

In vitro anticancer effects of a RAGE inhibitor discovered using a structure-based drug design system

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Abstract. Receptor for advanced glycation end-products (RAGE) is a pattern recognition receptor implicated in the pathogenesis of certain types of cancer. In the present study, papaverine was identified as a RAGE inhibitor using the conversion to small molecules through optimized-peptide strategy drug design system. Papaverine significantly inhibited RAGE-dependent nuclear factor κ -B activation driven by high mobility group box-1, a RAGE ligand. Using RAGE- or dominant-negative RAGE-expressing HT1080 human fibrosarcoma cells, the present study revealed that papaverine suppressed RAGE-dependent cell proliferation and migration dose-dependently. Furthermore, papaverine significantly inhibited cell invasion. The results of the present study

suggested that papaverine could inhibit RAGE, and provided novel insights into the field of RAGE biology, particularly anticancer therapies.

Introduction

Receptor for advanced glycation end products (RAGE) is a pattern recognition receptor that binds multiple ligands, including AGE (1), S100 proteins (2), lipopolysaccharides (3), phosphatidylserine (4), amyloid- β (A β) (5), and high mobility group box (HMGB)-1 (6). Interactions of these diverse ligands with RAGE result in intracellular signaling, including nuclear factor κ -B (NF- κ B) activation, which results in pathogenic processes such as diabetic complications (7), inflammatory diseases, Alzheimer's disease (AD) (8) and cancer (9). Takeuchi *et al* (10) demonstrated that RAGE expression in HT1080 human fibrosarcoma cells induced tumor cells to proliferate, migrate, invade and metastasize. HMGB-1 was revealed to induce RAS-related C3 botulinum toxin substrate (Rac)1 and cell division control protein 42 homolog (Cdc42) functions in RAGE-expressing HT1080 fibrosarcoma cells (10). Epidemiological studies also demonstrated that RAGE expression was associated with tumor malignancies of the stomach (11), colon and rectum (12-14), prostate (15), breast (16) and bone (17). Therefore, these previous studies suggested that RAGE represents a potential therapeutic target, and that inhibiting RAGE may be useful to anticancer strategies.

Previously, Sakai *et al* (18) developed a novel drug design system, involving the conversion of optimized binding peptide to non-peptidic small molecules by structure-based virtual screening (SBVS), followed by optimization of the small molecules using a structure-based drug design system, namely conversion to small molecules through optimized-peptide strategy (COSMOS). Using this strategy, the most optimized binding peptide is first computationally designed on a hot spot

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Abbreviations: RAGE, receptor for advanced glycation end-products; COSMOS, conversion to small molecules through optimized-peptide strategy; NF- κ B, nuclear factor κ B; AGE, advanced glycation end-products; A β , amyloid- β ; HMGB, high-mobility group box; Rac1, RAS-related C3 botulinum toxin substrate 1; Cdc42, cell division control protein 42 homolog; SBVS, structure-based virtual screening; ERK, extracellular signal-regulated kinase

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in the target protein. Subsequently, the optimized binding peptide may be converted to small molecules by SBVS based on its pharmacophore. Then, the selected candidates are evaluated using *in vitro* assays. Therefore, this strategy decreases the cost and time required to search for effective lead compounds, for drug design and for optimization (18). The present study identified a RAGE inhibitor, papaverine, using this drug design system. Papaverine is an opiate alkaloid, originally isolated from the plant *Papaver somniferum* and now synthetically produced as a direct-acting smooth muscle relaxant. Its mechanism of action may be associated with non-selective inhibition of phosphodiesterases and direct inhibition of calcium channels (19,20).

The present study assessed whether papaverine functioned as a RAGE inhibitor using *in vitro* cell culture systems of RAGE- and dominant-negative (dn)RAGE-expressing HT1080 fibrosarcoma cells.

Materials and methods

Papaverine. Papaverine hydrochloride (molecular weight, 375.85 Da; product number, P0016) was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan).

Cell lines. HT1080 human fibrosarcoma cells (American Type Culture Collection, Manassas, VA, USA) were transfected with a plasmid containing human full-length RAGE cDNA or cytoplasmic domain-deleted dnRAGE cDNA, or with the vector alone, as previously described (21,22). The cells were designated as RAGE-expressing, dnRAGE-expressing or mock-transfected (mock) HT1080 cells, respectively. Cells were maintained in RPMI-1640 medium (Nakarai Tesque, Kyoto, Japan) supplemented with 10% fetal bovine serum (FBS; Nichirei Biosciences Inc., Tokyo, Japan), 100 U/ml penicillin and 100 µg/ml streptomycin in the presence of G418 (geneticin, 200 µg/ml; Roche Applied Science, Mannheim, Germany).

