B1 but not B2 bradykinin receptor agonists promote DU145 prostate cancer cell proliferation and migration

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Abstract

Background: The kallikrein-kinin system (KKS) is an endogenous pathway involved in angiogenesis and tumourigenesis, both vital for cancer growth and progression.

Objectives: To investigate the effect of two bradykinin receptor (B1R and B2R) agonists on growth and motility of prostate tumour (DU145) and micro-vascular endothelial cells (dMVECs).

Methods: Increasing concentrations of selective B1R and B2R agonists were added to cultured cells. Cell proliferation and migration were assessed using the 3-[4,5 dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) and modified Boyden Chamber assays, respectively. Where significant stimulation was found, the influence of an antagonist was also investigated.

Results: Neither growth nor motility of endothelial cells was affected by either agonist. In DU145 cells, while the B2R agonist was without any significant effect, the B1R agonist stimulated proliferation and migration at concentrations of 10nM and 50nM respectively. Further, this effect was abrogated when cells were pre-incubated with a B1R antagonist.

Conclusions: Unlike the physiologically-active B2R, the pathologically-inducible B1R may be implicated in prostate tumourigenic events. The involvement of the KKS in malignant prostate pathology supports on-going exploration of bradykinin receptor antagonists as target candidates in the development of alternate approaches to cancer therapy.

Keywords: Bradykinin receptor antagonists, Kallikrein, Prostate tumour, Angiogenesis, Endothelial, Conditioned medium

DOI: http://dx.doi.org/10.4314/ahs.v14i3.22

Introduction

The kallikrein-kinin system (KKS) consists of two serine proteases, plasma and tissue kallikrein (TK), that release bio-active kinin peptides by enzymatic cleavage of hepatically-derived kininogens [1]. Stimulation of G protein-coupled bradykinin receptors (BR), B1R and B2R, mediate the effects of bradykinin (BK), which include promotion of cell growth, proliferation and migration [1-3]. Further, TK activates matrix metallo-proteinases (MMPs) involved in extra-cellular matrix (ECM) degradation [4,5].

The expression profiles of TK and BK are altered in numerous human cancers, and several studies have implicated the KKS in growth and metastasis of prostate tumours: BK and BR agonists have been shown to stimulate proliferation, migration and invasion of PC3 prostate cancer cells *in vitro* [3,6,7], while some have reported that an interaction with both BR sub-types may be vital for the proliferation of prostate cancer cells [6]. In addition, BR antagonist peptides have shown anti-cancer activity in athymic nude mice implanted with prostate cancer PC3 cells [8].

The KKS has been implicated in angiogenesis, the growth of new blood vessels from the pre-existing vasculature [9,10]. Several studies have demonstrated a role for TK in neo-vascularisation following hind-limb ischemia in murine models [11-13]. The KKS may also be involved in tumour-associated angiogenesis, which is a pre-requisite for tumour growth and metastasis [14-16]. The Ishihara group showed that a selective B2R, but not B1R, antagonist suppressed tumour-angiogenesis in mice implanted with sarcoma 180 cells [15]. They later

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suggested that BK enhanced angiogenesis by promoting tumour vascular permeability[16]. More recently TK, B1R and B2R have been shown to be present in the membrane projections of both endothelial and prostate cancer cells *in vitro* [10].

This study further explores the role of the KKS in prostate cancer and angiogenesis by investigating the effect of kinin receptor agonists and antagonists on prostate tumour (DU145) and micro-vascular endothelial (dMVEC) cell lines *in vitro*.

Methods

Ethics

Ethical approval was granted by the Biomedical Research Ethics Committee, University of KwaZulu-Natal (reference number BE152/08).

Cell Culture

Micro-vascular endothelial cells (dMVECs) and prostate cancer (DU145) cells were obtained from Clonetics (BioWhittaker, Walkersville, USA) and Highveld Biological (Sandringham, South Africa) respectively. dMVECs and DU145 cells were cultured in endothelial basal medium (EBM-2) and Dulbecco's Modified Eagle's Medium (DMEM), respectively, as previously described [10].

