beta-Adrenoreceptor antagonists reduce cancer cell proliferation, invasion, and migration

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ORIGINAL ARTICLE

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Abstract

Context: Propranolol, atenolol, and ICI118,551 are non-selective β-adrenergic receptor (AR), β_1-AR, and β_2-AR antagonists, respectively.

Objective: We investigated the efficacy of propranolol, atenolol, and ICI118,551 on proliferation, migration, and invasion of non-stimulated breast (MCF7), colon (HT-29), and hepatocellular (HepG2) cancer cells.

Materials and methods: β-AR expression profiling of cells was performed by real time PCR. Cell proliferation was determined by MTT. Boyden chamber and scratch assays were performed to evaluate invasion and migration.

Results and discussion: All cell lines expressed β-ARs. ICI118,551 was the most cytotoxic, whereas atenolol was the least effective β-AR antagonist for 24, 48, and 72 h. Cell invasion was inhibited by ICI118,551 (45, 46, and 50% for MCF7, HT29, and HepG2, respectively) and propranolol (72, 65, and 90% for MCF7, HT29, and HepG2, respectively). Propranolol, atenolol, and ICI118,551 reduced migration of MCF7, HT-29, and HepG2 cells to varying extents depending on the application concentration and duration. Propranolol and atenolol reduced migration of MCF7 and HT-29 in a concentration-dependent manner, whereas migration of these cells decreased after 48 and 72 h of ICI118,551 applications.

Conclusion: Beta$_2$-AR antagonist seemed to be the most cytotoxic β-blocker on non-stimulated cancer cells. Propranolol and ICI118,551 were more effective than atenolol in inhibiting invasion and migration of non-stimulated MCF7 and HT-29 cells; ICI118,551 being the most potent. Concordantly, β_2-selective blockage seemed to be more effective for non-stimulated cells. Effect of the selective β-AR antagonists showed variation depending on the concentration, incubation time, and histological origin of cells.

Introduction

Norepinephrine and epinephrine, the catecholamines released from the central and sympathetic nervous system neurons, and from the adrenal medulla, are the endogenous natural ligands for adrenergic receptors (ARs). ARs are members of a large seven-transmembrane (7TM) superfamily of receptors linked to guanine–nucleotide-binding proteins (reviewed in Insel, 1996). Two principal types of ARs with nine subtypes, six subtypes of α-adrenergic (α_{1A}, α_{1B}, α_{1D}, α_{2A}, α_{2B}, and α_{2C}), and two subtypes of β-adrenergic (β_1, β_2, and β_3), have been identified based on the tissue distribution, function, and structure (Insel, 1996; Kyprianou & Benning, 2000; Zhang et al., 2009). In addition to their crucial roles in the neuroendocrine and hemodynamic functions, they modulate diverse cellular processes, such as DNA synthesis and cytoskeletal protein function. Binding of a ligand to the extracellular face of a 7TM receptor causes transformation of the signal into a complex network of intracellular signals through interaction with transducer G proteins and their effectors at the intracellular face (reviewed in Nygaard et al., 2009; Wallukat, 2002). Beta-AR stimulation through this transmembrane allosteric mechanism can initiate multiple signaling cascades, including the cAMP/PKA, MAPK/ERK1/2, p38/MAPK, PI3K/AKT, VEGF, Src/STAT pathways, and arachidonic acid (AA) cascade (reviewed in Wallukat, 2002). Stimulation of β-ARs in tumor cells induces cancer cell growth and invasion via arachidonic acid cascade, particularly through the activation of cyclooxygenase-2 (COX-2) (Wong et al., 2007; Yuan et al., 2010).

Although the complex process and mechanism of metastasis have not been well understood to date, host factors in the tumor environment are believed to be involved. Norepinephrine-induced migration and invasion of carcinoma cells through β-AR stimulation with increased expression of

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vascular endothelial growth factor, matrix metalloproteinase (MMP)-2, and MMP-9 have been demonstrated in a number of cell lines (Drell et al., 2003; Masur et al., 2001; Schuller & Cole, 1989; Sood et al., 2006; Yang et al., 2006).

β-AR antagonists block receptor activation. There have been three generations of β-AR antagonists, classified based on their pharmacological properties. Among these, first generation β-AR antagonists constitute non-selective β_1/β_2 blockers, such as propranolol which is commonly used for the treatment of hypertension. There is growing clinical evidence on the efficacy and use of propranolol in treating severe infantile hemangiomas (Erbay et al., 2010; Leaute-Labreze et al., 2008; Sarialioglu et al., 2010). Emergence of evidence on the role of β-AR stimulation in hemangiomas and tumor cells raised the question whether β-AR blockers play regulatory roles in the cancer cell proliferation and migration/invasiveness. Pre-clinical studies demonstrated that inhibition of norepinephrine stimulation of lung adenocarcinoma and colon carcinoma, ovarian, and pancreatic cancer cells by propranolol exerted anti-proliferative, anti-migratory, and cytotoxic effects on cells (Guo et al., 2009; Masur et al., 2001; Schuller & Cole, 1989; Sood et al., 2006). Moreover, both β_1-selective (atenolol) and β_2-selective (ICI118,551) adrenoceptor antagonists have been shown to reverse nicotine-induced tumor growth in colon cancer xenograft mice (Wong et al., 2007). Recent clinical evidence revealed that the use of beta-blockers, prior to diagnosis or concurrently with chemotherapy, could increase survival, and decrease metastasis rates in cancer patients (Barron et al., 2011; London & McGlynn, 2012; Powe et al., 2010).