NF-κB luciferase assay. Stably transfected rat C6 glioma cells that expressed human RAGE and the NF-κB enhancer-luciferase system (pNF-κB-Luc; Agilent Technologies, Inc., Santa Clara, CA, USA) were used in this assay, as previously described (21). After a 4 h incubation at 37°C in a humidified 5% CO₂ atmosphere in Dulbecco's modified Eagle's medium supplemented with 0.1% FBS, the cells were stimulated with 1 µg/ml HMGB-1 (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) or with 100 µg/ml glyceraldehyde-derived AGE-modified bovine serum albumin (BSA) (Sigma-Aldrich; Merck KGaA) (21) with/without 10 or 20 µM papaverine or 0.08% dimethyl sulfoxide (DMSO) as a negative control for 4 h at 37°C in a humidified 5% CO₂ atmosphere. Luciferase activity was determined using the luciferase assay system (Promega Corporation, Madison, WI, USA) and an LB 941 Multimode Reader TriStar (Berthold Technologies GmbH & Co. KG, Bad Wildbad, Germany). These experiments were repeated three times.

Western blot analysis. RAGE- and dnRAGE-expressing HT1080 cells and mock control cells which were incubated with 0, 10 and 20 µM papaverine for 72 h at 37°C in a

humidified 5% CO₂ atmosphere, were washed with ice cold PBS, scraped off in PBS and pelleted by centrifugation at 300 x g for 5 min at 4°C. The cells were lysed immediately by sonication in 1% Triton X-100 (Sigma-Aldrich; Merck KGaA), 50 mM Tris-HCl, pH 7.5, 150 mM NaCl and protease inhibitor cocktail (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), and then centrifuged at 10,000 x g for 10 min at 4°C. The cell lysates (50 µg of protein) were separated via 12.5% SDS-PAGE and electroblotted onto polyvinylidene fluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were blocked at room temperature for 1 h with 5% (w/v) non-fat dried milk in PBS, and then incubated at room temperature for 1 h with either a rabbit anti-human RAGE-specific polyclonal antibody (1:1,000) produced as described previously (21) or a mouse anti β-actin antibody (cat. no. A5441, 1:10,000; Sigma-Aldrich; Merck KGaA). Goat anti-rabbit IRDye 680 (cat. no. P/N 926-32221) and goat anti-mouse IRDye 800CW (P/N 925-32210) were diluted 10,000-fold and used as the secondary antibodies. The antigen-antibody complex was visualized using the Odyssey Infrared Imaging System version 3.0 (LI-COR Biosciences, Lincoln, NE, USA). These experiments were repeated two times. Secondary antibody only was used as a negative control.

Plate-binding assays. Competitive binding inhibition assay with papaverine was performed using a 96-well AGE-BSA-coated plate as previously described (21). Briefly, 50 ng/ml soluble RAGE (sRAGE) was incubated with or without 10 or 20 µM of papaverine or 0.08% DMSO as a negative control on an AGE-BSA-coated plate at room temperature for 1 h. Following incubation and washed three times with 0.01% Tween-20, 0.15 M NaCl, 20 mM Tris-HCl (pH 7.5), horseradish peroxidase (HRP)-labeled anti-RAGE antibody (in human esRAGE ELISA kit; ready-to-use; cat. no. K1009-1; B-Bridge International, Inc., Santa Clara, CA, USA) was added and the plate was further incubated at room temperature for 1 h. The HRP-labeled antibody-sRAGE-AGE complex was then detected by measuring the absorbance at 450 nm using the microplate reader iMark (Bio-Rad Laboratories, Inc., Hercules, CA, USA). These experiments were repeated three times.

Cell proliferation assay. RAGE- and dnRAGE-expressing HT1080 cells and mock control cells were inoculated (1x10³ cells/well) at 37°C in a humidified 5% CO₂ atmosphere in a 96-well plate (BD Biosciences, Franklin Lakes, NJ, USA) containing RPMI-1640 medium supplemented with 10% FBS and 0, 10 or 20 µM papaverine or 0.08% DMSO as a negative control. Following inoculation, the total viable cell number was counted using a hemocytometer using the dye exclusion method with 0.2% Trypan blue at room temperature (Thermo Fisher Scientific, Inc.) using an inverted light microscope (Primovert, Zeiss, Carl Zeiss Industrielle Messtechnik GmbH, Oberkochen, Germany) at magnification, x4 objective at 0, 24, 48 and 72 h. These experiments were repeated three times.

