International Journal of Drug Delivery 6 (2014) 133-139
http://www.arjournals.org/index.php/ijdd/index

Original Research Article

ISSN: 0975-0215

Inhibitory effect of gemcitabine and brucine on MDA MB-231human breast cancer cells

Mamatha. Serasanambati[1,2], Shanmuga Reddy Chilakapati[1,2], Jhansi Rani Vangavaragu[3], Damodar Reddy Chilakapati[1*]

***Corresponding author:**

Damodar Reddy Chilakapati

[1]Sugen Life Sciences Pvt Ltd, Andhra Pradesh, India-517 505.
[2]Sri Venkateswara University, Department of Biochemistry, Andhra Pradesh, India-517 502.
[3]University of Kansas, Department of Pharmacology & Toxicology, Lawrence KS-66044, USA

Abstract

Combination of natural compounds and cytotoxic drugs represents a logical therapeutic approach for breast cancer. Brucine, a natural plant alkaloid is reported to possess cytotoxic, anti-inflammatory and anti-cancer activities. Gemcitabine is a nucleoside analog, commonly used in the treatment of several solid tumors, including breast cancer. In the present study we have examined the anticancer effect of brucine and gemcitabine on MDA MB-231 human breast cancer cells. Cell proliferation was assessed using MTT assay. Soft agar assay was used to evaluate the *in-vitro* clonogencity of MDA MB-231 cells. Cell migration was determined by *in-vitro* scratch assay and expression of p65 (NF-kB subunit) was evaluated by western blot analysis. Combination treatment with brucine and gemcitabine resulted in a significant inhibition of cell proliferation than either brucine or gemcitabine alone. Cells treated with combination of brucine and gemcitabine showed additive inhibition in colony formation and cell migration than treated with individual agents. The cells treated with brucine at 300 µM showed a significant decrease in p65-NF-kB expression but in combination treatment there was no additive inhibition of p65 expression compared to brucine treated cells. Overall, our results suggested that brucine in combination with gemcitabine showed supra-additive anticancer effects in MDA MB-231 cells and warrants further *in-vivo* studies in experimental animal models.

Keywords Brucine, Gemcitabine, MDA MB-231, p65, in vitro scratch assay

Introduction

Breast cancer is the common cause of cancer death in women [1]. Globally, every year more than one million women are diagnosed with breast cancer and more than 410000 are likely to die from the disease [2]. Present therapies include surgery, radiation and chemotherapeutic agents and there are limitations with these and side effects are common. Currently gemcitabine is used for breast cancer [3]. However gemcitabine treatment results in only marginal survival advantage and is associated with many side effects including development of drug resistance [3]. Thus there is need for the development of new therapeutic agents and testing new compounds isolated from plants with the existing anticancer agents are anticipated to show better outcome.

Many compounds derived from plants, such as taxanes (docetaxel, paclitaxel), vinca alkaloids (vindesine) and anthracyclines (epirubicin) are known anticancer agents [4, 5]. Brucine is a natural plant alkaloid isolated from the seeds of medicinal herb *Strychnos nux-vomica* [6]. It has been used for the treatment of liver cancer in Chinese medicine. Recent studies found that brucine has anti-proliferative effects in different cancer cell lines [7-11] and it induces cell death by arresting the cell cycle at G1 phase in colon cancer cells [12]. Previous reports revealed brucine has analgesic and anti-inflammation [13] properties.

Gemcitabine (2'2'-difluorodeoxycytidine) is an analogue of deoxycytidine which is used as a chemotherapeutic drug for the treatment of various cancers. Although it is inactive in native form, it is progressively phosphorylated to the active triphosphate form [14] which is incorporated into as fraudulent base in competition with dCTP resulting in DNA chain termination. Gemcitabine is used for the treatment of pancreatic cancer [15] and in combination for non-small cell lung cancer [16]. Gemcitabine in combination with natural compounds such as curcumin showed enhanced anticancer activity in pancreatic [17] and bladder cancer [18]. A major reason for failure of breast cancer therapy is due to development of drug resistance. Cell proliferation and chemoresistance in breast cancer cells have been linked to transcription factor nuclear factor-kB (NF-kB) [19]. Brucine chemo-sensitizes human lung cancer cells by inhibition of NF-kB [20]. The combination of brucine and gemcitabine has not been tested. In the present study we investigated the anticancer effect of brucine in combination with gemcitabine in MDA MB-231 breast cancer cells.

