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Rebamipide inhibits gastric cancer growth by targeting survivin and Aurora-B

A. Tarnawski [a,b,*], R. Pai [b], S.-K. Chiou [a,b], J. Chai [a,b], E.C. Chu [a,b]

[a] Department of Medicine, VA Long Beach Healthcare System, 5901 E. 7th Street, Long Beach, CA 90822, USA
[b] University of California, Irvine, CA 92697, USA

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Abstract

Rebamipide accelerates healing of gastric ulcers and gastritis but its actions on gastric cancer are not known. Survivin, an anti-apoptosis protein, is overexpressed in stem, progenitor, and cancer cells. In gastric cancer, increased and sustained survivin expression provides survival advantage and facilitates tumor progression and resistance to anti-cancer drugs. Aurora-B kinase is essential for chromosome alignment and mitosis progression but surprisingly its role in gastric cancer has not been explored. We examined in human gastric cancer AGS cells: (1) survivin expression, (2) localization of survivin and Aurora-B, (3) cell proliferation, and (4) effects of specific survivin siRNA and/or rebamipide (free radical scavenging drug) on survivin and Aurora-B expression and cell proliferation. Survivin and Aurora-B are strongly expressed in human AGS gastric cancer cells and co-localize during mitosis. Survivin siRNA significantly reduces AGS cell viability. Rebamipide significantly downregulates in AGS cell survivin expression, its association with Aurora-B and cell proliferation. Rebamipide-induced downregulation of survivin is at the transcription level and does not involve ubiquitin–proteasome pathway.
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Rebamipide, an amino acid analog of 2(1*H*)-quinolinone, a novel drug developed in Japan, is clinically used for mucosal protection, healing of gastroduodenal ulcers, and treatment of gastritis [1,2]. The mechanisms responsible for its healing actions include enhancement of mucosal defense, scavenging free radicals, and a temporary activation of genes encoding cyclooxygenase-2 and growth factors [1–3]. Surprisingly, the effect of rebamipide on growth of human gastric cancer cells remains unexplored. Survivin, the 16.5 kDa protein, first described in 1997, is a member of the inhibitor of apoptosis protein (IAP) family [4]. Survivin is overexpressed in fetal tissue, rapidly dividing cells, such as stem and progenitor cells, and in a variety of human malignancies [4,5]. It suppresses apoptosis by inhibiting pro-apoptotic caspases-3 and -7, and promotes cell cycle progression by acting as a microtubule stabilizer during mitosis [6–9]. A sustained overexpression of survivin is a characteristic feature of gastric cancer, where by inhibiting apoptosis and facilitating mitosis, it gives cancer cells, a survival and growth advantage [10–14]. Limited studies demonstrated that in gastric cancer expression of survivin plays an important role in tumor progression and resistance of malignant cells to anti-cancer drugs [10–14]. Our previous studies demonstrated that survivin is expressed in normal human gastric mucosa and is temporarily overexpressed in the epithelial cells of gastric ulcer margin where it plays protective and ulcer healing promoting roles [15,16]. Aurora family of serine/threonine kinases is highly conserved in eukaryotes,

* Corresponding author. Fax: +1 562 826 8016.
E-mail addresses: andrzej.tarnawski@med.va.gov, atarnawski@yahoo.com (A. Tarnawski).

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is essential in some cells for a proper progression of mitosis, and is involved in many processes involved in cell division [17–23]. Aurora-B is a chromosomal passenger protein essential for chromosome alignment and cytokinesis [17–23]. It concentrates at centromeres and relocates to the central spindle in anaphase [17–23]. Aurora-B plays roles in spindle dynamics, chromosome condensation, and cytokinesis by interacting with other proteins such as INCENP, survivin, and intermediate filaments [17–23]. Overexpression of both Aurora-A and Aurora-B frequently occurs in a variety of human cancers [22,23]. Surprisingly, the expression of Aurora-B in human gastric cancer has not been explored before.