Cell migration assay. Cell migration was evaluated using the monolayer denudation assay as previously described (10). Briefly, RAGE- and dnRAGE-expressing HT1080 and mock control cells were inoculated (2x10⁵ cells/well) and were

cultured to 100% confluence in a 12-well plate. Cells were then wounded by denuding a strip of the monolayer (width, ~1 mm) with a 200 μ l pipette tip. Cells were washed twice with serum-free RPMI-1640 medium, and then incubated for 20 h at 37°C under a humidified 5% CO₂ atmosphere in RPMI-1640 containing 0.1% FBS with/without 10 or 20 μ M papaverine or 0.08% DMSO as negative control. The rate of wound closure was assessed in four separate fields of view 20 h after denudation using a light microscope (magnification, x4 objective). These experiments were repeated three times.

Cell invasion assay. A total of mg/ml of matrigel-coated porous filters (pore size, 8 μ m) in a 24-well format (BD Biosciences) were used as barriers in Boyden chambers to assess the extent of invasion by RAGE- and dnRAGE-expressing HT1080 cells and mock controls. Cells were plated (2×10^5 cells) in the upper chambers with RPMI-1640 containing 0.1% BSA in the presence or absence of 10 or 20 μ M papaverine or 0.08% DMSO as negative control. The lower chambers were filled with 750 μ l RPMI-1640 containing 1% FBS in the presence or absence of 10 or 20 μ M papaverine. Following a 20-h incubation at 37°C in a humidified 5% CO₂ atmosphere, membranes were cut and removed from the insert housings. The filter membrane was fixed in 4% paraformaldehyde at room temperature for 2 min following the removal of non-invasive cells from the upper surface using a cotton swab. The bottom surface with the invasive cells was stained with 0.1% crystal violet at room temperature for 1 min, and the invasive cells were counted in six fields of view using a light microscope (magnification, x4 objective) as previously described (10). These experiments were repeated three times.

Statistical analysis. Statistical analysis was performed using one-way analysis of variance with the Tukey-Kramer post-hoc test. These tests were conducted using Ekuseru-Toukei 2015 (Social Survey Research Information Co., Ltd., Tokyo, Japan). Data are presented as mean \pm standard error of the mean. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Inhibitory effects of papaverine on RAGE-dependent NF- κ B activity. The present study assessed the inhibitory effects of papaverine on the RAGE-dependent NF- κ B intracellular signaling pathway. Using the C6 glioma system, which reflected RAGE-dependent NF- κ B activity (21), HMGB-1-induced activation of NF- κ B was evaluated in the presence or absence of papaverine in the culture media. Adding HMGB-1 significantly induced intracellular NF- κ B activation ($P < 0.01$); however, this upregulation was inhibited by papaverine treatment (10 or 20 μ M; Fig. 1A). In addition, the increase in NF- κ B activity induced by glyceraldehyde-derived AGE-BSA, another RAGE ligand, was significantly inhibited by papaverine (Fig. 1B). The results of the present study suggested that papaverine could inhibit the RAGE-dependent intracellular signaling pathway. The present study subsequently confirmed the papaverine-RAGE binding using a plate assay (21). Papaverine significantly and dose-dependently competed for the binding between glyceraldehyde-derived AGE-BSA and recombinant