Materials and methods

Cell lines and culture conditions

The human breast cancer cell line (MDA MB-231) was acquired from the American Type Culture Collection (ATTC, Manassas, VA), and was maintained in DMEM culture medium supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin at 37 C in incubator supplied with 5% CO_2.

Drugs and reagents

Brucine and gemcitabine were received as a powder from Sigma-Aldrich (St. Louis, MO, USA). Brucine (50 mM) and gemcitabine (10 mM) stocks were prepared in dimethyl sulfoxide (DMSO; Merck, Darmstadt, Germany) and phosphate buffer saline pH 7.2 (PBS), respectively, and stored at -20°C until use. Dulbecco's modified Eagle's medium (DMEM), Fetal Bovine Serum (FBS), trypsin, penicillin and streptomycin were purchased from Gibco-BRL (Grand Island, NY, USA). MTT was purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibodies against p65 (NF-kB) and goat anti-rabbit IgG were obtained from Santa Cruz Biotechnology (SantaCruz, CA, US). All other chemicals of analytical grade were purchased from Sigma, USA.

Determination of anti-proliferative effects by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method

The effect of brucine and/or gemcitabine on the proliferation of MDA MB-231 cells was determined by MTT assay [21]. Briefly, 5000 cells were seeded in a 96-well plate and treated with desired concentrations of brucine (10, 50, 100, 150, 200, 250 and 300 μM) or gemcitabine (0.001, 0.05, 0.1, 10, 25, 50 and 100 μM). Combination treatments were performed in three sets. First set: Cells were treated with brucine and gemcitabine at the same time for 48 h. Second set: Cells were pre-treated with gemcitabine for 12 h and then exposed to brucine for 48 h. Third set: Cells were pre-treated with brucine (50,100 and 300 μM) for 12 h and then exposed to gemcitabine (25, 50 and 100 μM) for 48 h. Cytotoxicity was determined by adding 10 μl of MTT (0.5 mg/ml in PBS) to each well and incubated for 4 h. The medium was removed and 100 μl DMSO was added to each well and after 10 min of mechanical shaking, the optical density was measured at 570 nm in plate reader (Spectra Max, M5, Bio-Rad, USA).

Colony formation assay in soft agar

The standard colony formation assay was performed as described [22]. MDA MB-231 cells (10 10^4) were plated in 60 mm culture plates and incubated overnight at 37ºC. The cells were treated either brucine (50 and 100 μM) or gemcitabine (25 and 50 μM) alone and in combination with both the drugs at the specified concentrations for 48 h. After treatment with drugs, the cells were harvested, 0.4 10^5 cells were counted from each concentration by using trypan blue assay and the cells were suspended in 0.3% top agar (Difco, Noble agar Detroit, MI) in the medium. Base agar (0.5%) was poured in 35 mm culture plates and top agar containing cells was poured on the base agar. The cells were incubated at 37ºC in CO_2 incubator (5% CO_2) for three weeks. The medium was added every three days. After three weeks, colonies were stained with 0.5% crystal violet and washed with PBS to remove excessive dye. Stained colonies were counted individually using inverted phase microscope (Zeiss, Axiovert 25, Germany).

In vitro Scratch Assay

MDA MB-231 cells (5 10^4) were seeded in 60 mm culture plates and the monolayer of 80% confluent cells were subjected to serum starvation for 2 h and treated for 48 h either with brucine (50 and 100 μM) or gemcitabine (25 and 50 μM) and their combinations. After incubation for 48 h, the drug containing medium was removed, scratch was created by sterile p200 tip and washed (x2) with PBS to remove floating cells and 2% FBS media was added. Photographic images were taken at 0, 12, 24 and 48 h using inverted phase microscope (Zeiss, Axiovert 25, Germany). Cell migration was expressed as the percentage of the gap relative to the total area of the cell-free region using Image-J software (National Institutes of Health, Bethesda, MD, USA) [23].

