**Bortezomib induces apoptosis and suppresses cell growth and metastasis by inactivation of Stat3 signaling in chondrosarcoma**

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Received August 17, 2016; Accepted October 7, 2016

DOI: 10.3892/ijo.2016.3806

**Abstract.** Bortezomib, formerly known as PS341, is a novel proteasome inhibitor with *in vitro* and *in vivo* antineoplastic effects in many malignancies. However, diverse antitumor mechanisms of bortezomib have been identified in many investigations and preclinical studies. Understanding the molecular and cellular mechanisms through which bortezomib acts will improve the therapeutic utility of this drug in different cancer types. In the present study, we investigated the *in vitro* and *in vivo* effects of bortezomib on chondrosarcoma. Bortezomib selectively inhibited cell growth in chondrosarcoma cells but not in normal articular cartilage cells. In addition to growth inhibition, apoptosis and cell cycle arrest, bortezomib triggered alleviation of migratory and invasive properties of chondrosarcoma cells. Mechanistically, signal transducer and activator of transcription 3 (Stat3) and its downstream targets Bcl-2, cyclin D1 and c-Myc was inactivated by bortezomib treatment. Accordingly, small interfering RNA (siRNA)-mediated Stat3 knockdown enhanced bortezomib-induced apoptosis, and concomitantly enhanced the inhibitory effect of bortezomib on cell viability, migration and invasion. Moreover, while Slug, MMP9, MMP2, CD44, N-cadherin and vimentin, the mesenchymal cell markers, were repressed by bortezomib concomitant increased expression of E-cadherin was observed. *In vivo*, bortezomib downregulated Stat3 activity and mesenchymal cell marker expression, induced apoptosis and inhibition of metastasis and tumor growth. Together, inactivation of Stat3 signaling contributes to bortezomib-induced inhibition of tumor growth, migration and invasion on chondrosarcoma. Bortezomib demonstrates an antineoplastic role on chondrosarcoma both *in vitro* and *in vivo*. These beneficial effects can be explained by bortezomib-mediated Stat3 suppression. The present study suggests a promising therapeutics target in chondrosarcoma and probably in other kinds of metastatic malignant tumors.

**Introduction**

Chondrosarcoma is the second most commonly occurring primary bone tumor (1-3). At present, wide-margin surgical resection remains the main curative method of treatment for chondrosarcoma, which continues to have poor prognosis due to resistance to conventional chemo- and radiotherapy (4-6). Consequently, there exists a critical clinical need to explore novel molecular-targeted therapies for this disease.

The transcription factor signal transducer and activator of transcription 3 (Stat3) is a point of convergence for numerous oncogenic and inflammatory signaling pathways, including cytokines, growth factors and oncogenes (7-9). Stat3 participates in cell growth and survival regulating cell proliferation, apoptosis and autophagy (9,10). Constitutive activation of Stat3 represents a key player in tumor angiogenesis and metastasis and has been observed in other cancers of the bone including osteosarcoma (10-15). As such, Stat3 has emerged as a promising molecular target for cancer therapy.

Bortezomib, formerly known as PS341, is a selective proteasome inhibitor, which has been approved by the US Food and Drug Administration for the treatment of patients with multiple myeloma and mantle cell lymphoma (16,17). Recently it was indicated that bortezomib also has crucial antineoplastic activity against some solid tumors, including ovarian and prostate cancers as well as squamous cell cancer of the head and neck, Ewing's sarcoma and osteosarcoma. Preclinical data have suggested that bortezomib's antineoplastic effect occurs via diverse mechanisms, exerting significantly different roles in a tissue and cancer-specific context (18-21). Moreover, it has been shown that inactivation of Stat3 plays an important role in bortezomib-mediated treatment of myeloma (22). However, the effects of bortezomib on human chondrosarcoma have not been adequately studied and, in particular, the mechanisms by which bortezomib exerts its antitumor functions in human chondrosarcoma have not been previously investigated.
While the majority of published studies on the antineoplastic actions of bortezomib have focused on suppression of proliferation and promotion of apoptosis (17,19), little is known about the effects of bortezomib on modulating the cell cycle progression and metastasis in cancer.