This study was aimed to determine: (1) expression and cellular localization of survivin and Aurora-B in human gastric cancer AGS cells and (2) to examine in gastric cancer AGS cells the effect of: (a) downregulation of survivin with specific siRNA and (b) treatment with rebamipide on survivin and Aurora-B expression and cell proliferation. Since ubiquitin–proteasome pathway is a major cellular process of survivin degradation [24], we examined whether rebamipide-induced downregulation of survivin occurs via the ubiquitin–proteasome mechanism.

This study demonstrates for the first time that Aurora-B is strongly expressed in human gastric cancer AGS cells and binds in these cells to survivin in the mitotic spindle. It further shows that anti-ulcer drug rebamipide arrests growth and proliferation of human gastric cancer cells by reducing survivin and Aurora-B expression. Rebamipide-induced downregulation of survivin is at the transcription level and does not involve ubiquitin–proteasome degradation pathway.

Materials and methods

RPMI-1640 medium was obtained from Gibco-BRL (Grand Island, NY). Dulbecco's modified Eagle's medium/F12 was obtained from Invitrogen (Carlsbad, CA). Antibiotic, antimycotic supplement, and other tissue culture reagents were obtained from Fisher Scientific (Tustin, CA). Fetal bovine serum (FBS) was obtained from Atlanta Biologicals (Norcross, GA). Rabbit polyclonal survivin antibody (FL-142) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and Aurora-B mouse monoclonal antibody (AIM-1) was obtained from BD Biosciences Pharmingen (San Diego, CA). All other chemicals including proteasome inhibitor MG-132 and secondary antibodies for survivin and Aurora-B immunostaining were obtained from Sigma Chemical (St. Louis, MO).

Gastric cancer AGS cell line. Human gastric cancer cell line (AGS) (from ATCC, Rockville, Maryland CRL 1739) was routinely cultured in RPMI-1640 medium supplemented with 10% FBS, 1% glutamine, antibiotics, and anti-fungal agents. This cell line is derived from gastric adenocarcinoma resected from a female patient who had received not prior therapy. This is a hyperdiploid human cell line. The modal chromosome number was 49, occurring in 60% of cells. For each experiment, cells were serum-deprived for 24 h prior to treatment with either: (a) medium only (control) or (b) medium-containing rebamipide (1.4, 2.7, 5.4, and 13.5 mM; equivalent to 0.5, 1, 2, and 5 mg/ml medium) for 3–48 h.

To determine whether ubiquitin–proteasome pathway is involved in rebamipide action on survivin expression level, AGS cells were pretreated with proteasome inhibitor, MG-132, 10 μM, 30 min prior to treatment with either medium or rebamipide 5 mg/ml for 6 h.

mRNA expression of survivin by RT/PCR. Human survivin specific RT-PCR primers (GenBank Accession No. NM-001168) used for analysis of survivin mRNA expression by RT/PCR were purchased from R&D System (Minneapolis, Minnesota) (RPP-204-025). Cells were directly lysed in Trizol Reagent for a RNA isolation. Total RNA (0.3 μg) from each sample was used in the reverse transcription reaction to synthesize first strand cDNA. Ten microliters of cDNA was used in each PCR.

Western blot analysis. Cell lysates containing equal amount of proteins were subjected to SDS–PAGE and transferred onto a nitrocellulose membrane. The membrane was incubated with a specific, primary polyclonal survivin antibody followed by peroxidase-conjugated secondary antibody. Immunoreactive proteins were visualized using ECL detection system (Amersham, Arlington Heights, IL). Where indicated, the membrane was stripped and reprobed with another specific primary antibody. Protein signals were quantified using Metamorph Imaging System, version 3.0 (Universal Imaging, Downingtown, PA). Indicated protein signal intensities from the Western blots were subtracted from background signal intensities.