Previous findings mainly emphasize the inhibitory effect of β-AR blockers on the norepinephrine/epinephrine-induced cell proliferation and migration. However, there is limited evidence on the cytotoxicity of these selective and non-selective β-AR antagonists and sole chemotherapeutic effect on cancer cells. In the present study, we aimed to investigate direct effects of propranolol, a non-selective β-AR antagonist, atenolol, a selective β_1-AR antagonist, and ICI118,551, a selective β_2-AR antagonist on cell proliferation, migration, and invasion of breast (MCF7), colon (HT-29), and hepatocellular (HepG2) non-stimulated cancer cells for the *in vitro* demonstration of potential cytotoxicity and efficiency of beta-blockers.

Materials and methods

Cell lines and culture conditions

HTB-22 (MCF7) human breast adenocarcinoma cells were obtained from HUKUK (ŞAP Institute, Ankara, Turkey), HTB-38 (HT-29) human colorectal adenocarcinoma, and HB-8065 (HepG2) human hepatocellular carcinoma cells were obtained from ATCC (Rockville, MD). MCF7 cells were maintained in RPMI-1640 medium, HT-29 cells were maintained in McCoy's-5A medium (Biochrom AG, Berlin, Germany) and HepG2 cells were maintained in Dulbecco's minimal essential medium (DMEM) (Biochrom AG). All media were supplemented with 10% heat-inactivated fetal bovine serum (FBS, Biochrom AG), L-glutamine (Biochrom AG), and streptomycin–penicillin (Biological Industries, Kibbutz Beit-Haemek, Israel). Incubation conditions at

37 °C in a humidified atmosphere of 5% CO_2 were maintained in a Heraeus incubator (Hanau, Germany).

Assay for antiproliferative effect (MTT assay)

The effects of propranolol (Sigma Aldrich, St. Louis, MO), atenolol (Sigma), and ICI118,551 (Sigma) on the proliferation of all cell lines were colorimetrically tested by biochemical reduction of MTT [3-(4,5-dimethyl-2-thiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; Sigma-Aldrich, St. Louis, MO]. The compounds were diluted from high to low concentrations horizontally in 96-well microtiter plates. Cells were seeded to each well (1×10^5) with the exception of medium controls. The plates were incubated for 24, 48, or 72 h and then 20 μL of MTT solution (5 mg/mL) was added to each well. After incubation for 4 h, 100 μL acidic sodium dodecyl sulfate (Sigma-Aldrich, St. Louis, MO) solution (10% w/v) was added to each well. The plates were further incubated overnight to allow the dissolution of formazan crystals that were produced by mitochondrial activity of the viable cells. The inhibition of cell proliferation was determined by measuring the optical density of the chromogenic product at 540 nm with an ELISA reader (Biotek Instrument ELx800, Winooski, VT). Inhibitory concentration 50 (IC_{50}; the drug concentration at which 50% of the cells are viable) values were calculated from the logarithmic trend lines of cell proliferation graphs. Assays were performed as at least triplicate independent experiments.

Analyses of AR gene expression (real-time PCR analyses)

Total RNA was isolated from MCF7, HT-29, and HepG2 cell lines with TriPure isolation reagent according to the manufacturer's instructions (Roche Diagnostics GmbH, Mannheim, Germany). The quality and quantity of RNA were determined by using NanoDrop 2000 Spectrophotometer (Thermo Scientific, Wilmington, DE). One microgram of total RNA was reverse transcribed using Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics GmbH, Mannheim, Germany). Real-time ready catalog assays which are short FAM-labeled hydrolysis probes containing locked nucleic acid were used for RT-PCR reactions (Roche Diagnostics GmbH, Mannheim, Germany). Expression of beta (ADRA1B-Assay ID: 100800 and ADRA2B-Assay ID: 100801) and alpha (ADRA1A-Assay ID: 113884 and ADRA2A-Assay ID: 114264) ARs were determined using a semi-quantitative RT-PCR by Light Cycler 480 II system (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions with a pre-incubation step at 95 °C for 10 min, followed by 45 cycles at 95 °C for 10 s, 60 °C for 30 s, and 72 °C for 1 s. TBP (Assay ID: 101145) were used for normalization of the expression data. Semi-quantitative PCR reactions were run in triplicate, and the expressions of *ADRA1A*, *ADRA2A*, *ADRA1B*, and *ADRA2B* were determined in MCF7, HT-29, and HepG2 cell lines.