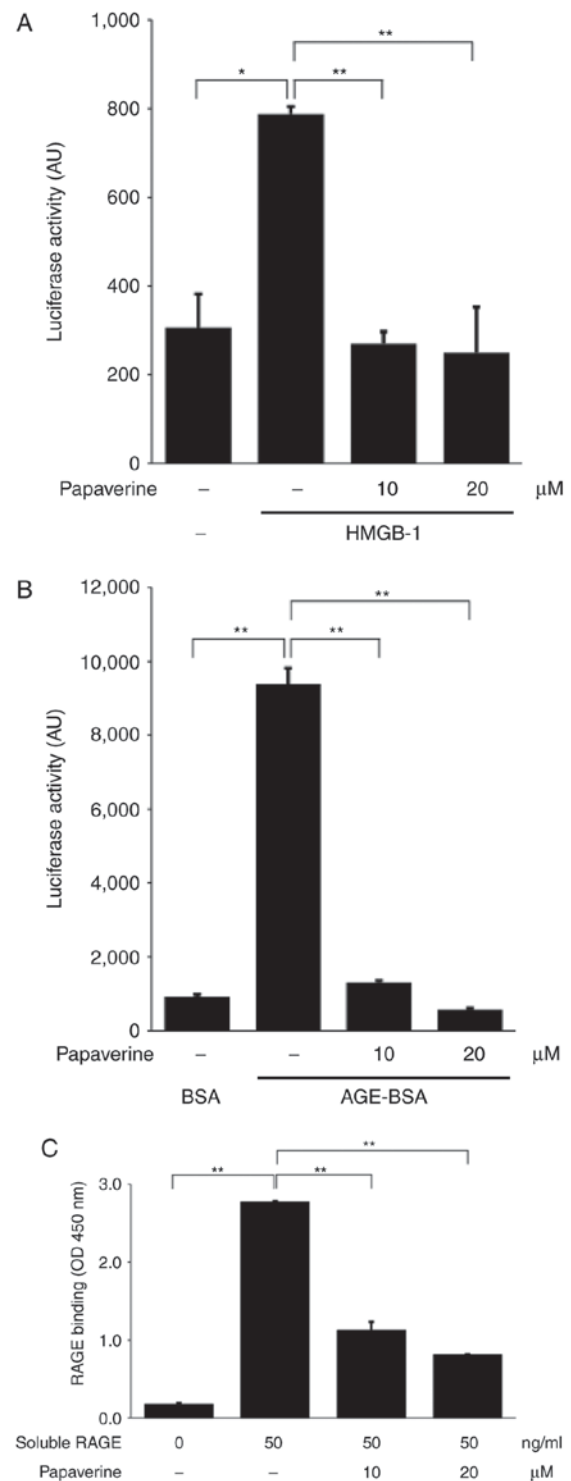


Figure 1. Papaverine inhibits HMGB-1 and AGE-BSA-induced RAGE signaling. Cells were stimulated with (A) 1 μ g/ml HMGB-1 or (B) 100 μ g/ml AGE-BSA with or without 10 or 20 μ M papaverine for 4 h. The luciferase activity was measured and expressed as arbitrary units (AU). Values represent the mean \pm standard error of the mean ($n=3$). BSA, 100 μ g/ml non-glycated BSA as a negative control; ** $P < 0.01$ vs. 1 μ g/ml HMGB-1; * $P < 0.05$ vs. 1 μ g/ml HMGB-1. (C) A plate binding assay. AGE-BSA-RAGE binding was competitively inhibited by papaverine. ** $P < 0.01$ vs. 50 ng/ml soluble RAGE. HMGB, high mobility group box; AGE, advanced glycation end-products; BSA, bovine serum albumin; RAGE, receptor for advanced glycation end-products; OD, optical density; soluble RAGE, recombinant soluble RAGE.

soluble RAGE (Fig. 1C), which suggested that the binding site of papaverine to RAGE may be shared with that of AGE-BSA.