Immunohistochemistry. AGS cells were plated on coverslips and grown to ~70% confluence. Cells were subjected to various treatments as indicated, fixed with 4% paraformaldehyde for 20 min at room temperature, then with cold (−20 °C) acetone for 5 min, and blocked with Superblock (SCYTEK, Logan, Utah) for 7 min at room temperature. After washing with PBS, coverslips were incubated with anti-survivin or anti-Aurora-B antibodies (1:200 dilution) for 2 h at room temperature. After washing with PBS, coverslips were incubated with secondary antibodies: anti-rabbit Texas Red-conjugated for survivin (dilution 1:400) and anti-mouse FITC-conjugated for Aurora-B (1:100 dilution) for 40 min at room temperature. Coverslips were then mounted and images were captured using a Nikon digital camera DXM1200 (Nikon, Tokyo, Japan) attached to a Nikon Optiphot fluorescence microscope.

siRNA studies. Human survivin siRNA (silencer validated siRNA, targeting exon-3 of human survivin) was custom synthesized by Ambion (Houston, TX). AGS cells were seeded into 6-well plates at 10^5 cells per well 24 h before transfection. Three wells were used per each treatment and each condition. A total of 200 nmol/L of survivin siRNA or equimolars of control nonspecific RNA were transfected into RGM-1 cells using oligofectamine reagent (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. Sixteen hours after transfection, cells were incubated in serum-free medium. At 8, 32, and 56 h after serum starvation, cultures were incubated for another 24 h. Media were collected for assessment of cell injury using LDH release assay [25] as described below. Additional transfected cells were lysed after the same incubation time to examine survivin expression by Western blot analysis. Cellular damage was determined and quantified using the Cytoscan LDH Colorimetric Assay Kit (Geno Technology, St. Louis, MO), according to the manufacturer's instructions. Briefly, release of cytosolic LDH enzyme into the culture supernatant upon cell membrane damage triggered an enzymatic reaction resulting in conversion of a tetrazolium salt into a red color formazan. The amount of formazan is proportional to the amount of damaged cells and was measured at 490 nm using a DU640B spectrophotometer (Beckman). Percent cell injury was determined using the following methods:

$$\%\ \text{cell injury} = \frac{(\text{experimental OD}_{490} - \text{spontaneous OD}_{490})}{\text{maximum LDH release}(\text{OD}_{490})} \times 100.$$

Spontaneous OD was obtained from the culture supernatant of vehicle-treated controls, and maximum OD was read from the positive control (cells disintegrated by sonication).

Cell proliferation assay. Cell proliferation was studied by determining the incorporation of [^{3}H]thymidine into cellular DNA similar to our previous study [26]. In brief, gastric cancer AGS cells were grown in 24-well culture plates to attain 75% confluence. Cells were serum starved for 24 h and treated with rebamipide 0.5–5 mg/ml for 3–48 h. Two hours prior to termination of the experiment, 2 μCi of methyl-[^{3}H]thymidine was added to each well. After incubation, the cells were washed three times with ice-cold PBS, lysed with 0.5 N NaOH, and neutralized with 0.5 N HCl. The radioactivity was then measured [26].

Statistical analysis. Student's t test was used to compare data between two groups. One-way ANOVA followed by Bonferroni correction was used to compare data between three or more groups. Values are expressed as means ± SD. A value of $P < 0.05$ was considered statistically significant.

Results

Survivin mRNA and protein are strongly expressed in gastric cancer AGS cells as reflected by RT/PCR (Fig. 1A), Western blotting (Fig. 1B), and immunostaining (Figs. 1C and 2A). Immunostaining demonstrated expression of survivin in ~52% of cancer cells, strong staining predominantly localized to the nuclei (Figs. 1C and 2A). Aurora-B is also strongly expressed in AGS cells, often co-expressed and co-localized with survivin, especially in the mitotic spindle of cells undergoing divisions (Figs. 2B and C).