Cell invasion assay

Cell invasion was determined using the CytoSelect cell invasion assay (Cell Biolabs, San Diego, CA) according to the

manufacturer's instructions. Briefly, cells were serum-starved for 24 h. Basement membranes of Boyden chambers were rehydrated with 300 μL serum-free medium, and 1×10^6 cells in appropriate serum-free medium were seeded into the upper chamber containing antagonists or agonist (isoproterenol, a non-selective agonist) for β adrenoceptors. Bottom wells were filled with medium supplemented with 10% FBS. Propranolol concentrations were 150, 50, and 15 μM for MCF7, HT-29, and HepG2 cells, respectively. Atenolol concentrations were 600 μM MCF7 and HT-29, and 2400 μM for HepG2 cells. About 90, 20, and 10 μM ICI118,551 were applied to MCF7, HT-29, and HepG2 cells, respectively. Ten μM isoproterenol (Sigma) were applied (Lung et al., 2005; Yang et al., 2009). After 24 h incubation (37 °C, 5% CO$_2$), non-invasive cells were removed from the upper chamber and cell invasion was assessed by light microscopy after staining of invaded cells with cell stain solution. For colorimetric quantification of invasion, inserts were then placed in extraction buffer (200 μL, for 10 min), and absorbance (A) at 560 nm was determined after transfer to a 96-well plate (100 μL/well) using an ELISA reader (Biotek Instrument ELx800, Winooski, VT). Changes in cell invasion due to antagonist and isoproterenol applications were expressed as percent relative to untreated control as follows:

$$\text{Invasion } (\%) = (A_{\text{treatment}}/A_{\text{control}}) \times 100$$

All assays were performed as duplicates.

In vitro scratch assay

The *in vitro* scratch-wound healing assay was used to study cell migration. Cells were seeded in a six-well plate at a density of 1×10^6 cells per well. Linear scratch wounds were made in the cell monolayer using a pipette tip. Scratch wounds were then visualized using an Olympus CK40 inverted microscope, and baseline photomicrographs were captured by a digital camera (Eclips E600, Nikon, Kanagawa, Japan). Receptor antagonists and isoproterenol were applied into separate wells. Propranolol concentrations were 100, 150, and 200 μM for MCF7; 50, 100, and 150 μM for HT-29 and 10, 20, and 50 μM for HepG2 cells. Atenolol concentrations were 200, 400, and 600 μM for MCF7; 400, 600, and 800 μM for HT-29; and 800, 1000, and 1500 μM for HepG2 cells. About 50 and 100 μM ICI118,551 for MCF7; 25 and 50 μM for HT-29; and 10 and 20 μM for HepG2 cells were applied. Isoproterenol was applied at 10 μM concentration (Lung et al., 2005; Yang et al., 2009) to all cell lines. Scratch wounds were captured at 24 h intervals during 72 h time. The areas of the scratch-wounds were analyzed by ImageJ software (National Institutes of Health, Bethesda, MD). For quantification, the distance between the wound edges was measured at least 10 random points, and the mean values were calculated (d). The percentage of wound healing (WH) was calculated as

$$\% \text{ WH} = \left[(d_{\text{original wound}} - d_{\text{healing}})/d_{\text{original wound}} \right] \times 100$$

Statistical analyses

Homogeneities of data were controlled by Levene's test. The normality of distributions of the variables was analyzed by the Kolmogorov–Smirnov test. The results of the normality test and homogeneity test were used to decide which statistical methods to apply in comparing groups. Normal distributed groups were compared by means of independent samples and Student's t-test, and the results were expressed as $\chi \pm S_x$: mean $\pm$ standard error of the means. Non-normal distributed groups with heterogeneous variances were compared by means of the Mann–Whitney U-test, and the results were expressed as $\chi \pm S_x$: mean $\pm$ standard error of mean. A two-proportion z-test and the Fisher exact test were used for comparing the ratios. Value of p less than 0.05 was considered to be statistically significant. Statistical analyses were performed by SPSS 17.0 and MINITAB 13.0 statistical software programs (SPSS Inc., Chicago, IL).

Results

Antiproliferative effects of β-AR antagonists on MCF7, HT-29, and HepG2 cells

MTT findings demonstrated that propranolol caused a concentration-dependent reduction in proliferation of MCF7, HT-29, and HepG2 cells. As displayed in Table 1, after 24 h incubation, propranolol was more cytotoxic on HT-29 and HepG2 cells than it was on MCF7 ($p < 0.05$). However, cytotoxicity of propranolol on MCF7 and HT-29 cell lines increased with increasing incubation periods ($p < 0.05$). Results obtained after 72 h of incubation were indifferent for the cell lines.

Atenolol application caused concentration-dependent reductions in cell proliferation in all cell lines. However, according to IC$_{50}$ values, atenolol was found to be less cytotoxic at all cell lines tested when compared with propranolol (Table 1) ($p < 0.05$). In addition, cytotoxicity of atenolol increased with increasing incubation periods ($p < 0.05$). IC$_{50}$ obtained with HepG2 cell line was significantly higher than that of MCF7 and HT-29 cell lines ($p < 0.05$).