Agonists and Antagonists

Selective B1R (Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe) and B2R (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-ψ(CH-NH)-Arg) agonists were commercially obtained from Tocris Bioscience, USA. These were added to both cell types in increasing concentrations to test their effect on proliferation and migration. Concentration ranges were 10 to 100nM for DU145 and 50 to 1000nM for dMVECs cells. Pre-treatment with the B1R antagonist, des-Arg^9-[Leu^8]-BK acetate salt, at a concentration of 10□ M, was used to test the specificity of stimulation observed with the B1R agonist. In addition, vascular endothelial growth factor (VEGF) was tested as a positive proliferation control. BR antagonists and VEGF were purchased from Sigma-Aldrich, USA.

Cell Proliferation

The MTT assay was performed as previously described [17]. Briefly, dMVEC and DU145 cells were seeded in 96-well culture plates (Corning, NY, USA) at a density of 4500 cells/cm^2. At 60% confluency, cell-specific growth medium was replaced with BR agonists or VEGF. For antagonist experiments, the B1R antagonist was added to cells 30 minutes prior to the addition of agonists. Cell proliferation was determined after 24 hours incubation at 37^{o}C, 5% CO_2 using the colorimetric MTT assay. Each experiment was repeated a number of times and four replicates were done for each sample.

Cell Migration

Cell migration assays were performed using a modified Boyden chamber assay in 96-well HTS Transwell® plates (Corning) containing 8 μm polyester membranes. Briefly, dMVEC or DU145 cells were re-suspended in their respective serum-free, growth factor-free medium and added to the upper chambers (20 000 cells/well). BR agonists were prepared in 10% FBS/DMEM and 5% FBS/DMEM for dMVEC and DU145 migration assays, respectively, and added to the bottom chamber of the modified Boyden chamber. For antagonist experiments, cells were pre-treated with 10μM BR antagonist for 30 minutes before addition to the upper chambers. The plate was incubated overnight at 37^{o}C, 5% CO_2. Cells that had migrated were then dissociated from the porous membrane and quantified following 1 hour incubation with 1x cell dissociation solution (CDS; Sigma, St. Louis, USA) and 2mM fluorescent Calcein-AM working solution (BD Biosciences, USA) at a ratio of 1ml: 1μl. The receiver plate was then read using a fluorescent top reader at 485 nm excitation, 520nm emission (BMG, Germany). A standard curve was run concurrently with each experiment to extrapolate and quantify the number of cells that had migrated. Each experiment was repeated a number of times and three replicates were done for each sample.

Statistical Analysis

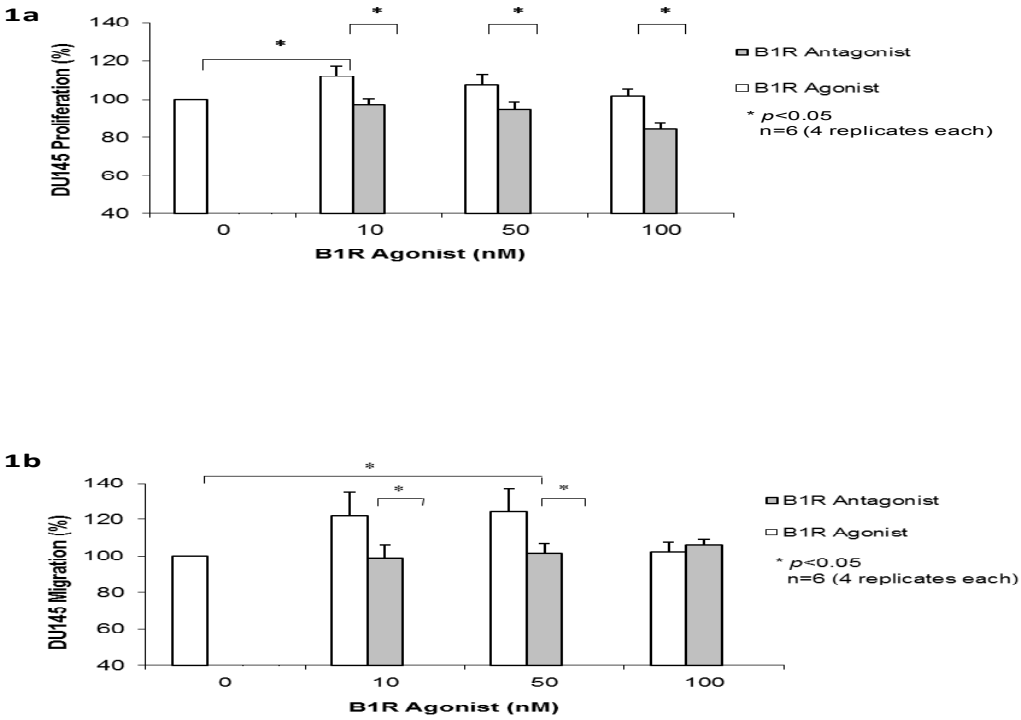
One-way analysis of variance (ANOVA) was used to test for statistical significance of differences between multiple concentration groups followed by Dunnett's post-hoc testing. Student's t-test was used for comparison between two groups. A p-value ≤ 0.05 was considered statistically significant. Statistical analysis was performed using the software package SPSS 18 (IBM, USA).