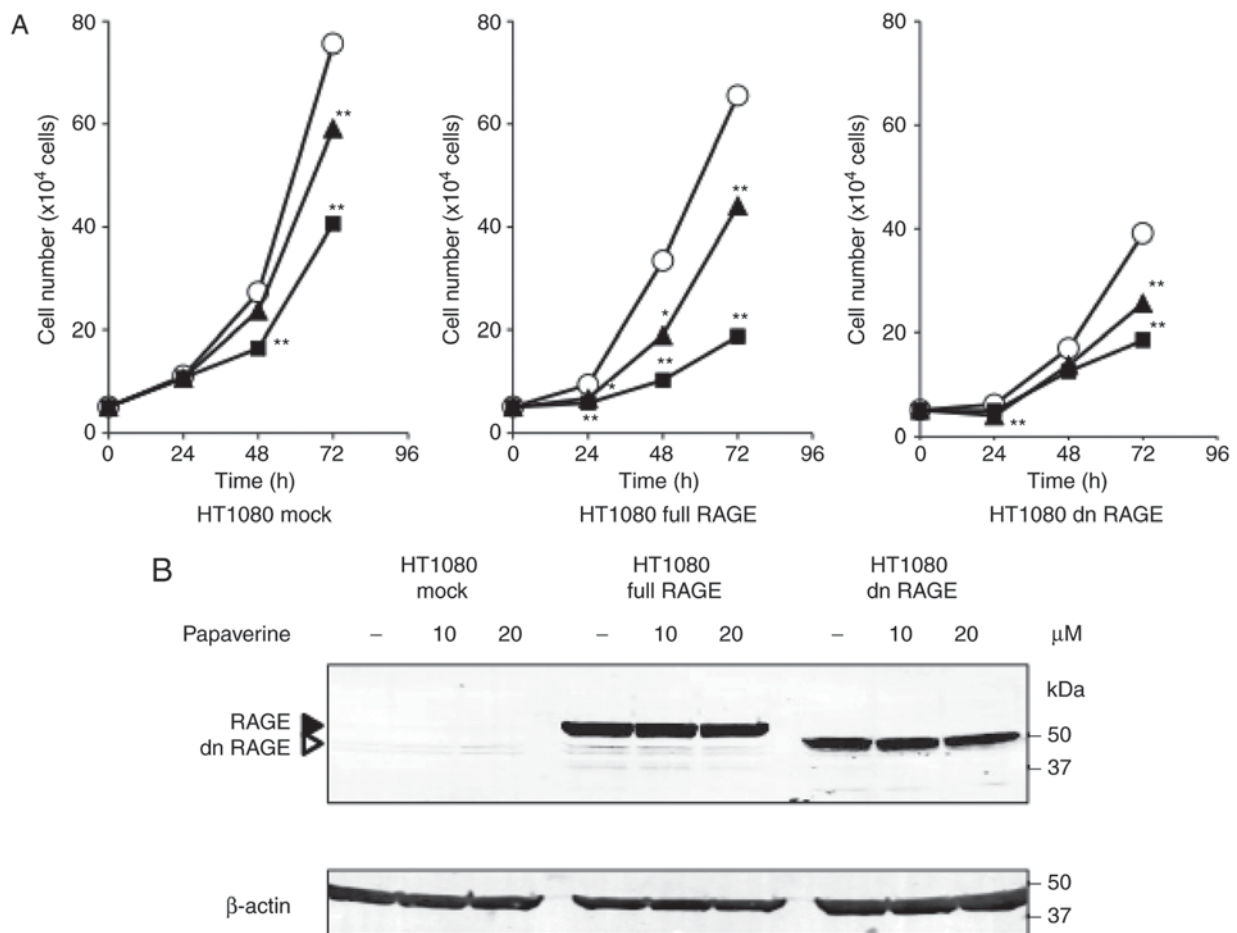


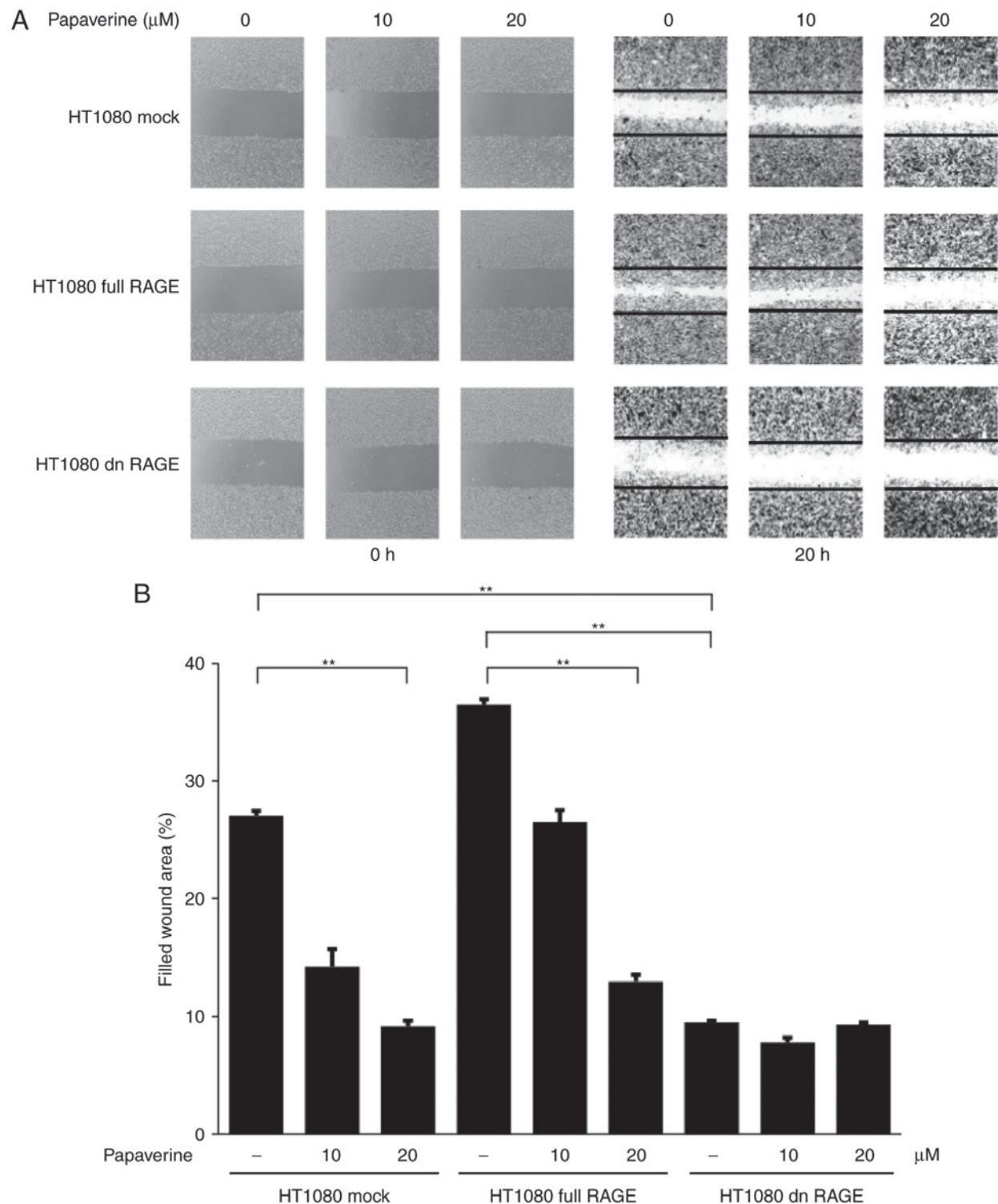
Figure 2. Papaverine decreases RAGE-induced cell proliferation in HT1080 fibrosarcoma cells. (A) Total viable cell number was determined at 0, 24, 48 and 72 h following inoculation of HT1080 RAGE, HT1080 dnRAGE and HT1080 mock cells in RPMI-1640 medium supplemented with 10% fetal bovine serum with or without 10 or 20 μ M papaverine. Values represent the mean \pm standard error of the mean ($n=3$). Open circle, non-treated control; closed triangle, 10 μ M papaverine; closed square, 20 μ M papaverine. * $P<0.05$ and ** $P<0.01$ vs. non-treated control. (B) Western blotting. Human RAGE- and dnRAGE-expressing HT1080 and mock control cells were treated with or without papaverine for 72 h. Closed and open arrow heads indicate RAGE and dnRAGE proteins, respectively. RAGE, receptor for advanced glycation end-products; dn, dominant-negative.

Inhibitory effects of papaverine on RAGE-dependent cell proliferation, migration and invasion. To mimic RAGE-dependent tumor malignant behaviors and assess the inhibitory effects of papaverine, the present study used an established human fibrosarcoma cell line HT1080 that expressed human RAGE and dnRAGE and the mock control cells. The HT1080 cells expressed HMGB1 and secreted HMGB1 levels did not differ among the mock-transfected, RAGE-expressing and dnRAGE-expressing HT1080 cells (10). The present study assessed the effects of papaverine treatment on HT1080 cell proliferation. Papaverine significantly and dose-dependently decreased the proliferation rate of RAGE-expressing HT1080 cells ($P<0.05$ vs. non-treated HT1080 cells; $P<0.01$ vs. non-treated HT1080 cells). In particular, at 20 μ M it was most effective between treated and non-treated conditions in RAGE-expressing HT1080 cells (65.5% reduction) at 48 h (Fig. 2A). A reduction (39.8%) was also observed between treated and non-treated conditions in the mock control cells at 48 h; however, its effectiveness was decreased in dnRAGE-expressing HT1080 cells (26.2% reduction) (Fig. 2A). Papaverine did not change the protein expression levels of RAGE and dnRAGE in the RAGE-expressing and dnRAGE-expressing HT1080 cells, respectively (Fig. 2B). Whether papaverine could inhibit the RAGE-dependent migration and invasion