Treatment with specific survivin siRNA significantly knock down survivin expression (Figs. 3A and B) and significantly reduced cell viability (Fig. 3C). Treatment with rebamipide significantly reduced survivin mRNA and protein expression (Figs. 4A, B and 5) and reduced Aurora-B (Fig. 5) and cell proliferation (Fig. 6). Pretreatment with the proteasome inhibitor, MG-132, did not affect rebamipide-induced downregulation of survivin in AGS cells (data not shown), indicating that ubiquitin–proteasome pathway is not involved in the mechanism of rebamipide action on survivin in AGS cells.

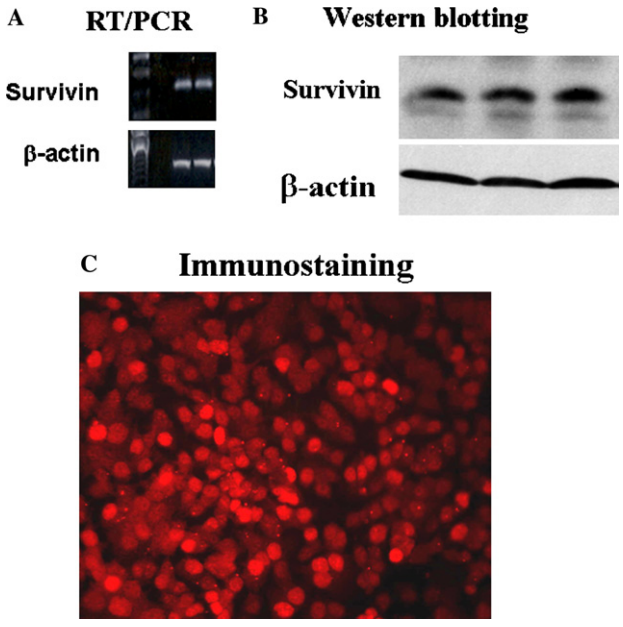

Fig. 1. Expression of survivin mRNA and protein in human gastric cancer AGS cells. (A) RT/PCR detection of survivin mRNA, (B) Western blotting for survivin, and (C) immunostaining using specific antibody and Texas Red-conjugated secondary antibody. In AGS cells, survivin is expressed in ~52% of cells (red staining) predominantly in the nuclei.