Western blot analysis

To detect the expression levels of p65-NF-kB protein by western blot analysis. MDA MB-231 cells (1 10^6) were seeded in 60 mm culture plates and allowed to adhere overnight at 37ºC. The cells were treated either with brucine (300 μM) or gemcitabine (100 μM) or combinations of both drugs for 48 h. The cells were washed twice with ice-cold PBS buffer (pH 7.4) and incubated with RIPA buffer containing protease inhibitor cocktail for 10 min at 4ºC. The cell lysates were centrifuged at 14000 rpm for 10 min at 4ºC and the protein concentration was determined using the Bio-Rad protein assay. An equal volume of loading buffer was added to the samples and were boiled at 95ºC for 10 min. Protein (20 μg) samples were loaded and separated using 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and proteins were transferred onto nitrocellulose membrane. The membrane was blocked with 5% non-fat dry milk in 1X TBST buffer for 1 h followed by incubation with p65 (NF-kB) primary antibody (1:1000 dilutions in TBST) overnight at 4ºC. After washing with TBST, the membrane was incubated with goat anti-rabbit IgG conjugated secondary antibody (1:5000 dilutions in TBST) for 1h at room temperature followed by washing with TBST. The protein bands were determined by incubation with electro-chemiluminescence (ECL- Bio-Rad) reagent for 1 min. The band intensity was determined by Image-J software (National Institutes of Health, Bethesda, MD, USA) MDA [24].

Statistical analysis

Experimental results are presented as the mean ± standard deviation (SD) obtained from three independent experiments. Differences were evaluated by the one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. The level of significance was set at p 0.05.

Results

Brucine and gemcitabine treatment suppresses proliferation of MDA MB-231 cells

To determine whether combination treatment with brucine and gemcitabine enhances the inhibition of MDA MB-231 cell proliferation MTT assay was performed. Individual treatment with brucine or gemcitabine showed significant inhibition of cell proliferation in a dose dependent manner. Cells treated with brucine and gemcitabine individually at 200 µM and 100 µM suppressed the cell proliferation by 45 and 50% respectively (p 0.001, Fig. 1A, 1B) compared to untreated cells. The IC_{50} values of brucine and gemcitabine in MDA MB-231 cells were 116.4 and 70 µM respectively. Combination treatment with both brucine and gemcitabine at 300 µM and 100 µM showed significant (p 0.001) decrease in cell proliferation (79%) compared to untreated cells indicating an additive effect.

The inhibition of cell proliferation in the combination of gemcitabine and brucine was significantly higher (Figure 2A) than pre-treatment with either gemcitabine or brucine (Figures 2B and C). Although, inhibition of cell proliferation in cells pre-treated with gemcitabine for 12 h followed by brucine treatment were statistically significant (p 0.001) compared with cells pre-treated with brucine and followed by gemcitabine (Figure 2B and 2C).

Figure 1: Brucine and gemcitabine suppresses the proliferation of MDA MB-231 cells