In the present study, we investigated the in vitro and in vivo effects of bortezomib on human chondrosarcoma. In particular, we demonstrated the importance of Stat3 signaling for the antitumor actions of this agent, including apoptosis promotion, cell cycle arrest, inhibition of migration and invasion. Thus, we report on the therapeutic potential of bortezomib on human chondrosarcoma, based on our improved understanding of this agent.

Materials and methods

Animals and ethical statement. Six-week-old BALB/c female athymic nude mice were obtained (Vital River Laboratories Co., Ltd., Beijing, China). The present study was carried out in accordance with the recommendations in the Guide for the Chinese Ethics Review Committees. The protocol was approved by the Ethics Committee of Peking University People's Hospital. All experiments was carried out under the ethics approval of Peking University People's Hospital and was conducted according to the National Institutes of Health guidelines. Nude mice were maintained under specific pathogen-free (SPF) conditions, and bedding, water and daily rations were sterilized.

Tissue specimen. The paraffin-embedded pathological specimens from 12 patients with chondrosarcoma and adjacent non-tumor tissues were obtained from the Department of Pathology and the Musculoskeletal Tumor Center, Peking University Peoples Hospital (Beijing, China). Informed consents (written in the light of the ethical guidelines) were obtained from all the patients. All human specimens were approved by the Research Ethics Committee of Peking University Peoples Hospital (Beijing, China).

Cell culture, cell viability assay and colony formation assay. The human articular chondrocyte cell line HC-a (ScienCell Research Laboratories, Carlsbad, CA, USA) was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% fetal bovine serum (FBS), plus antibiotics. SW1353 cells were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA) and were maintained in L-15 medium (Gibco, Grand Island, NY, USA). OUMS-27 cells, HCS-2/8 cells and JJ012 cells were respectively harvested, washed and suspended with indicated condition, which included gene knockdown. The cell viability was examined by CCK-8 assay. For colony formation assay, following treatment, adherent cells were trypsinized and 1,000 viable cells were subcultured in 6-well plates (in triplicate). Cells were allowed to adhere and colonize for 14 days. Media were removed and cells were fixed in methyl alcohol for 15 min and stained with crystal violet staining solution for visualizing colonies, as previously described (23).

Western blot analysis. Equal amounts of proteins collected from cell lysates were loaded on 10-15% SDS-PAGE gels using a NuPAGE system (Invitrogen, Carlsbad, CA, USA) and then transferred onto PVDF membranes as previously described (24). The following antibodies were used in the experiments: anti-p-STAT3, anti-STAT3, anti-c-Myc, anti-E-cadherin, anti-vimentin, anti-cyclin D1, anti-Bax, anti-Bcl-2, and anti-GAPDH were all from Cell Signaling Technology (Beverly, MA, USA). Anti-N-cadherin, anti-Slug, anti-MMP9 and anti-CD44 were from Abcam (Cambridge, MA, USA).

Immunohistochemistry. Paraffin sections were reacted with rabbit polyclonal anti-p-Stat3, anti-vimentin, anti-N-cadherin and anti-E-cadherin antibodies (1:200 dilution). Sections stained with non-immune rabbit serum (1:200 dilution) in phosphate-buffered saline (PBS) instead of primary antibody served as negative controls. Cells exhibiting positive staining on cell membranes and in the cytoplasm and nucleus were counted in at least 10 representative fields (x400 magnification) and the mean percentage of positive cells was calculated. Immunostaining was assessed by two independent pathologists blinded to clinical characteristics and outcomes.

Cell cycle and apoptosis analysis by flow cytometry. Cells for cell cycle analysis were fixed in 70% ethanol, digested with RNaseA and labeled with propidium iodide (PI). Apoptotic cells were analyzed with Annexin V/FITC kit (BD Biosciences, San Jose, CA, USA) according to the manufacturer's instructions and were analyzed by flow cytometry after compound treatment as previously described (25).

Wound healing assay. Cells (2x10⁴) OUMS-27, HCS-2/8 and SW1353 were seeded into a 24-well plate. The tumor cells grew to confluence 24 h later. An artificial wound was introduced with a P10 pipette tip/well. Data of the wounded area were taken at 0 and 24 h with a microscope (Olympus Corp). The assay was repeated three times.