Results

Addition of the B1R agonist enhanced both proliferation and migration of DU145 cells, the effect being statistically significant at 10 and 50nM respectively. In addition, pre-treatment with a B1R antagonist abolished these effects (Figures 1a and 1b). The B2R agonist produced a much smaller but non-significant effect on

Figure 1 (a). Effect of B1R agonist on DU145 proliferation.
In the absence and presence of the B1R antagonist, a significant abrogation of agonist-induced proliferation was demonstrated with B1R blocking, as indicated in the B1R agonist concentration range of 10 to 100nM. Agonist-antagonist interaction was measured against a non-induced control.

Figure 1(b). Effect of B1R agonist on DU145 migration.
In the absence and presence of B1R antagonist, it was demonstrated that the receptor blockade results in a reduction of cell migration compared to both non-induced and agonist-induced cell motility. Thus, there was significant induction of cell migration at agonist concentrations of 10 and 50nM compared with non-induced controls, which were subsequently inhibited by the receptor antagonist.

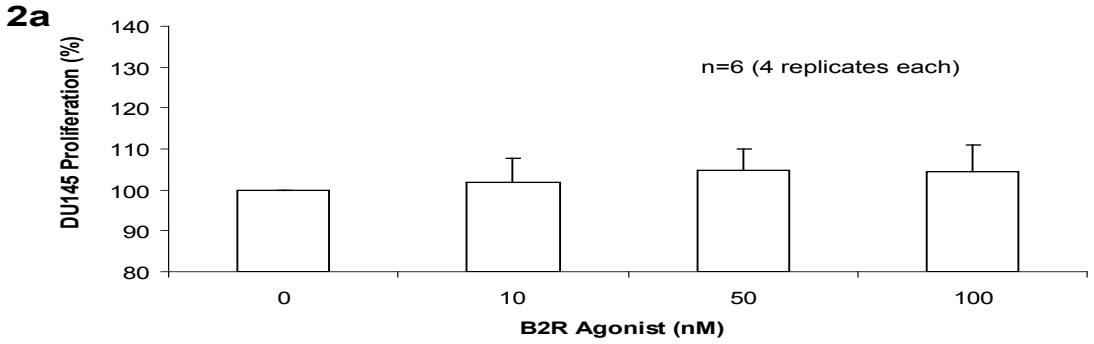


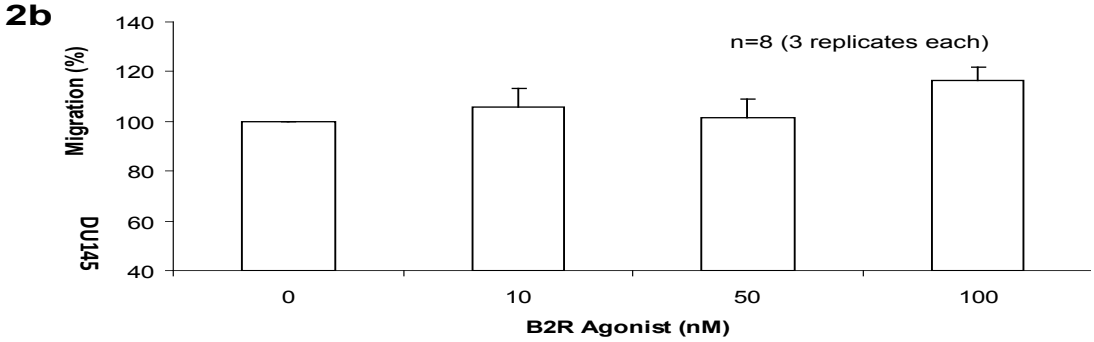
growth and migration of prostate tumour cells (Figures 2a and 2b).