MTT results demonstrated high cytotoxicity of ICI118,551 on three cell lines tested. Cytotoxicity of ICI118,551 on MCF7 cell line increased after 48 and 72 h when compared with 24 h ($p < 0.05$). In contrast, IC$_{50}$ values obtained after 24, 48, and 72 h for HT-29 and HepG2 cell lines were statistically indifferent ($p > 0.05$).

Table 1. IC$_{50}$ values for propranolol, atenolol, and ICI118,551.

		IC$_{50}$ (μM) $\pm$ SEM[a]		
		24 h	48 h	72 h
MCF7	Propranolol	157.6 ± 2.6	51.3 ± 5.2	30.0 ± 6.2
	Atenolol	579.0 ± 38.9	437.5 ± 8.9	223.3 ± 29.9
	ICI118,551	87.6 ± 5.5	25.0 ± 3.3	25.7 ± 0.2
HT-29	Propranolol	56.9 ± 2.9	42.8 ± 2.6	20.10 ± 4.9
	Atenolol	599.2 ± 87.7	470.5 ± 26.1	277.2 ± 41.9
	ICI118,551	21.9 ± 1.5	25.2 ± 0.7	25.8 ± 1.0
HepG2	Propranolol	15.40 ± 2.4	21.0 ± 5.8	27.81 ± 5.2
	Atenolol	2023.8 ± 108.2	1540.0 ± 276.8	1346.5 ± 92.9
	ICI118,551	11.6 ± 0.4	12.0 ± 0.6	11.6 ± 0.4

[a]SEM (standard error of the means) was derived from at least three independent experiments.

MCF7, HT-29, and HepG2 cells expressed both β₁- and β₂-AR genes

The real-time PCR analysis demonstrated that all the cell lines expressed β₁- and β₂-AR genes (*ADRA1B* and *ADRA2B*, respectively). In addition, MCF7 and HepG2 cells expressed α₁- and α₂-AR genes (*ADRA1A* and *ADRA2A*, respectively). *ADRA1A* expression was not determined in HT-29 cells.

qRT-PCR analysis (Figure 1) revealed that alpha and beta ARs show different expression pattern in MCF7, HT-29, and HepG2 cell lines. Low level of *ADRA1A* expression was detected in MCF7 and HepG2 cells, but we could not detect *ADRA1A* expression in HT-29 cells. The highest expression of *ADRA2A* and *ADRA2B* was found in HT-29 cell line. *ADRA2A* and *ADRA2B* genes expressed in HT-29 cells approximately 110- and 3-fold higher than MCF7 cells, and 570- and 14-fold higher than HepG2 cells, respectively. In contrast, the *ADRA1B* expression was found to be higher in MCF7 cells, which is expressed 2-fold higher than HT-29 cells and 10-fold higher than HepG2 cells.
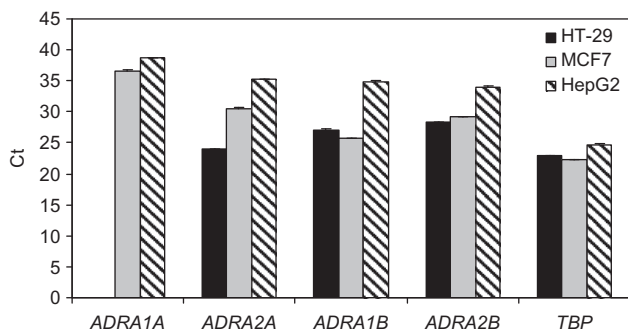

Figure 1. qRT-PCR analysis of ARs in MCF7, HT-29, and HepG2 cell lines.

Table 2. Effects of β-AR antagonists and isoprotrenol on invasion of MCF7, HT-29 and HepG2 cells incubated for 24 h. Changes in cell invasion due to antagonist and isoproterenol applications were expressed as percent relative to untreated control.

Cell line	% Invasion (relative to non-treatment control) ± SEM[a]			
	Propranolol	Atenolol	ICI118,551	Isoproterenol
MCF7	72.0 ± 0.2*	93.8 ± 0.6	44.3 ± 5.2*	104.1 ± 14.8
HT-29	65.5 ± 0.4*	93.7 ± 2.9	46.0 ± 3.8*	109.5 ± 9.50
HepG2	88.8 ± 1.2*	95.9 ± 2.3	50.6 ± 0.8*	126.9 ± 11.6*

[a]SEM (standard error of the means) was derived from two experiments.
*Represents significant difference ($p < 0.05$) in mean invasiveness of the treatment group in comparison to control.

Propranolol and ICI118,551 caused reduction in carcinoma cell invasion

The invasiveness of tumor cells was measured by the Boyden chamber-based cell invasion assay. Invasive cells were discriminated from non-invasive cells based on their ability to degrade basement membrane-coated layer and pass through the pores of membrane (Albini et al., 1987). The findings obtained from the assay were expressed in terms of percent invasion relative to invasion of non-treated control cells after 24 h (Table 2). Accordingly, the invasiveness of MCF7, HT-29, and HepG2 cells was significantly inhibited ($p < 0.05$) by 24 h propranolol and ICI118,551 applications. Among the cell lines tested, propranolol exerted highest reduction in invasiveness of HT-29 cells (66%). In contrast, the effects of ICI118,551 on invasiveness of three cell lines were similar, but higher than that of propranolol (in a range of 44–51%). Atenolol, in contrast, did not seem to exert significant effect ($p < 0.05$) on invasiveness of MCF7, HT-29, and HepG2 cells (i.e., invasiveness were higher than 93% all the cell lines).