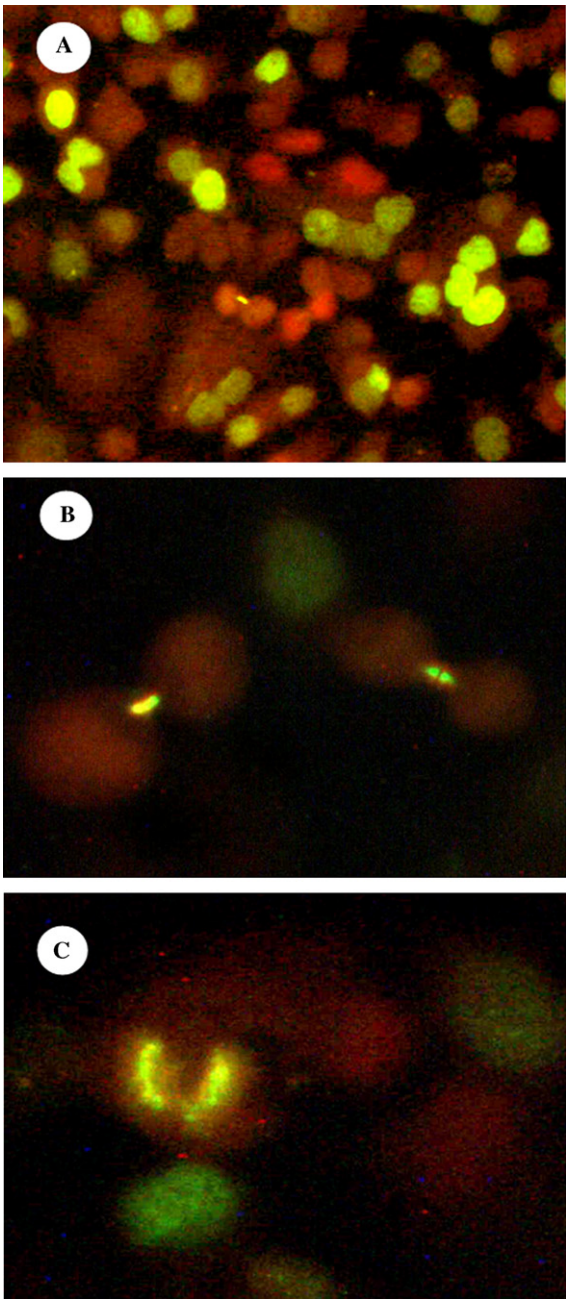

Fig. 2. Double immunostaining for survivin and Aurora-B using specific antibodies. Survivin is stained red, Aurora-B green, and co-localization of survivin with Aurora-B is stained yellow (A). Survivin and Aurora-B are co-localized to the mitotic spindle of cells undergoing division (B,C).

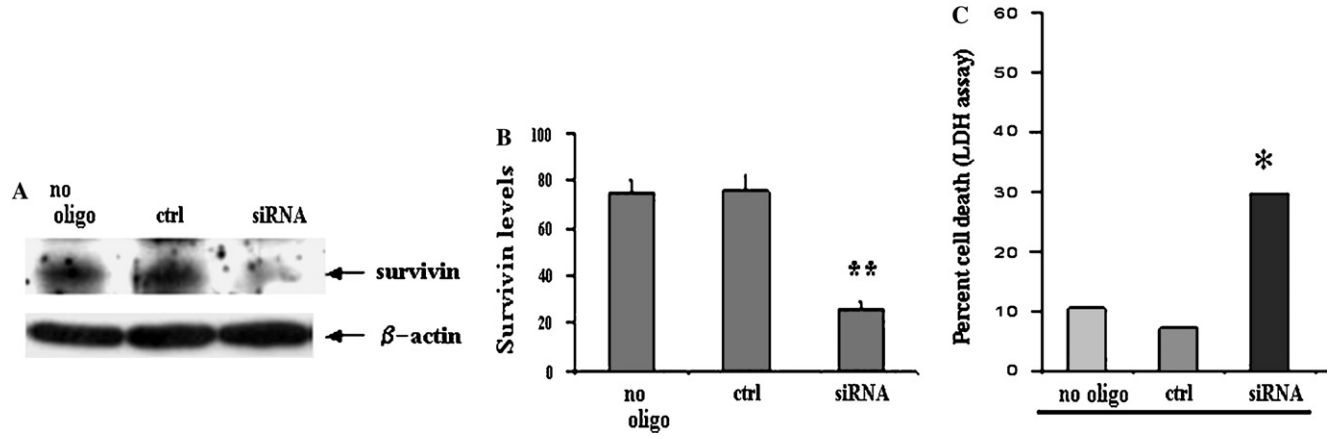

Fig. 3. Effect of treatment with either specific human survivin siRNA, no oligo or nonspecific RNA (control) on AGS cell survivin protein expression level determined by Western blotting (A,B) and cell viability is determined by LDH release into the medium (C).

