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Carbonic anhydrase inhibition boosts the antitumor effects of Imatinib mesylate via potentiating the antiangiogenic and antimetastatic machineries

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ABSTRACT

Carbonic anhydrase inhibitors have emerged in the past few years as an interesting candidate for the development of novel unconventional strategies. Despite their effect in tumor regression via inhibition of tumor acidification, their potential role is not yet fully elucidated. Herein, we investigated whether acetazolamide (AZ) could modulate imatinib (IM) anticancer activity, both in breast cancer cells (T47D) and in isolated tumor specimens of Ehrlich ascites carcinoma (EAC). The impact of this combination on angiogenesis was evidenced by decreasing PDGF-A expression and enhancing that of TSP-1. In the meantime, AZ significantly suppressed IM-induced attenuation of VEGF secretion in T47D cells, most probably due to NO inhibition. The combination also dramatically decreased the metastatic activity of T47D cells by mitigating the protein levels of MMP-2 and -9 and phosphorylation of p38 MAPK, while increasing the expression of TIMP-1 and -2. In addition, a strong proapoptotic effect was observed in T47D cells after combining AZ and IM in terms of increased caspase-9 and -3 activities. Interestingly, these results were confirmed by the reduction in the isolated tumor volume, MVD, Ki-67 and VEGF expression. Eventually, the study provides a new therapeutic strategy for treating cancer.

1. Introduction

Tumor metastases are responsible for approximately 90% of all cancer-related deaths (Spina et al., 2015). Recognizing and understanding the mechanisms concerned in tumor cell invasion may limit tumor progression, and, reduce mortality for many cancer patients. Tumor hypoxia and angiogenesis are considered one of the critical players involved in metastasis (Angelica and Fong, 2008). Tumor hypoxia arises due to increased metabolic activity and oxygen consumption by rapidly proliferating tumor cells, leading to alterations of local pH of surrounding microenvironment (Justus et al., 2015). The extracellular space of most tumors is mildly acidic, owing to exuberant production of lactic acid and activation of hypoxia-inducible factor-1 (HIF-1). There is growing evidence that extracellular acidity can boost the invasiveness and metastatic capacity of cancer cells, render cancer cells relatively resistant to chemotherapeutic drugs, and impede immune rejection of tumors (McCarty and Whitaker, 2010).

Manipulation of the extracellular and/or intracellular pH of tumors may have considerable potential in cancer therapy. Carbonic anhydrases (CAs) are a family of zinc-binding metalloproteinases that catalyze reversible hydration of carbon dioxide to produce H^+ and HCO_3^-, thereby regulating the acid-base balance and respiration (Juhász et al., 2003). Some transmembrane CA isozymes (CA IX and CA XII) are prominently found to be expressed only in tumor cells and are functionally related to oncogenesis (Parkkila et al., 2000). Previous studies have indicated that inhibition of CAs may be a target for the treatment of cancers overexpressing CAs (Gondi et al., 2013).

Acetazolamide (AZ) (5-acetamido-1,3,4-thiadiazole-2-sulphonamide) is a member of the sulphonamide drug family and is a broad-spectrum CA inhibitor. In fact, AZ was shown to function as a modulator in anticancer therapies. AZ suppressed tumor growth and metastasis and reduced the invasive capacity of several renal carcinoma cell lines (Parkkila et al., 2000). Moreover, AZ delayed the development of tumor, especially, when administered in combination with various chemotherapeutic agents (McDonald et al., 2012).

Imatinib (IM) is a well-known tyrosine kinase inhibitor, used as a 'magic bullet' for the treatment of leukemia and gastrointestinal tumors. IM inhibits the activity of a number of receptor tyrosine kinases, which

Abbreviations: AZ, acetazolamide; IM, imatinib; EAC, Ehrlich ascites carcinoma; PDGF-A, platelet derived growth factor-A; TSP-1, thrombospondin-1; VEGF, vascular endothelial growth factor; NO, nitric oxide; MMP, matrix metalloproteinase; MAPK, mitogen activated protein kinase; TIMP, tissue inhibitor of metalloproteinase; MVD, microvessel density.