Although isoproterenol seemed to increase invasiveness of cells, only the finding obtained with HepG2 cells was statistically significant (127%; $p < 0.05$).

Effects of antagonists on carcinoma cell migration

In vitro migration properties of tumor cells were determined by the wound-healing assay. The assay mimics cell migration during wound healing *in vivo* (Figure 2). Wound-healing at every 24 h for 72 h was expressed as percent wound-healing relative to the original scratch (Figure 3). Accordingly, 100 μM propranolol caused significant reduction ($p < 0.05$) in migration of MCF7 cells after 48 and 72 h. Increasing propranolol concentration to 150 and 200 μM caused further reduction ($p < 0.05$) in migration. Atenolol (200 μM) significantly inhibited MCF7 migration at all application periods, and WH of cells were significantly lower when 600 μM atenolol was applied ($p < 0.05$). ICI118,551 (100 μM) inhibited MCF7 cell migration after 48 and 72 h ($p < 0.05$). Propranolol inhibited migration of HT-29 cells ($p < 0.05$). Doubling the propranolol concentration from 50 to 100 μM caused significant reduction in migration ($p < 0.05$). Atenolol (400 μM) application caused significant reduction of HT-29 migration in all time periods tested ($p < 0.05$). Doubling the atenolol concentration from 400 to 800 μM caused significant reduction in migration after 24 and 72 h ($p < 0.05$). ICI118,551 inhibited HT-29 cell migration after 48 and 72 h ($p < 0.05$) of application periods. Doubling the ICI118,551
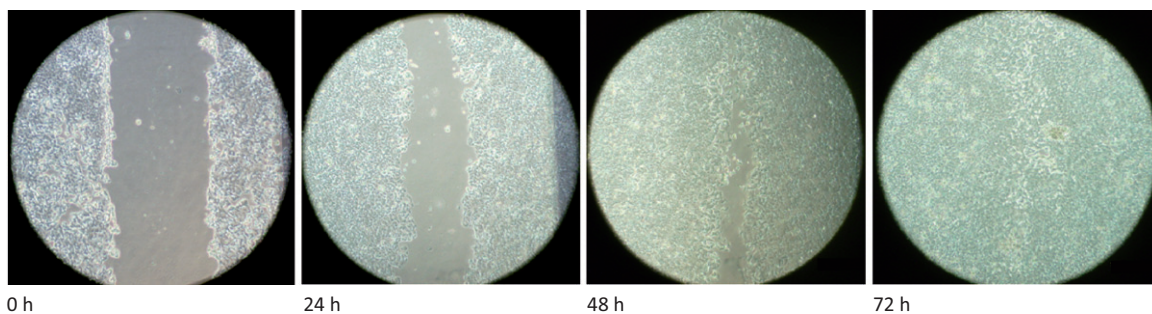

