduction and the expression of immediate-early oncogenes such as *c-fos* and *c-jun* are involved in the anti-proliferative effect of antagonists of BN/GRP and of GHRH on PC-3 and DU-145 human androgen-independent prostate cancers. Previously, we reported that these antagonists significantly suppressed the growth of PC-3 and DU-145 tumours, the combined therapy with GHRH antagonist MZ-J-7-118 and BN/GRP antagonist RC-3940-II causing the greatest tumour inhibition [25].

PKC isoforms that are overexpressed in advanced prostate cancer [34] appear to be involved in cell function, growth, apoptosis and neoplastic transformation in the prostate gland [7]. PKC is implicated in prostate cancer etiology because its activation is vital for the growth of androgen-independent prostate cancer cells [35]. In recent years, there has been a growing interest in the application of PKC inhibitors in cancer treatment. This is reflected in the fact that at present at least 4 PKC inhibitors with different isoform specificities are in clinical trials for cancer treatment [36]. Growth inhibition of PC-3 and DU-145 prostate cancer cells by tamoxifen was also associated with inhibition of PKC and induction of $p21^{(waf1/cip1)}$ [37].

In our present work, we evaluated the expression of PKC isoforms in PC-3 tumours by immunoblotting and found the expression of α, δ, η and ζ isoforms. Our results clearly demonstrate a significant down-regulation of α-, η- and ζ-PKC isoforms and a significant up-regulation of the δ-PKC isoform after treatment with antagonist of BN/GRP, GHRH, or their combination. These findings suggest that the inhibitory effect of BN/GRP- and GHRH antagonists on the tumour growth involves changes in the expression of some PKC isoforms.

The inhibition of PKC α could contribute to the tumour inhibitory effect of our antagonists, since the role of PKC α in the proliferation, tumourigenicity and metastasis of prostatic and other cancers including melanomas and lung carcinomas, is well established [11,36,38,39].

Depending on its responsiveness to hormones, prostate cancer can show PKC α-dependent pro-apoptotic or antiapoptotic properties [38]. An overexpression of PKC α was found in androgen-independent human prostate cancer [39]. If constitutive PKC α activity is required for the survival and growth of androgen-independent human prostate cancer, the inhibition of PKC α could be useful for the clinical treatment of prostate cancer. An oligodeoxynucleotide directed against human PKC α (ISIS 3521) inhibited tumour growth of PC-3 prostate cancers and increased the effect of cisplatin [40]. ISIS 3521 is currently in clinical trials for the treatment of patients with hormone-refractory prostate cancer and other advanced solid tumours [11,36].

In contrast to PKC α, the effect of other PKC isoforms on the growth of androgen-independent prostate cancer is not well described. Therefore, the possible

significance of up-regulation of PKC δ and the decrease of PKC η and ζ isoforms for the antitumour mechanism of antagonists of BN/GRP and GHRH remains to be elucidated. It was shown that overexpression of PKC δ reduced the growth of LNCaP androgen-independent prostate cancer cells by enhancing the apoptotic effect of phorbol esters [41]. Down-regulation of PKC η potentiated the cytotoxic effects of exogenous tumour necrosis factor-related apoptosis-inducing ligand in PC-3 prostate cancer cells [42]. Significantly higher levels of PKC isoforms α, β, ε, and η were found in samples of prostate carcinoma compared to benign prostate hyperplasia [43]. PKC ζ levels are also significantly increased in tumour specimens of prostate cancer patients [34].

In the present study, we also investigated the effects of PKC activators and inhibitors on the growth of PC-3 and DU-145 cells *in vitro* and found that the PKC inhibitor BIM significantly decreased cell growth while another PKC inhibitor PKC (19–31) did not affect the cell growth *in vitro*. This could be due to the selectivity of different PKC inhibitors to different isoforms. Proliferation of PC-3 and DU-145 cells was significantly decreased after 85 h of incubation with PKC activators PMA and PDBu, probably due to a down-regulation of PKC isoforms. The antiproliferative effect of PKC activator TPA on PC-3 and DU-145 cells was also reported by others [44].