In dMVECs, the B1R agonist produced a small (<6%) and statistically-insignificant proliferative effect at concentrations greater than 250nM (n=4, 4 replicates each), while the B2R agonist did not stimulate proliferation at any of the concentrations tested (n=4, 4 replicates each).

Figure 2(a): DU145 proliferationin response to B2R agonist
The B2R receptor agonist had no effect on prostate tumour cell proliferation .

Figure 2(b): DU145 migration in response to B2R agonist
The B2R receptor agonist had no effect on prostate tumour cell migration (b).





These results are shown in Figure 3. In contrast to these findings, VEGF, a well-known mitogen, stimulated dMVEC proliferation. Migration assays revealed that both BR agonists failed to stimulate endothelial cell motility (Figure 4).

Figure 3. Effect of B1R and B2R agonists on dMVEC proliferation.
No effect was observed when either bradykinin receptor agonist was used to stimulate endothelial proliferation.

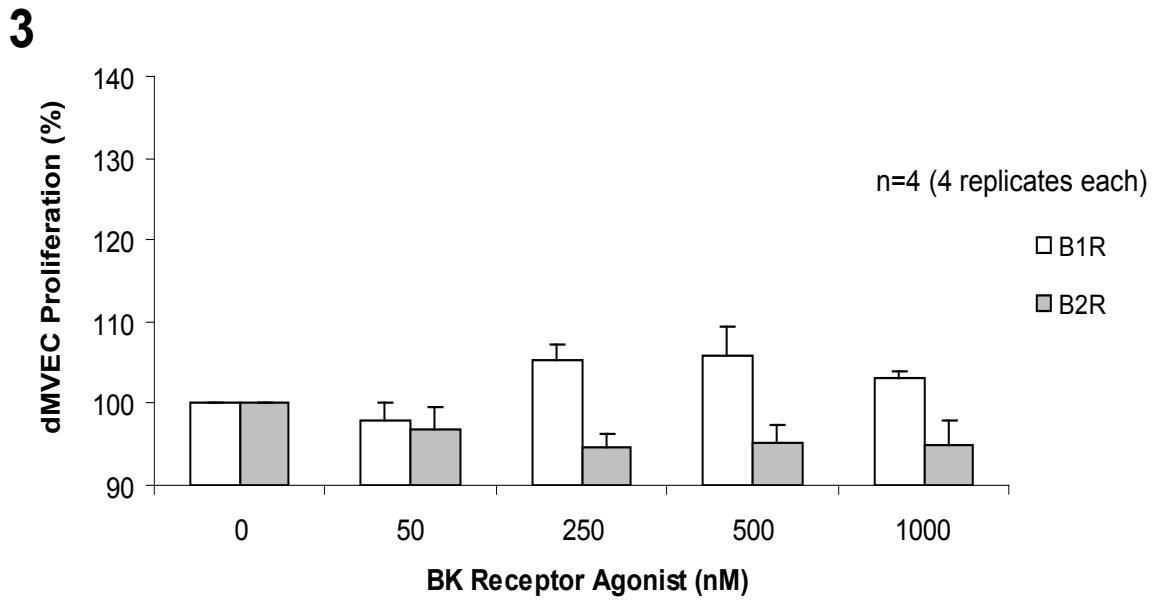
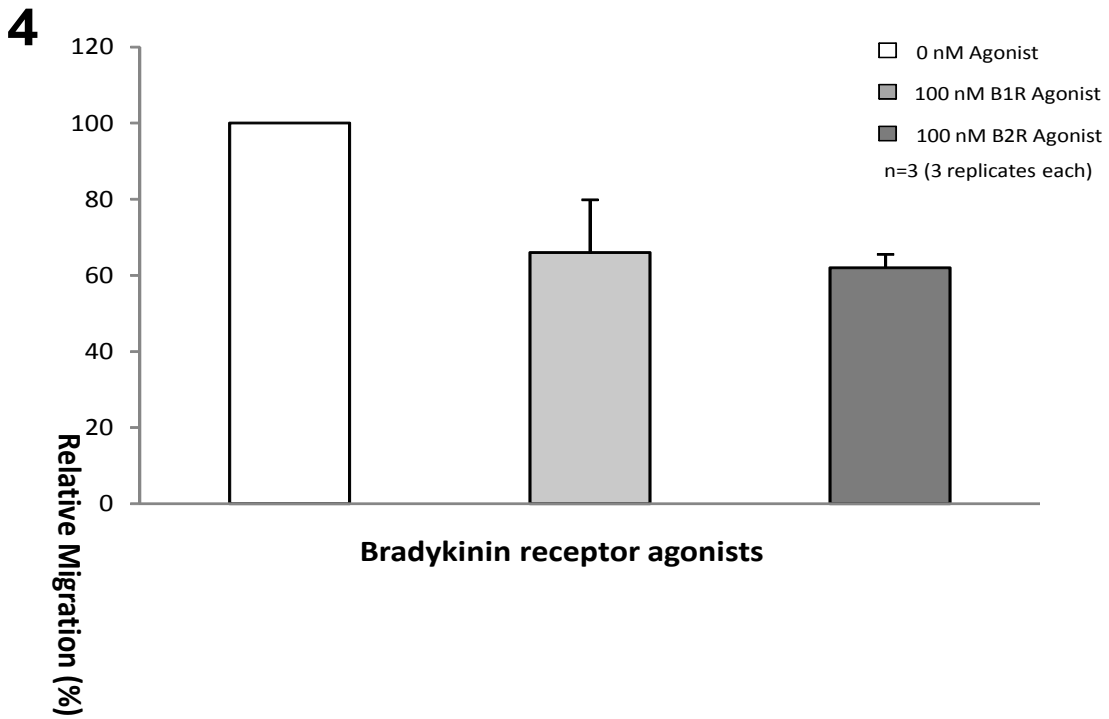