of HT1080 cells was also assessed *in vitro*. The inhibitory effects of papaverine on cell migration were observed between treated and non-treated RAGE-expressing HT1080 cells. Additionally, the inhibitory effects were observed between treated and non-treated conditions in mock control cells (Fig. 3). However, no inhibitory effects of papaverine on migration were observed in dnRAGE-expressing HT1080 cells (Fig. 3), which indicated a selective inhibitory action of papaverine against RAGE. Furthermore, papaverine treatment significantly and dose-dependently inhibited HT1080 cell invasion in the Matrigel assay ($P<0.01$; Fig. 4). A statistically significant inhibitory effect of papaverine treatment was also identified between treated and non-treated dnRAGE-expressing HT1080 cells ($P<0.01$). The results of the present study demonstrated that papaverine may inhibit RAGE-dependent and RAGE-independent malignant phenotypes of cancer cells; the latter may include an antitumor effect of papaverine via an elevation of intracellular cyclic AMP by phosphodiesterase inhibition (8).

Discussion

RAGE is a multi-ligand, pattern recognition receptor that has been implicated in the growth, progression and metastasis of



multiple types of human cancer (23,24). RAGE ligands include S100/calgranulins (S100A4, S100A6, S100A7, S100A8/9, S100A14, S100B and S100P) (2,25-29) and HMGB-1 protein, which have been demonstrated to be upregulated in glioma, melanoma, bladder, liver, pancreatic, prostate, colorectal, gastric and lung cancer (30-34). Ligand-RAGE signaling pathways enhance the properties associated with malignant tumor

phenotypes (10,22,35-37), including by activating members of the small GTPase family (Cdc42 and Rac1), members of the mitogen-activated protein kinase family (extracellular signal-regulated kinase, p38 and stress-activated protein kinases/c-Jun amino-terminal kinases), NF- κ B and the formin homology 1 domain (10,22,35-37). Therefore, blocking RAGE and ligand-RAGE signaling could represent a potential strategy