Mitogen activated kinases are multifunctional effectors that participate in cellular responses to external stimuli. MAP kinase activity correlated with the growth of prostate cancer in *in vitro* studies [45] and an overexpression of MAPK is found in patients with advanced and androgen-independent prostate cancers [14]. It was also reported that androgen receptor activation by growth-promoting compounds requires a functional MAPK signalling pathway [46]. In our study, we detected a very low expression of phosphorylated MAPK in PC-3 cells grown *in vitro* as well as in PC-3 tumours grown in nude mice as compared to DU-145 cells and tumours. This difference in constitutive activation of MAPK in PC-3 and DU-145 cells is in line with earlier findings as described by others [17,47]. However, Zelivianski and colleagues [48] found a higher expression of phosphorylated MAPK in PC-3 cells. These differences might have been affected by a variability of MAPK expression in different PC-3 cell clones and may be changed by several passages of tumour cells. The basal pERK expression in PC-3 cells used in the present study was very low and could be even lower in tumours passaged in nude mice.

The proliferation of PC-3 cells *in vitro* was not affected by the MAPK inhibitor PD98059. Due to the very low basal expression of MAPK in PC-3 tumours, we could not investigate in this model the effects of antagonists of BN/GRP and GHRH on the MAPK expression levels. In contrast, MAPK is constitutively activated in the DU-145 model [16,17]. We found readily detectable levels of MAPK in DU-145 tumours and the proliferation of DU-145 cells was significantly inhibited by PD98059, indicating that the MAPK pathway is important for the proliferation of this cell line. In DU-145 tumours, RC-3940-II, MZ-J-7-118 and their combination powerfully decreased the expression of MAPK compared to controls. Thus, in the DU-145 model, inhibition of MAPK could be one of the main intracellular mechanisms by which BN/GRP and GHRH antagonists inhibit proliferation of tumours. However, other intracellular mechanisms must be involved in the antitumour action of these growth factor antagonists in the PC-3 model which expresses very low levels of MAPK and is unresponsive to treatment with MAPK inhibitor PD98059. Our findings demonstrate the effect of GHRH- and BN/GRP antagonists on MAPK.

Pituitary GHRH receptors are coupled to adenylylcyclase and have similar signal transduction pathways to mitogenic stimuli comprising protein kinase A (PKA), the Ras/Raf kinases complex, and MAPK [49–51]. Similarly, bombesin, which is overexpressed in prostate cancer, has been found to activate MAPK [16]. Increased PKC and MAPK signalling was reported to be associated with an accumulation of mRNA for *c-fos* and *c-jun* proto-oncogenes which are the most distal components of diverse converging mitogenic signal transduction pathways [52–54]. In a recent study, we showed that BN/GRP- and GHRH antagonists produced a down-regulation of PKC isoforms, MAPK and *c-fos* and *c-jun* transcription factors in lung cancer [6]. BN/GRP antagonists have also been shown to decrease mRNA for immediate early genes such as *c-fos* and *c-jun* in human breast cancer [12] and exposure of ovarian cancer cells to GRP results in a stimulation of mRNAs for *c-fos* and *c-jun* [55]. In the present study, treatment with antagonists of BN/GRP and GHRH tended to decrease the expression of *c-jun* in PC-3 and DU-145 tumours but *c-fos* was not affected. However, it should be mentioned that effects on immediate early oncogenes are difficult to be demonstrated *in vivo*, because these effects are very transient and could be abolished in the time between the last treatment with antagonists and the harvesting of tumours [56].

We utilised the PC-3 and DU-145 models for the investigation of intracellular signalling in this work since these cell lines represent human androgen-independent prostate cancers at an advanced stage and have been extensively used for the evaluation of BN/GRP and GHRH antagonists in our previous studies. Our results indicate that antagonists of BN/GRP and GHRH antagonists exert their antiproliferative effects in part by inhibiting the expression of PKC isoforms and by affecting subsequent steps of intracellular signalling including MAPK, and *c-jun* oncogene. Further studies are necessary to elucidate the full mechanisms of action

of antagonists of bombesin and of GHRH and to develop more effective analogs for prostate cancer treatment.

Conflict of interest statement

None declared.

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