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are implicated in tumorigenesis and metastasis as Bcr-ABL, c-Kit, platelet-derived growth factor receptor (PDGF-R), and epidermal growth factor receptor (EGF-R) (Bilir et al., 2011). Its success encouraged the search for its antineoplastic effects on other cancer types including endometrial, anaplastic thyroid and prostate cancers (Bilir et al., 2011). However, treatment failures due to resistance to monotherapy and relapse are still common.

Consequently, the current study sought to investigate whether AZ can potentiate the anticancer effects of IM both *in vitro* on T47D breast cancer cells and in the isolated tumor of EAC. The study also aimed to highlight the underlying mechanisms of this interaction, focusing on multiple targets as the angiogenic, metastatic and apoptotic machineries.

2. Materials and methods

2.1. Drugs

IM mesylate was obtained from Eton Bioscience, Inc. (San Diego, CA, USA). It was stored at −20 °C and was freshly dissolved in RPMI-1640 medium. AZ was purchased from Sigma Aldrich Chemical Co. (ST. Louis, MO, USA). It was stored at room temperature and was freshly dissolved in DMSO, and then serially diluted in RPMI-1640 medium. Final concentration of DMSO never exceeded 0.1% (v/v) both in control and in treated samples.

2.2. Chemicals

The primer sequences for HIF-1α, CA IX, CA XII, PDGF-A, thrombospondin-1 (TSP-1), matrix metalloproteinases-2 and -9 (MMP-2 and -9), tissue inhibitor of metalloproteinases-1 and -2 (TIMP-1 and -2) and GAPDH were supplied by Invitrogen (USA). Antibodies used for Western blot analysis of CA XII (primary goat anti-human CA XII polyclonal antibody), MMP-2 (primary mouse anti-human MMP-2 monoclonal antibody), MMP-9 (primary mouse anti-human MMP-9 monoclonal antibody), phospho-p38 mitogen-activated protein kinase (primary rabbit anti-human phospho-p38 MAPK oligoclonal antibody) and β-actin (primary mouse anti-human β-actin monoclonal antibody) were obtained from Invitrogen. The antibodies used in immunohistochemistry; anti-CA IX, anti-Ki-67, anti-CD34, and anti-vascular endothelial growth factor (VEGF) were supplied by Thermo Fisher Scientific (USA). All other chemicals and solvents were of highest analytical grade commercially available.

2.3. Human cancer cell lines and cell culture

Human breast cancer cells (T47D and MCF-7) were obtained from American Type Culture Collection (ATCC, Manassas, VA). They were maintained as monolayer cultures in RPMI-1640 supplemented with 10% FBS and 1% penicillin-streptomycin.

2.4. Cytotoxicity assay

Cytotoxicity was determined using sulforhodamine-B (SRB) method as described bySkehan et al.(1990). In brief, T47D and MCF-7 cells were seeded at a density of 3×10^3 cells/well in 96-well microtiter plates. They were left to attach for 24 h before incubation with drugs. The cells were then treated with either IM alone (20–80 μM), or AZ alone (400–800 μM), or the combination of IM and AZ for 48 h. The optical density (O.D) of each well was measured spectrophotometrically at 570 nm using ELISA microplate reader (TECAN Sunrise™, Germany).

The mean values were estimated as percentage of cell viability as follows: O.D (treated cells) / O.D (control cells) × 100. The IC_{50} value (the concentration that produces 50% inhibition of cell growth) of each drug was calculated using sigmoidal dose response curve-fitting models (Graph-Pad Prism software, version 5).