Transwell assay. HCS-2/8, OUMS-27 and SW1353 cells were respectively harvested, washed and suspended with DMEM (Gibco/Life Technologies, 11965-092), L-15 medium (Gibco) and seeded to the upper chambers of Transwell inserts (8 µm pore size; Corning Incorporated, Corning, NY, USA) with/without low concentration bortezomib (10 nM) in the migration assay. The upper chambers were coated with Matrigel (BD Biosciences, 354234) prior to the inoculation of the cancer cells and bortezomib in the invasion assay. The lower compartments were filled with DMEM or L-15 medium supplemented with 5% FBS. The cells in the upper chamber were removed with a swab after incubation for 12 h in the migration assay or 24 h in the invasion assay. The cells that migrated to the lower layer and attached to the membrane were stained with crystal violet and were numbered in five
fields per well under a microscope. The assay was repeated three times.

**Mesenchymal-epithelial transition (MET) induction.** OUMS-27 and HCS-2/8 cells were cultured as adherent cells in the complete medium overnight. The cells were maintained in either medium alone or medium supplemented with 2.5 ng/ml TGF-β1 (R&D Systems, Minneapolis, MN, USA), with or without 20 nM bortezomib in a humidified 5% CO₂ incubator at 37˚C. The morphological images of the cells were taken seven days after the incubation. The experiments were repeated three times.

**Gene knockdown using siRNA.** The siRNAs to Stat3 or control siRNA were all purchased from Suzhou GenePharma, Co., Ltd. (Suzhou, China). Cells were transfected with siRNA using Lipofectamine 3000, purchased from Origene Technologies Inc. (Rockville, MD, USA) according to the manufacturer’s instructions. Cells were incubated for 48 h before further treatment.

**Generation of xenografts.** Six-week-old BALB/c female athymic nude mice (Vital River Laboratories) were subcutaneously injected in the right flank with cells (2×10⁶ in 0.1 ml PBS). Once a palpable tumor developed, the mice were randomly divided into two groups and intraperitoneally administered dimethyl sulfoxide (DMSO) or bortezomib at a dose of 0.5 mg/kg every other day for 30 days. The volume of xenografts was measured every 5 days (tumor volume = (length × width²)/2). The mice were sacrificed after 30 days. The tumor samples were processed for routine IHC. The tumor metastatic ability of HCS-2/8 cells (5×10⁶) was observed following cell injection intravenously into the tail vein. Four weeks later, the mice were randomly divided into two groups and intraperitoneally administered with DMSO or bortezomib at a dose of 0.5 mg/kg every other day for 30 days (n=6 per group), the mice were sacrificed and the number of metastatic nodules on the lung surface was counted. Metastatic lungs were fixed with 4% paraformaldehyde before dehydration and paraffin embedding. Paraffin sections were stained with hematoxylin and eosin according to the standard protocols.

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**Figure 1.** Expression of p-Stat3, vimentin and N-cadherin in normal articular cartilage, in chondrosarcoma and in their corresponding cell lines HC-a, HCS-2/8, OUMS-27, SW1353 and JJo12. (A) Western blot analysis demonstrates that p-Stat3 was highly expressed in chondrosarcomas but low in the normal articular cartilage tissues (left panel). Average expression levels of p-Stat3 of normal articular cartilage and chondrosarcoma tissues (right panel). Data are presented as mean ± SD (n=12). *P<0.05. (B) Proteins were examined by western blotting in human articular chondrocyte cell line HC-a and chondrosarcoma cell lines HCS-2/8, OUMS-27, SW1353 and JJo12. (C) The expression of p-Stat3, vimentin and N-cadherin a cohort of 42 human chondrosarcoma specimens was determined by IHC staining. Representative images of p-Stat3, vimentin and N-cadherin normal and chondrosarcoma specimens are shown.
Statistical analysis. Kaplan-Meier analysis was used to estimate the cumulative cause-specific survival rate and the differences in mouse survival with DMSO/bortezomib (n=5 per group). The influence of bortezomib on the growth, apoptosis, migration, invasion, cell cycle and tumor formation of chondrosarcoma cells were analyzed by the Student's t-test. In all statistical analyses, statistical significance in the two-sided test was indicated with P≤0.05 and P<0.01 was remarkably significant.