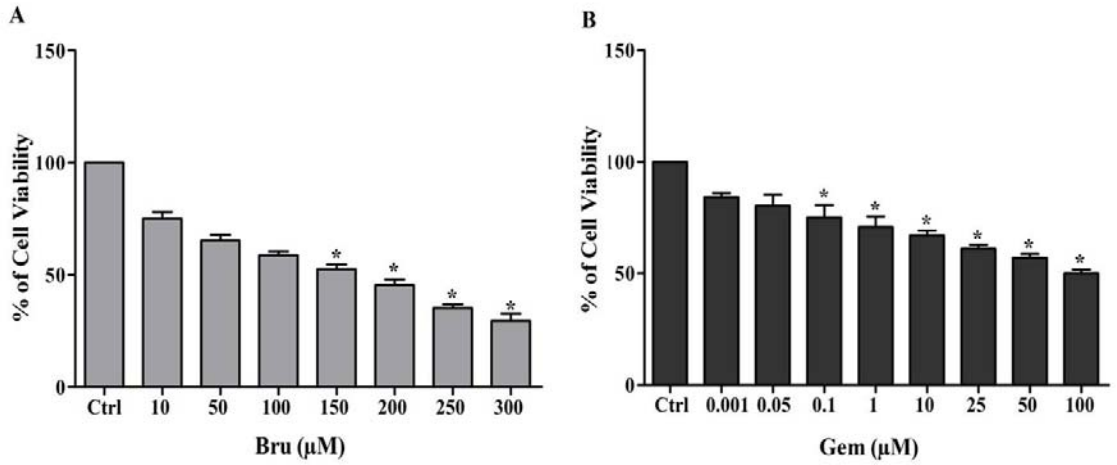
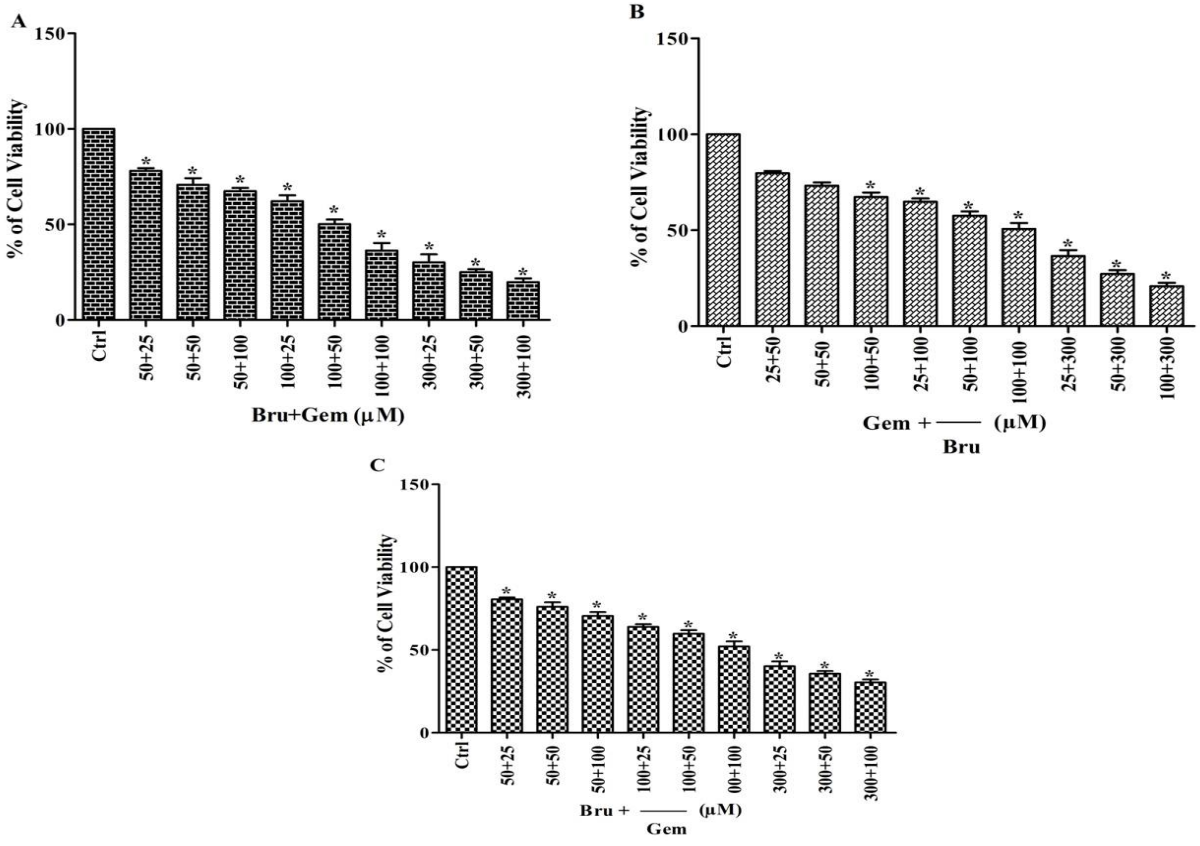