| 0 h | 24 h | 48 h | 72 h |

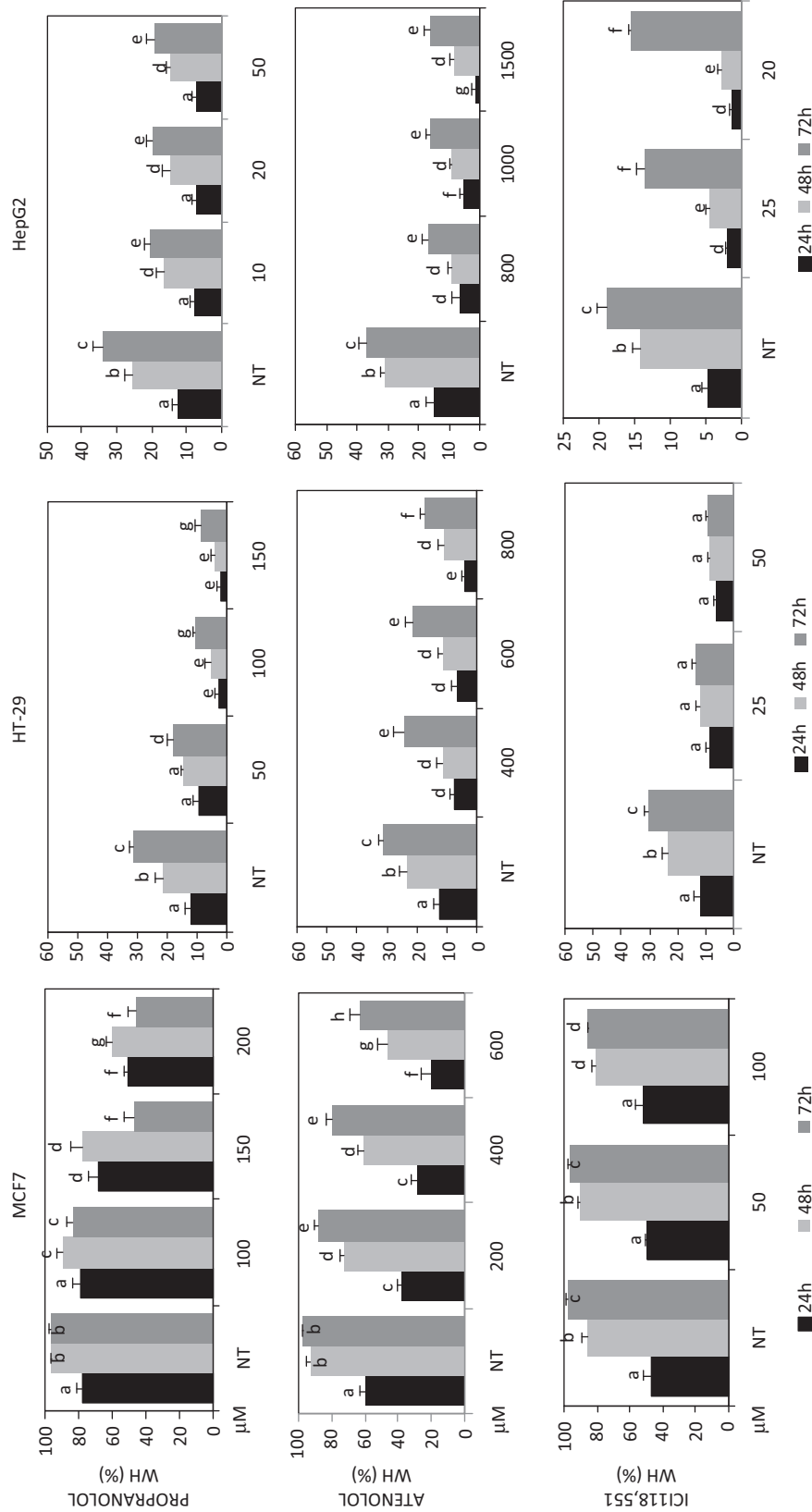Figure 2. Representative micrographs for wound healing assay (4×).

Figure 3. Effect of propranolol, atenolol, and ICI118,551 on wound healing (%WH = [($d_{\text{original wound}} - d_{\text{healing}}$)/$d_{\text{original wound}}$] × 100, where d represents distance between wound edges of MCF7, HT-29, and HepG2 cells. Each d was obtained by the measurement of at least 10 random points. Mean d and SEM were derived from eight d values. Black, grey, and dark grey bars represent WH after 24, 48, and 72 h of treatment, respectively. Different letters represent significant difference between groups with $p < 0.05$ (i.e. control versus treatments and treatments in between at specific application periods and in between application periods at specific concentrations).

concentration from 25 to 50 μM did not cause reduction in migration ($p < 0.05$). Propranolol caused reduction in wound-healing of HepG2 cells in 48 and 72 h ($p < 0.05$), and the inhibitory effects were concentration independent ($p > 0.05$). Eight hundred micromolar atenolol inhibited HepG2 cell migration at all time periods tested ($p < 0.05$). Increased atenolol concentrations had no effect on migration of HepG2 cells ($p > 0.05$) except for the 24 h applications. In addition, ICI118,551 inhibited HepG2 cell migration in all time periods tested ($p < 0.05$). WH of cells were differed among 24, 48, and 72 h applications at both 10 and 20 μM ($p < 0.05$), although there was no significant difference between two concentrations ($p > 0.05$). Effects of 10 μM isoproterenol on migration characteristics in the cell lines were also tested. Isoproterenol seemed to have no significant effect on wound-healing capacity of the cell lines tested (data not shown).

Discussion

The cytotoxic potential of β-blockers has been studied previously on different cell lines. In particular, propranolol has been shown to reduce mitogenic activity of pancreatic, leukemia, and oral squamous cancer cells mainly via β₂-ARs (Hajighasemi & Mirshafiey, 2009; Shang et al., 2009; Zhang et al., 2009). Previous studies showed that propranolol acts as G_i-independent manner (Galandrin et al., 2008) to inhibit cell proliferation and induce apoptosis through different downstream molecules which participate in Ras/Akt, Src tyrosine kinase, MAPKs, ERK1/2, and PKA/AA pathways (Hajighasemi & Mirshafiey, 2009; Shang et al., 2009; Zhang et al., 2009). In particular, induction of apoptosis of pancreatic cancer cell line by propranolol was well characterized by stimulation of proteolytic activities of caspase-3 and caspase-9, and the intrinsic pathway (Zhang et al., 2009).