Results

p-Stat3 and EMT-related protein expression in the normal articular cartilage, in chondrosarcoma and in their corresponding cell lines HC-a, OUMS-27, HCS-2/8, SW1353 and JJ012. We examined the expression of p-Stat3 using western blot analysis in 12 samples taken from the normal articular cartilage and chondrosarcoma tissues, respectively. Western blot analysis showed that p-Stat3 was highly expressed in chondrosarcomas and considerably lower in the normal articular cartilage tissues (Fig. 1A and B). These results indicated that p-Stat3 might have tumor-promoting roles in chondrosarcoma. To validate our results, we analyzed the expression of p-Stat3 in the corresponding cell lines HC-a, HCS-2/8, OUMS-27, SW1353 and JJ012 by western blotting and observed similar results (Fig. 1C).

We also assessed the expression of p-Stat3 and vimentin with immunohistochemistry (IHC). Weak expression of
p-Stat3, N-cadherin and vimentin was observed in the normal articular cartilage, but strong expression was recorded in the chondrosarcoma tissues (Fig. 1D).

Bortezomib specifically inhibits the growth and promotes apoptosis of human chondrosarcoma cells. To investigate the effect of bortezomib on growth of human chondrosarcoma cells, we first examined the function of bortezomib in three human chondrosarcoma cell lines (OUMS-27, HCS-2/8 and SW1353) and human articular chondrocyte cells (HC-a), respectively. We cultured each cell line with different concentration of bortezomib for 12, 24 or 48 h and analyzed cell viability. The growth of human chondrosarcoma cells was inhibited by bortezomib in a dose- and time-dependent manner. By contrast, the viability of HC-a cells only decreased slightly (Fig. 2A). As shown in Fig. 2B and C, the IC_{50} of bortezomib in HC-a cells was higher than that needed for chondrosarcoma cells (HCS-2/8, OUMS-27 and SW1353). In colony formation assays, bortezomib decreased colony formation compared with control (Fig. 2D and E). These data showed that bortezomib specifically suppressed the growth of human chondrosarcoma cells but not normal cells in vitro.

Bortezomib induces apoptotic cell death and promotes G0/G1 phase arrest. To investigate the role of bortezomib in cell death of chondrosarcoma cells, chondrosarcoma cells were analyzed by flow cytometry following Annexin V-FITC and propidium iodide (PI) dual staining. As shown in Fig. 3A, bortezomib significantly induced apoptosis. As key executors of cell apoptosis, the ratio of Bax to Bcl-2 proteins increased following a 48-h treatment with bortezomib, or with 20 nM bortezomib for indicated time-points (Fig. 3C). As previously reported, siRNA-mediated inhibition of STAT3 promoted G0/G1 cell cycle arrest (26). We evaluated...
the effects of bortezomib on cell proliferation and cell cycle arrest by performing cell cycle analysis. Our analysis demonstrated that chondrosarcoma cells were mostly arrested within the G0/G1 phase, indicating there was a reduced number of dividing tumor cells following bortezomib treatment. There were also fewer cells under S phase, demonstrating that bortezomib could also inhibit DNA replication (Fig. 3B). In addition, cyclin D1, a G0/G1 phase-related protein, was decreased following treatment, as analyzed by western blotting assay (Fig. 3C). Together, these results illustrate that apoptosis, as well as cell G0/G1 phase arrest, are involved in the response of chondrosarcoma to bortezomib treatment.

**Bortezomib attenuates the migratory and invasive capacities of chondrosarcoma cells.** Chondrosarcoma cells display high migration and invasion capacities, that can be attenuated by bortezomib, as determined by wound healing assay (Fig. 4A) and transwell assays (Fig. 4B), respectively. In the wound-healing assay, bortezomib could very effectively suppress the migration of chondrosarcoma cells. Moreover, treatment with bortezomib resulted in markedly decreased invasive abilities of HCS-2/8, OUMS-27 and SW1353 cells in Matrigel (Fig. 4C). These data show that, in human chondrosarcoma cells, bortezomib effectively attenuates the migratory and invasive capacities.