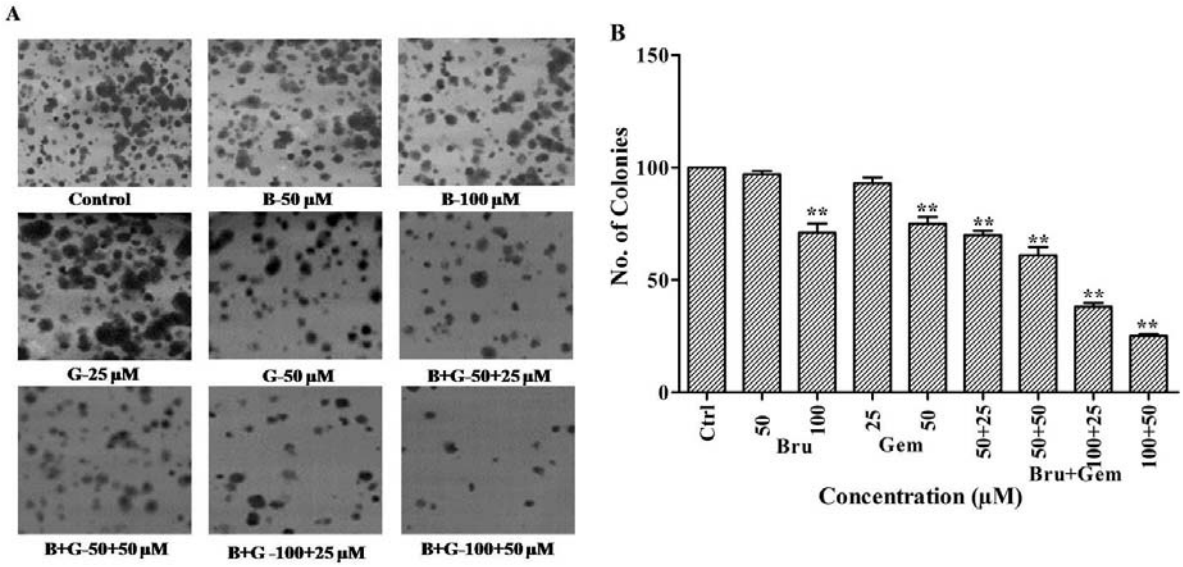
Figure 2: Treat at same time and pre-treatment with brucine and gemcitabine suppresses the proliferation of MDA MB-231 cells



Effect of brucine and/or gemcitabine on anchorage-independent growth of MDA MB-231 cells

Soft agar assay was performed to determine the colony formation ability of MDA MB-231 cells treated with brucine and/or gemcitabine. The number of colonies in untreated cells and cells treated with both the agents, alone or in combination are counted and summarized in Figure 3. Individual treatment with brucine (100 µM) and gemcitabine (50 µM) showed significant inhibition in colony formation, 29% and 25% respectively compared to untreated cells. Combination treatment with 100 µM brucine and 50 µM gemcitabine resulted in additive inhibition (75%) of colony formation compared to treatment with either brucine or gemcitabine alone. These results were consistent with the results observed in MTT assay.

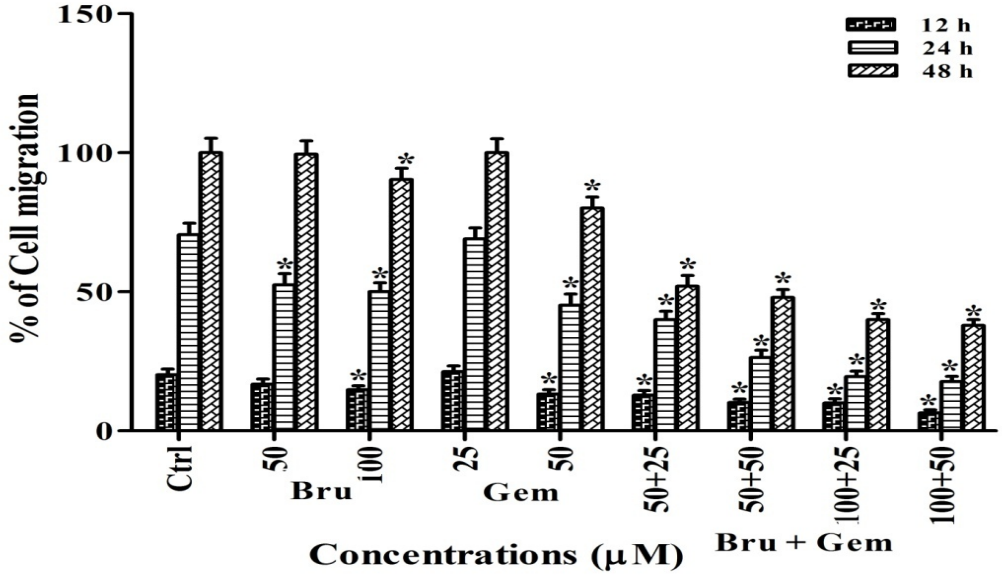
Figure 3: Effect of brucine and gemcitabine on colony formation of MDA MB-231 cells in soft agar



Brucine and gemcitabine inhibit the cell migration

The effect of brucine and/or gemcitabine on MDA MB-231 cell migration was measured by in vitro scratch assay. Cell migration was reduced by 10% and 20% with individual treatment with brucine (100 µM) and gemcitabine (50 µM), respectively (Figure 4). However, the cell migration was significantly inhibited (60%) when the cells were treated in combination of both the agents (100 µM brucine and 50 µM gemcitabine). These results suggest that in combinational therapy shows additive effect in inhibiting the migration of MDA MB-231 cells.