Isoproterenol is a non-selective β-AR agonist acting via adenylyl cyclase (AC) and MAPK. Both Gi-independent and dependent pathways contribute to the response to isoproterenol. For example, bucindolol and propranolol share the same Gi-independent MAPK signaling pathway, but they have opposite effects in terms of AC activity (Galandrin et al., 2007). So, two downstream signaling responses, by distinguishing two compounds, may further contribute to ligand selectivity. Although ERK1/2 activation is one of the downstream effectors of AC signaling, two pathways can be independently regulated. Isoproterenol is an agonist for these two systems, ERK1/2 activation via isoproterenol stimulation being independent of cAMP generation. Interestingly, propranolol acts as an inverse agonist for AC, and partial agonist for ERK1/2. Both isoproterenol and propranolol act on a common Gi-independent pathway; however, activation of different downstream effectors, like ERK1/2, may have distinct consequences on gene regulation and cell proliferation (Galandrin et al., 2008).

Results of the present study demonstrated that the non-selective β-blocker propranolol was more cytotoxic to all cell lines than β₁-blocker atenolol, but less cytotoxic than β₂-blocker ICI118,551 (Table 1). IC₅₀ of propranolol were approximately 2-fold of that of ICI118,551 for all cell lines and incubation periods. However, atenolol to

propranolol IC₅₀ ratios varied 4–80 fold, and atenolol to ICI118,551 IC₅₀ ratios varied 6–120 fold depending on the cell line and incubation period. Cheong et al. (2008) and Weddle et al. (2001) studied cytotoxicity of different β-blockers on immortalized retinal and corneal cells, and pancreatic cancer cells, respectively. Both the studies reported atenolol as the least cytotoxic among the β-blockers studied. In another study, ICI118,551 and atenolol were applied to nicotine-stimulated colon carcinoma cells, and both agents were shown to inhibit cell proliferation. β₂-Adrenergic receptor mediated more dominant effect in these applications (Wu et al., 2005). Likewise, ICI118,551 inhibited DNA synthesis of unstimulated pancreatic adenocarcinoma cells, whereas atenolol did not (Weddle et al., 2001). Furthermore, ICI118,551 was more effective in tumor growth inhibition than atenolol as demonstrated in mouse xenograft of colon carcinoma (Wong et al., 2007).

In the study of Zhang et al. (2011), ICI118,551 caused inhibition of pancreatic carcinoma cell proliferation, induction of apoptosis, and G1/S phase arrest. They also found that these effects were stronger than the β1-AR antagonist metoprolol. These effects were associated with the down-regulation of cyclin D1 and cyclin E by ICI118,551. Meanwhile, limited activation of Bax, caspase-9, and caspase-3, and suppression of the ERK and Akt pathways which phosphorylate Bad, and allows Bcl-2 to form homo-dimers which may affect antiapoptotic response of cells of different histological origins. Suppression of the NF-κB activation in cells through any upstream effectors may also cause attenuated sensitivity of cells at transcriptional level. In fact, cells of different histological origins have variable sensitivity to apoptosis which is also related to chemoresistance of cells due their differences at genomic or transcriptional level.

Although cytotoxicity ratios did not seem to correlate with β₁- and β₂-AR mRNA expression levels in cells, β₂-blocker seemed to be more cytotoxic in non-stimulated cancer cells than β₁-blocker and even non-selective β-blocker that act via both types of receptors. In fact, native (non-stimulated) β-ARs occurring in glioma cells were shown to be constitutively active (Sokołowska & Nowak, 2005). So, one possible mechanism of action may be the blockage of native β-adrenergic stimulation. Moreover, a ligand's pharmacologic efficacy, or its ability to produce an effect, may not be fully explained by its ability to stimulate a single receptor-mediated signaling pathway. Previous studies have demonstrated that stimulating a receptor with a ligand can expose different intrinsic effects on various effectors systems (reviewed in Kenakin, 1995; Urban et al., 2007). It has been also reported that after ligand binding many receptors such as β₂-ARs can exist in multiple ''active'' conformations. These variable conformations may reveal various different cellular responses and may help clarify the diverse signaling profiles (Ghanouni et al., 2001; Swaminath et al., 2005). Wisler et al. (2007) characterized diverse signaling profiles of non-selective β-blocker carvedilol, and postulated that ligand bias may play a role in determining the effectiveness of β-AR antagonists targeting 7TMRs.

Furthermore, various factors including histological origin of cells, hormone dependency, and intracellular cell signaling

pathways, as well as physicochemical properties of drugs may contribute to the differential effects of these β-blockers on non-stimulated cells. Liu et al. (2008) studied interaction of β_2-ARs with EGFR, and demonstrated that ICI118,551 was more effective than atenolol in inhibiting EGFR-stimulated cAMP increase in esophageal cancer cells. EGF increases cAMP level by stimulating AC, and cAMP affect downstream pathways via kinase A. EGFR signal transduction pathway is directly involved in processes related to tumor prognosis like angiogenesis, invasion and metastasis, and resistance to apoptosis.