**Bortezomib induces the mesenchymal-epithelial transition of chondrosarcoma cells.** Epithelial-mesenchymal transition (EMT) is a critical process for epithelial cells to harbor mesenchymal properties and is closely involved in cancer.
invasion and metastasis (27). To evaluate the effect of bortezomib on this process, we enhanced mesenchymal properties of chondrosarcoma cells with TGF-β1 (27,28) and found that the cell lines showed mesenchymal appearance, which was fibroblast-like. When treated with bortezomib, however, the chondrosarcoma cell lines maintained an epithelial appearance (Fig. 5A), indicating that bortezomib efficiently induced the mesenchymal-epithelial transition. Moreover, bortezomib impaired the expression of mesenchymal cell markers, including N-cadherin, vimentin and slug. However, the expression of E-cadherin, an epithelial cell marker, increased (Fig. 5B).

**Bortezomib inhibits the Stat3 signalling pathway.** As Stat3 is a well-known cancer therapeutic target, we studied whether Stat3 is engaged in bortezomib action in chondrosarcoma cells. Western blotting assay demonstrated that expression of p-Stat3 and its target c-Myc were decreased in SW1353 cells in dose- and time-dependent manner following bortezomib treatment (Fig. 6A). As shown in Fig. 3C, the other two direct downstream targets of Stat3, cyclin D1 and Bcl-2 decreased in the same way.

To further confirm the regulatory role of Stat3 signaling in bortezomib-treated chondrosarcoma, we characterized the effects of bortezomib in SW1353 cells in which Stat3

Figure 5. Bortezomib induces the mesenchymal-epithelial transition of chondrosarcoma cells. (A) Bortezomib suppressed the epithelial-related phenotypic traits induced by TGF-β1 in chondrosarcoma cells. (B) Protein expression levels of vimentin, N-cadherin and E-cadherin were carried out in HCS-2/8 and OUMS-27 cells with TGF-β1 and bortezomib treatment.

Figure 6. Inactivation of Stat3 signaling contributes to bortezomib-induced effects. (A) Bortezomib treatment repressed expression of p-Stat3 and c-Myc, as assayed by western blot analysis. (B) Western blotting was used to assess p-Stat3, Stat3 targets, apoptosis-related proteins, and EMT-related proteins in Stat3 siRNA- or control siRNA-transfected SW1353 cells treated with bortezomib. (C) The cell viability of siStat3 silenced SW1353 cells treated with 20 nM bortezomib for 48 h was decreased more sharply than that treated with siStat3 or 20 nM bortezomib only, assayed by CCK-8.
was silenced with siRNA. In accordance with bortezomib treatment, knockdown of Stat3 not only induced apoptosis, cell cycle arrest as indicated by increased bax and decreased cyclin D1, but also led to promotion of MET, as evidenced by enhanced E-cadherin expression and decreased N-cadherin, vimentin, MMP-9, CD44 and slug. The expression of p-Stat3 was decreased following siStat3 or bortezomib treatment for 48 h, while the level of p-Stat3 was more significantly repressed when treated both siStat3 and bortezomib, analyzed by western blot assay (Fig. 6B).

Moreover, silencing of Stat3 resulted in enhanced bortezomib-induced decreases in cell viability. As shown in Fig. 6C, the cell viability of siStat3 silenced SW1353 cells treated with 20 nM bortezomib for 48 h was decreased more significantly than that treated with siStat3 or only with 20 nM bortezomib. Thus, these data suggest that Stat3 inactivation is involved in bortezomib-mediated inhibition of growth, cell cycle pregression, metastasis and induced apoptosis.

Bortezomib inhibits chondrosarcoma tumor growth in vivo. Five days after HCS-2/8 and OUMS-27 cells were injected subcutaneously into the right armpit of nude BALB/c mice, the mice were randomly divided into two groups and intraperitoneally administered DMSO or bortezomib at a dose of 0.5 mg/kg.
every other day for 30 days. The volume of xenografts was measured every five days (tumor volume = (length x width$^3$)/2).

In line with the in vitro data, bortezomib administration was very effective in inhibiting tumor growth in vivo throughout the course of treatment, resulting in decreased tumor size (Fig. 7A). These data indicate that bortezomib reduces tumor volume and growth rate of chondrosarcoma cells in vivo.

The tumors were removed and evaluated by immunohistochemistry. Bortezomib decreased p-Stat3, vimentin and N-cadherin expression in tumors formed by HCS-2/8 and OUMS-27 cells (Fig. 7B).