2.5. Determination of IM uptake by T47D cells using liquid chromatography-tandem mass spectrometry (LC/MS/MS) analysis

T47D cells were seeded at a density of 2×10^4 cells/well of a 24-well plate and left for 24 h. The plate was divided into 2 groups as follows:

(a) Group I was treated with IM alone (23.25 μM).
(b) Group II was treated with both IM (23.25 μM) and AZ (800 μM).

The medium was then aspirated after 0, 3, 6, 24 and 48 h intervals, centrifuged and supernatant was stored at −20 °C till HPLC assay. Forty microliters of the supernatant were diluted 10× with deionized water, then mixed thoroughly with 1200 μl of methanol (Alliance Bio, USA) and centrifuged at 3000 rpm for 15 min at 4 °C using Eppendorf Centrifuge, 5804 R, USA. Ten microliters of the resultant clear supernatant were then injected into the LC/MS/MS system. Data acquisition was performed with analyst 4.0 software (ABSCIEX). Separation was performed using Agilent C18 (5 μm, 50 × 4.6 mm) reversed phase analytical column (Agilent, CA, USA). The mobile phase pumped at a flow rate of 700 μl/min consisted of 0.1% formic acid in methanol:water (55:45 v/v). Mass spectrometric analysis was performed in the positive ESI ion mode.

2.6. Quantitative real time RT-PCR analysis

Total RNA was extracted from cell culture using QIAamp® RNA Blood Mini kit (Qiagen, Germany), following the manufacturer's protocol. Conversion of RNA to cDNA and real time PCR quantification were carried out using QuantiTect® Reverse Transcription and QuantiTect™ SYBR® Green PCR kits. Primer sequences were shown in Table 1. A protocol including PCR activation at 95 °C for 15 min, denaturation at 95 °C for 5 s, and annealing/extension at 60 °C for 30 s was performed on a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The values of RT-PCR products were normalized with respect to GAPDH and were expressed as relative expression levels. Analysis of data was performed using the $2^{-\Delta\Delta CT}$ formula (Livak and Schmittgen, 2001).

2.7. Western blot analysis

Total protein from T47D cells with different treatments was harvested and extracted with protein lysis buffer (150 mM NaCl, 0.3% NP-40

Table 1
Primer sequences for quantitative real time PCR.

Gene	Sequences
HIF-1α	Forward 5′-CAA GAA CCT ACT GCT AAT GC-3′
	Reverse 5′-TTA TGT ATG TGG GTA GGA GAT G-3′
CA IX	Forward 5′-GTC TCG CTT GGA AGA AAT CG-3′
	Reverse5′-AGA GGG TGT GGA GCT GCT TA-3′
CA XII	Forward 5′-CTG CCA GCA ACA AGT CAG-3′
	Reverse 5′-ATA TTC AGC GGT CCT CTC-3′
PDGF-A	Forward 5′-CCC CTG CCC ATT CGG AGG AAG AG-3′
	Reverse 5′-TTG GCC ACC TTG ACG CTG CGG TG-3′
TSP-1	Forward 5′-CTG ATC TGG GTT GTG GTT GTA-3′
	Reverse5′-CCT GTG ATG ATG ACG ATG A-3′
MMP-2	Forward 5′-GGC TGG TCA GTG GCT TGG GGT A-3′
	Reverse 5′-AGA TCT TCT TCT TCA AGG ACC GGT T-3′
MMP-9	Forward 5′-GCG GAG ATT GGG AAC CAG CTG TA-3′
	Reverse 5′-GAC GCG CCT GTG TAC ACC CAC A-3′
TIMP-1	Forward 5′-ACC ATG GCC CCC TTTGAG CCC CTG-3′
	Reverse 5′-TCA GGC TAT CTG GGA CCG CAG GGA-3′
TIMP-2	Forward 5′-CTC GGC AGT GTG TGG GGT C-3′
	Reverse 5′-CGA GAA ACT CCT GCT TGG GG-3′
GAPDH	Forward 5′-GTC AGT GGT GGA CCT GAC CT-3′
	Reverse 5′-AGG GGT CTA CAT GGC AAC TG-3′