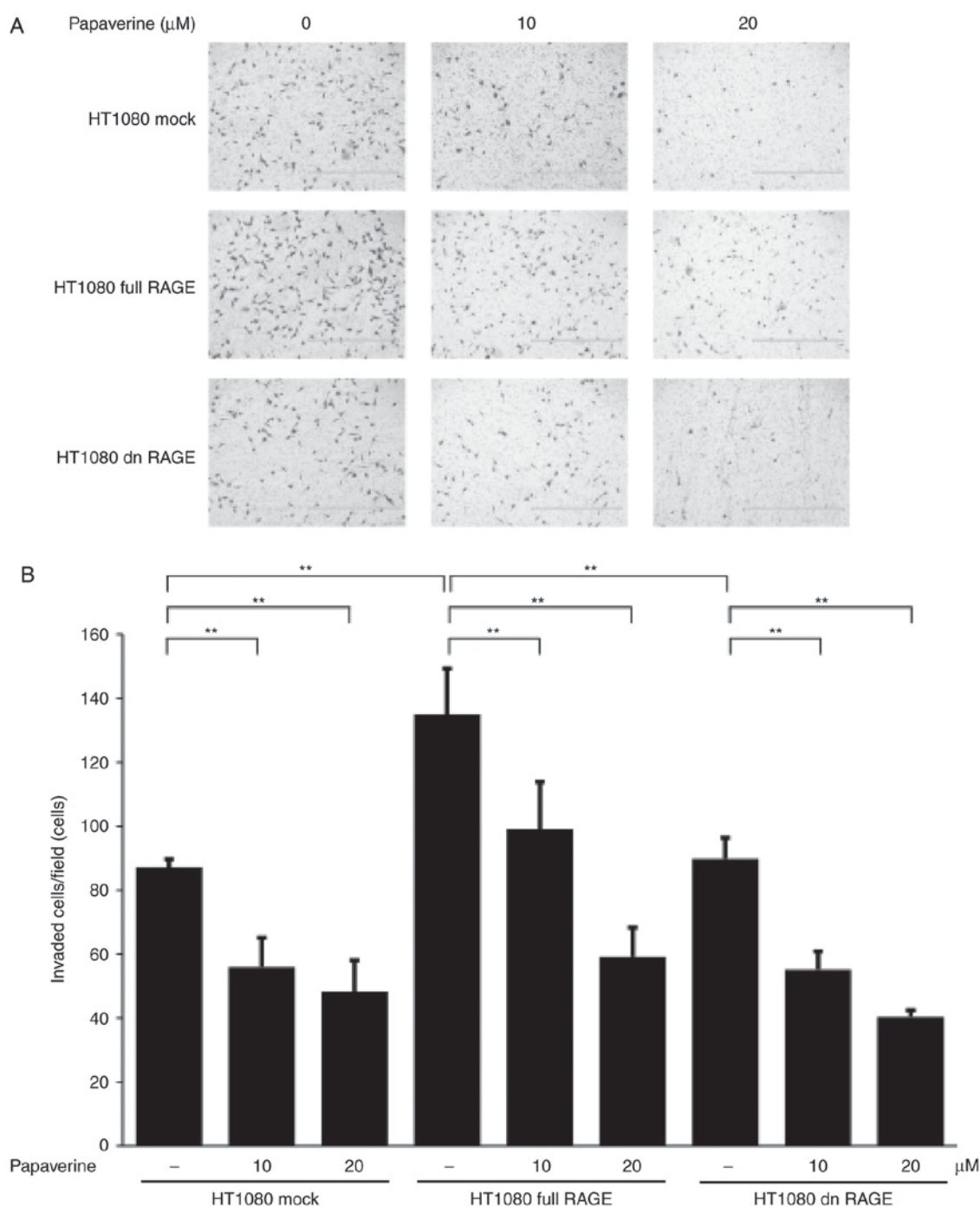


Figure 4. Papaverine inhibits RAGE-induced invasion of HT1080 fibrosarcoma cells. (A) Matrigel cell invasion assay was performed using HT1080 RAGE, HT1080 dnRAGE and HT1080 mock fibrosarcoma cells. Scale bar, 1 mm. (B) The number of migrating cells was determined. -, non-treated control; 10, 10 μM papaverine; 20, 20 μM papaverine. Values represent the mean \pm standard error of the mean (n=3). **P<0.01. RAGE, receptor for advanced glycation end-products; dn, dominant-negative.

for treating certain types of cancer. Previous experimental studies have revealed that inhibiting RAGE suppressed tumor growth, invasion and angiogenesis in multiple types of cancer (10,16,38). The therapeutic efficacy of blocking RAGE from interacting with HMGB-1 was initially demonstrated in glioma cells, in which this blockade inhibited tumor growth and invasion (16). Subsequently, strategies and components of RAGE inhibition have been reported in oncology and other fields, including neurology (39,40). For example, RAGE-neutralizing antibodies and sRAGE decreased the emergence of lung metastasis

following intracardiac injection of Lewis lung carcinoma cells (39,40). In addition, endogenous secretory receptor for advanced glycation end products, another soluble decoy form of RAGE, was demonstrated to inhibit A β -42 uptake into mouse brain; therefore, it may be effective in AD (41). Hong *et al* (42) evaluated the effects of the RAGE-specific inhibitor FPS-ZM1 on A β metabolism, AGE-induced inflammation and oxidative stress in rat hippocampus. In addition, blocking RAGE signaling in tumor-associated macrophages has been proposed as a potential anticancer strategy; the macrophages form the tumor

microenvironment, which could drive tumor angiogenesis (30). FPS-ZM1 directly inhibited primary tumor growth; in addition, it blocked RAGE signaling in tumor-associated macrophages, inhibited tumor angiogenesis and inflammatory cell recruitment and inhibited metastasis to the lungs and liver (16). Another RAGE-binding peptide, RP-1, was demonstrated to inhibit A β -induced cellular stress in human neuroblastoma cells *in vitro* (43). Furthermore, Han *et al* (44) reported that 4,6-bisphenyl-2-(3-alkoxyanilino) pyrimidine inhibited the binding of A β to RAGE.

The present study identified papaverine as a RAGE inhibitor using the drug design system COSMOS, an example of drug repositioning, the application of well-known existing drugs and compounds for novel indications. As a structure from which researchers could develop novel drugs, papaverine could represent a potential precursor to a therapeutic RAGE inhibitor. RAGE has been implicated in multiple pathogenic processes, in cancer and numerous other diseases, including diabetes, atherosclerosis, inflammatory and neurodegenerative diseases (7-9,45). Therefore, papaverine and its derivatives could be useful in preventing and treating multiple RAGE-associated diseases. To clarify our understanding of RAGE inhibition by papaverine, *in vivo* animal models should be used in future studies.

To conclude, the results of the present study suggested papaverine could inhibit RAGE and provided novel insights into the field of RAGE biology.

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