Furthermore, bortezomib-treated mice had a significantly longer survival time compared with control mice (Fig. 7C).

**Bortezomib inhibits metastasis in vivo.** To extend our in vitro observations, we investigated whether bortezomib could regulate tumor metastasis in vivo. After HCS-2/8 cells were injected intravenously into the tail vein at 4-weeks, the mice were intraperitoneally administered DMSO or bortezomib at a dose of 0.5 mg/kg every other day for another 30 days. Bortezomib-treated mice displayed statistically significantly lower numbers of lung metastases than those treated with DMSO (Fig. 7D). Hematoxylin and eosin staining, of the lungs revealed fewer lung metastatic nodes in the mice treated with bortezomib (Fig. 7E).

**Discussion**

Chondrosarcoma is the second most frequent type of primary bone cancer following osteosarcoma (1-3), with limited effective treatment options. At present, surgical resection remains the only effective means of treating chondrosarcomas, but remains associated with poor prognosis due to their resistance to adjuvant treatments such as radio- and chemotherapy. However, molecular targeted therapies have completely changed the treatment paradigm of some tumors due to its high efficiency and low toxicity.

Persistent activation of signal transducer and activator of transcription-3 (Stat3) is found in a wide range of solid malignancies of tumors, containing musculoskeletal tumor, such as Ewing’s sarcoma and osteosarcoma (13,29-31). In accordance with our studies, we found that the Stat3 signal pathway is abnormally activated in chondrosarcoma. In addition, inhibiting Stat3 by specific siRNA could suppress the growth of human chondrosarcoma cells.

Bortezomib is a dipeptidyl boronic acid, which is a selective inhibitor of the proteasome. Many studies have showed that bortezomib can reversibly suppress the proteasome pathway by binding with the 20S proteasome complex directly and blocking its enzymatic activity (17,32). However, the effects of bortezomib appear to be cell type-specific or context dependent.

In the present study we found that a low concentration of bortezomib (20 nM) inhibited the growth of chondrosarcoma cells alone and did not affect normal articular chondrocyte cells. Mesenchymal-related traits, which is the key to tumor metastasis, was also efficiently inhibited by bortezomib treatment. Moreover, bortezomib caused cell cycle arrest and apoptosis of chondrosarcoma cells at a low concentration (20 nM). The molecular mechanisms of action of bortezomib included: growth inhibition, apoptosis promotion, cell cycle arrest, promotion of MET and the inactivation of Stat3.

Our findings are consistent with studies which have revealed that bortezomib inhibited tumor growth via inactivation of the Stat3 pathway (33). While some studies have suggested that bortezomib facilitated GO/G1 arrest in cancer cells (23,34), we found that bortezomib markedly induced GO/G1 phase arrest by downregulating cyclin D1, consequently resulting in chondrosarcoma cell quiescent and impeding tumor progression.

EMT is thought to play a significant role in metastasis (35,36). Metastatic relapses are characterized by rapidly proliferating, drug-resistant tumors that are associated with a high mortality rate (37). Metastasis is a complicated process, during which cells undergo phenotypic alteration and migrate away from the primary tumor, survive in the vasculature or lymphatics, and colonize metastatic sites (38). Stat3 is activated to trigger the molecular events in the EMT of various types of human tumors (39-41).

In the present study, following exposure of chondrosarcoma cells to bortezomib for 6 days, the cells displayed an epithelial-like morphology. Furthermore, there were increased expression of E-cadherin (an epithelial marker) and decreased expressions of N-cadherin and vimentin (mesenchymal markers). In tumors formed by HCS-2/8 and OUMS-27 cells injected into nude BALB/c mice, bortezomib reduced the expression of N-cadherin and vimentin. These results demonstrate that, exposure to bortezomib leads to chondrosarcoma cells undergoing MET in vitro and in vivo.

In conclusion, bortezomib demonstrates an antineoplastic role on chondrosarcoma both in vitro and in vivo. These beneficial effects can be explained by bortezomib-mediated Stat3 suppression. This study suggests a promising therapeutic target in chondrosarcoma and probably in other kinds of metastatic malignant tumors.

**Acknowledgements**

The present study was supported by the National Natural Science Foundation of China (nos. 81572633 and 81472509).

**References**