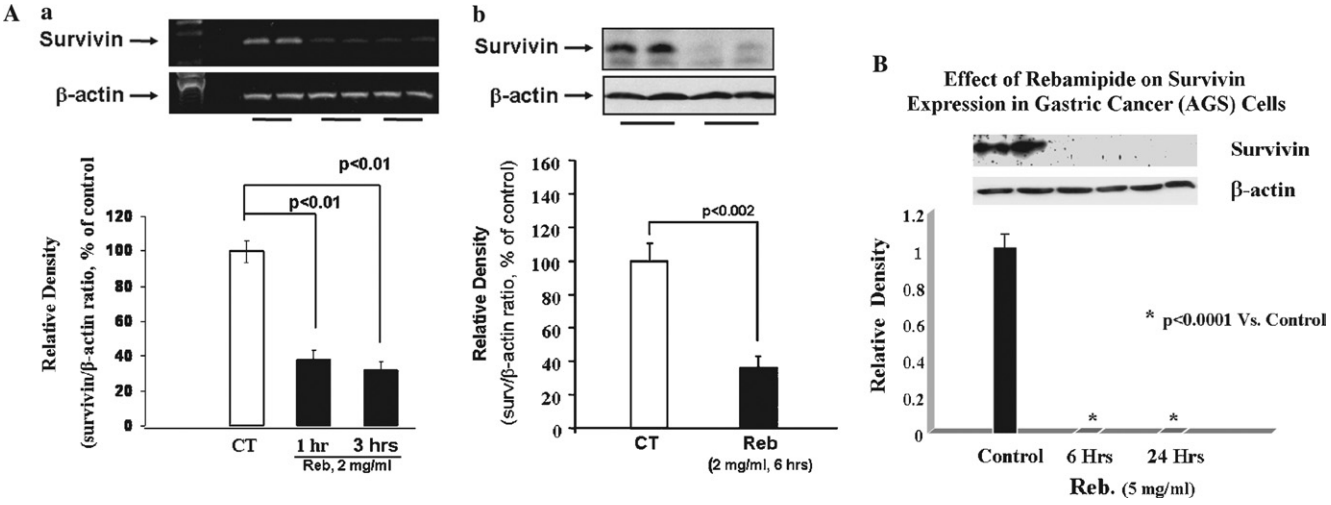

Fig. 4. (A,B) Treatment of AGS cells with rebamipide significantly reduces survivin mRNA and protein expression determined by RT/PCR (A,a) and Western blotting (A,b). (A) Two milligrams of rebamipide and (B) 5 mg rebamipide.

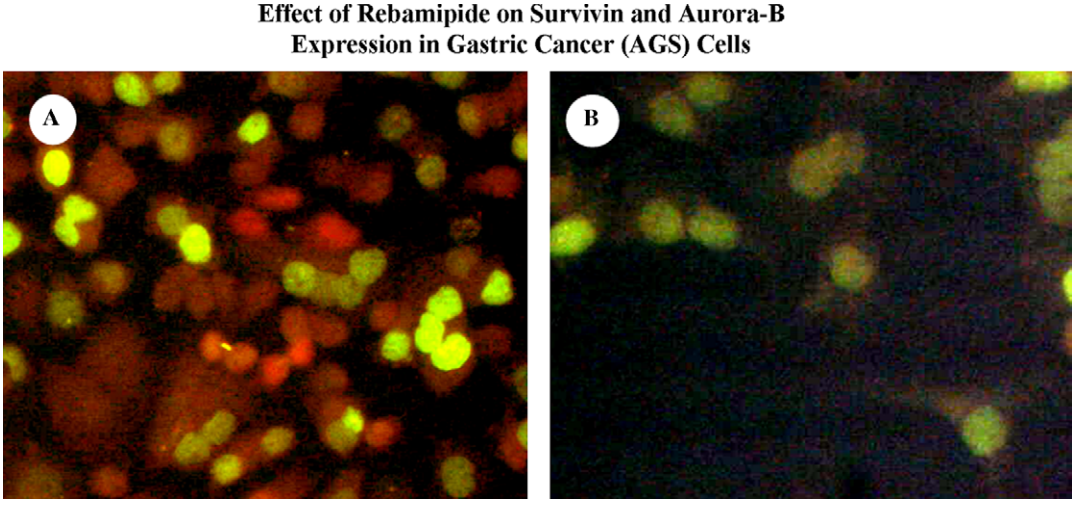

Fig. 5. Immunostaining of AGS cells for survivin and Aurora-B: (A) placebo treatment and (B) rebamipide treatment. Rebamipide treatment dramatically reduced expression of survivin and Aurora-B in AGS cells, both the number of cells and intensity of staining and inhibited mitoses as reflected by a virtual absence of mitotic figures.