Figure 4: dMVEC migration in response to B1R and B2R agonists
No effects were observed when either bradykinin receptor agonist was used to stimulate endothelial cell migration.



Discussion

Our study supports a role for KKS in tumourigenesis: Addition of a B1R agonist (Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe) to cultured prostate cancer cells enhanced proliferation and migration, an effect that was also abrogated by the relevant antagonist. Unlike Barki-Harrington et al. [6] who found increased proliferation with stimulation of the B2R, our study failed to show significant promotion of tumour growth or motility with a B2R agonist (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-ψ(CH-NH)-Arg). However, this was not entirely unexpected because it is thought that the ubiquitous B2R mediates physiological effects while the inducible B1R plays a role in prostate cancer pathology [3].

The extent to which the B1R agonist stimulated tumour cells in our study was less than that in previous work where 1.5 fold increases were noted [3,6]. However, this could be explained by the fact that both the prostate tumour cell line and BR agonists used in the studies were different. DU145 and PC3 cell lines are both classical epithelial cell lines of prostate cancer; however, they differ in terms of their DNA profiles [18]. The effect of BR agonists on tumour cells *in vitro* may depend on several factors including the particular agonist and tumour type, and varying conditions of the experimental models. The exposure period in our experiments was notably shorter (24 hours) than those in other studies (48 hours).

In the present study, neither selective B1R nor B2R agonists induced proliferation of micro-vascular endothelial cells (dMVECs). This was in contrast to the findings in other studies implicating the KKS in angiogenesis. For example, increased endothelial cell proliferation has been reported upon the addition of a B1R, though not B2R, agonist [9,19]. Parenti et al. found that activation of B1R was involved in endothelial cell proliferation, while B2R contributes indirectly to angiogenesis via mediation of inflammatory processes [20]. It is pertinent to note, however, that these studies used larger blood vessels such as human umbilical vein endothelial cells (HUVECs) and bovine coronary post-capillary venules (CVEC). In addition, exposure periods (48 and 72 hours) were far longer than the 24 hours in our study. Interestingly, despite the difference in cell lines and conditions, our study supported the finding of Morbidelli et al. that BR agonists do not affect migration [19].

In summary, this study, the first to investigate BR agonists and antagonists in DU145 tumourigenesis, supports previous authors who have suggested the involvement of the KKS in prostate cancer. The effect of KKS may be less marked in DU145 cells than in those of more aggressive and metastatic tumours such as PC3.

Acknowledgements

The authors wish to acknowledge the National Research Foundation (South Africa) for funding part(s) of this project, as well as the University of Kwa-Zulu Natal Research Office for their continued support.

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