Tumor cell migration and invasion, the prerequisite for metastasis development, are not only merely genetically determined but are also distinctly environmentally regulated (Guo et al., 2009). The ability to invade extracellular matrices plays an important role in metastasis, and in supplying blood flow to tumors. In this study, we evaluated effects of AR antagonists on the invasiveness and migration properties of MCF7, HepG2, and HT-29 cell lines. The findings form the Boyden chamber assay demonstrated that both propranolol and ICI118,551 significantly inhibited invasion of non-stimulated cancer cells (Table 2). Atenolol, in contrast, caused 4–7% non-significant reductions in invasion of all three cell lines. Likewise, ICI118,551 was shown to be more potent than metaprolol, another β_1-blocker, in inhibiting invasion of non-stimulated pancreatic cancer cells after 20 h of application via CREB, NFκB, and AP-1 pathways (Zhang et al., 2010). In contrast, Drell et al. (2003) and Lang et al. (2004) reported that ICI118,551 and atenolol did not affect invasion of non-stimulated mammary and prostate carcinoma cells. However, their application concentrations and durations were considerably lower than that of the present study and the study of Zhang et al. (2010) (i.e., 1 and 10 µM ICI118,551 and atenolol were applied for 12 and 20 h, respectively). In fact, concentrations empirically determined in the present study were compatible to application periods. In contrast, propranolol was more effective than β_1- and β_2-selective adrenoceptor blockers in the inhibition of invasiveness of MCF7 and HT-29 cells, in parallel to the results of other reports obtained with different cell lines (Lang et al., 2004; Masur et al., 2001). In previous in vitro studies, it has been shown that propranolol completely blocked norepinephrine-mediated invasiveness of ovarian and prostate cancer, although alone it had no effect on invasiveness of in these cells (Palm et al., 2006; Sood et al., 2006). β-ARs can enhance production of MMP-2 and MMP-9, and propranolol diminishes invasiveness of cells by MMP2 and MMP9 inhibition as well as VEGF and p38/MAPK (Guo et al., 2009; Huang et al., 2012). In fact, propranolol alone was found to inhibit VEGF production and MMP2 activity in human leukemic cell lines (Hajighasemi & Hajighasemi, 2009).

It was reported that propranolol inhibited the promigratory effect of norepinephrine and reduced metastases formation in BALB/c nude mice (Palm et al., 2006). Wound healing and tumor progression both involve similar processes such as cell proliferation, inflammation, and angiogenesis (Lee et al., 2009). One of the important characters related to process of tumor cell penetration is motility. Our findings from the in vitro scratch assay demonstrated that propranolol, atenolol, and ICI118,551 reduced migration of MCF7, HT-29, and HepG2 cells to varying extents depending on the application concentration and duration (Figure 2). About 24 h of incubation with propranolol and atenolol were effective for inhibition of MCF7 migration, whereas longer incubation periods were required to observe inhibitory effect of ICI118,551 on cell migration. Increasing concentrations of propranolol and atenolol affected in vitro wound healing of HT-29 cells at different incubation periods. WH of ICI118,551 applied HT-29 cells did not differ with varying incubation periods. All antagonist treatments except for the 24 h propranolol treatments caused reduction in WH of HepG2 cells, and inhibitions were concentration-independent (except for the 24 h atenolol treatments). In a recent study, it has been demonstrated that propranolol administration alone inhibited migration and invasiveness of epithelial progenitor cells via Akt and MAPK/ERK pathway (Zou et al., 2013). In the current study, the administration of propranolol alone inhibited both invasiveness and migration of cancer cells depending on the cell type, application concentration, and time. Conclusively, propranolol and ICI118,551 were found to be more effective than atenolol in inhibiting both invasion and migration of non-stimulated MCF7 and HT-29 cells. In particular, ICI118,551 was the most potent inhibitory agent of invasion.

On one hand, the more potent effect of ICI118,551 on cells demonstrates involvement of β2-AR and its downstream signalling pathways. On the other hand, the action of second messengers, phosphorylation of effectors, downstream transcriptional events, and regulation of cell cycle seem to happen in a cell type-specific manner. For example, both angiogenesis and signaling through the RAF/MEK/ERK cascade play critical roles in the development of HCC. In addition to being highly angiogenic, human HCC tumors have high expression and enhanced activity of MAP kinase (Schmidt et al., 1997). Therefore, inhibition of both angiogenesis and RAF/MEK/ERK signaling may cause high sensitivity of HepG2 cells to ICI118,551. In addition, mutational status of any of the downstream effector proteins of cancer cell lines may affect the sensitivity of cells to ICI118,551. Even cells of different origins may express a mutated β2-AR receptor rendering it less responsive to antagonists.

Finally, β_2-selective adrenoceptor blockage seems to be more effective for non-stimulated cells. Furthermore, the effect of the selective antagonists may show variation depending on the concentration and incubation period, as well as histological origin of cells. β_1-Selective blockers may act through cAMP/PKA pathway, whereas in addition to cAMP/PKA, RAS, MAPK, NFκB, and AP-1 act in concert in β_2-selective blockage of the inhibition of invasion (Schuller, 2002; Weddle et al., 2001). Pharmacoepidemiological studies support the suggestion that β-blockers could provide a clinical benefit through inhibition of the prometastatic effects of β-AR signaling on the tumor microenvironment. The use of agents for tumor remodeling, such as β-blockers, in combination with chemotherapeutic drugs may be a promising therapeutic strategy to treat patients with solid tumors.

Acknowledgements

Declaration of interest

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