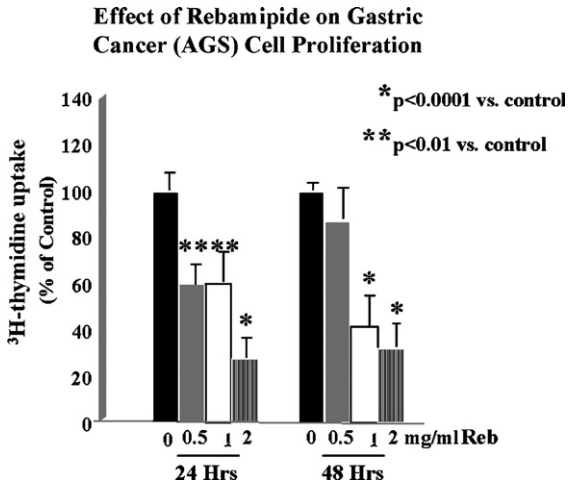

Fig. 6. Effect of rebamipide treatment on AGS cell proliferation determined by [^{3}H]thymidine uptake.

Discussion

This study demonstrated that survivin is strongly expressed in human gastric cancer AGS cells and that anti-ulcer drug, rebamipide, strongly downregulates survivin expression. This downregulation is at the transcription level, since rebamipide did significantly reduce survivin mRNA. Since the ubiquitin–proteasome pathway regulates survivin degradation in some cells [24] including human hepatocellular carcinoma cell lines [27], we examined whether proteasome inhibitor, MG-132, affects rebamipide-induced survivin downregulation. The proteasome inhibitor, MG-132, did not affect rebamipide-induced downregulation of survivin in AGS cells, which clearly indicates that proteasome degradation pathway is not involved in survivin downregulation by rebamipide. Downregulation of survivin preceded a significant inhibition of AGS cell proliferation reflected by reduced [^{3}H]thymidine uptake and a dramatic reduction in the number of mitotic figures. This finding underscores the essential role of survivin in mitotic spindle formation and promotion of mitosis.

This study also demonstrated for the first time a strong expression of Aurora-B in human gastric cancer AGS cells and its binding, association, and co-expression with survivin in the mitotic spindle in cancer cells undergoing division. Furthermore, it demonstrated the important role of survivin in gastric cancer cell growth and viability. Downregulation of survivin with specific siRNA significantly decreased AGS cell viability as reflected by increased LDH release into the medium (a sensitive index of cell viability [25]), which indicates increased gastric cancer apoptosis by downregulation of survivin. Moreover, this study demonstrated that anti-ulcer drug, rebamipide, reduces survivin and Aurora-B expression in AGS cells, decreases binding of Aurora-B to survivin in the mitotic spindle, and reduces cell proliferation. The concentrations of rebamipide used in this study are clinically relevant, since after oral ingestion, the drug has direct contact with gastric mucosa and therefore local concentrations are high [28]. The in vivo relevance of our findings with regard to effect of rebamipide on cancer cells is supported by a paper reporting that treatment with rebamipide significantly reduced duodenal carcinogenesis in mice [29]. However, that study did not provide any insight into the mechanisms. Since rebamipide is used in Japan, Korea, China, Philippines, and other Asian countries for treatment of gastritis, which in chronic stages may be associated with intestinal metaplasia and gastric cancer, our findings have important clinical implications. Overall, the present study provides a rationale for further testing of anti-cancer properties of rebamipide.

Conclusion

(1) Survivin and Aurora-B are strongly overexpressed in human gastric cancer cells, especially in the mitotic figures. (2) Survivin downregulation with siRNA inhibits gastric cancer cells viability and growth. (3) Rebamipide (anti-ulcer drug) arrests growth of human gastric cancer cells by reducing survivin and Aurora-B expression, preventing their translocation to the mitotic spindle and inhibiting cell proliferation. (4) The regulatory mechanism of rebamipide-induced downregulation of survivin operates at the transcription level and does not involve ubiquitin–proteasome pathway.

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