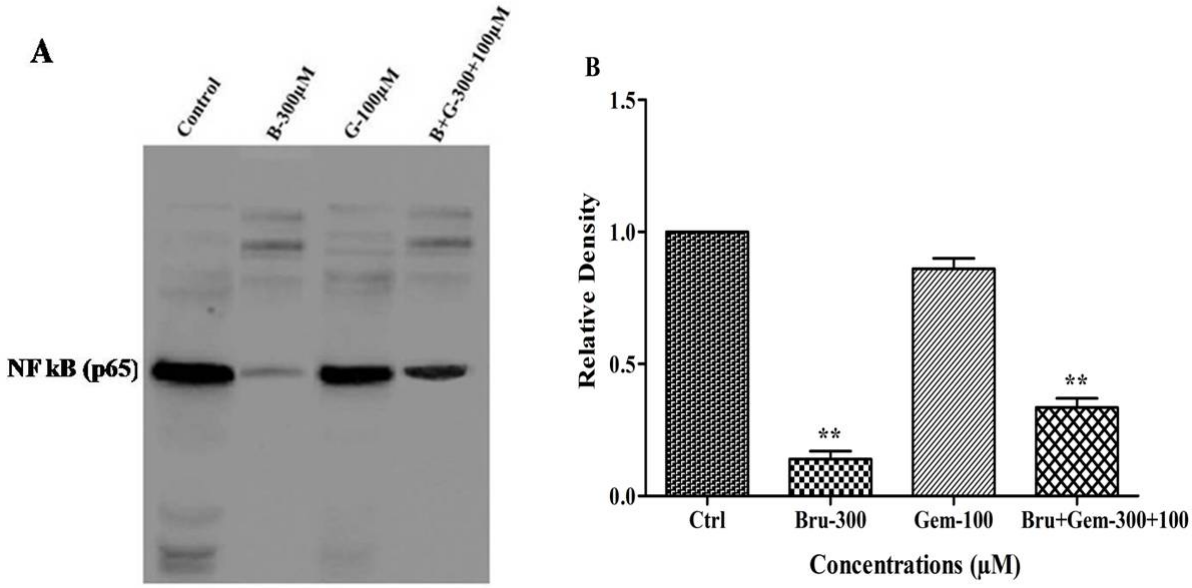
Figure 4: Effect of brucine and gemcitabine on cell migration



Effect of brucine and/or gemcitabine on the expression of NF-kB

We next investigated whether the inhibitory effect of brucine and gemcitabine on breast cancer cell is mediated through the alteration of NF-kB expression. Protein extracts of un-treated and brucine and gemcitabine treated MDA MB-231 cells were analyzed by Western blot assay to assess p65 expression. Cells treated with brucine at 300 µM showed significantly (76%) reduced level of p65 protein expression compared to untreated cells (Figure 5). Gemcitabine (100 µM) treatment did not show any effect on p65 protein expression compared to untreated cells. Combination treatment with 300 µM brucine and 100 µM gemcitabine inhibited the p65 expression by 62% compared to untreated cells. Therefore combination appears to have less inhibitory effect on p65 expression when compared to brucine treatment alone.

Figure 5: Effect of brucine and gemcitabine on p65 NF-kB expression.



Discussion

Plants have been an important source of potent drugs for the treatment of many types of diseases including cancer and provide leads for the development of novel agents. Combinations of phytochemicals with known anticancer drugs have been shown to be highly effective [25]. The efficacy of treatment would increase and the chance of adverse effects may be lowered due to the use of lower concentrations when combined together. The results in the present study indicated that brucine in combination with gemcitabine significantly decreased the MDA MB-231 breast cancer cell proliferation. Brucine has been used as anticancer agent in various types of cancers including hepatoma and lung cancer [26]. Previous studies indicated that brucine inhibits HepG2 cells by inducing apoptosis through increased caspase-3 [9]. Results of the present study revealed that combination exposure of both drugs at same time point exhibited highest inhibition in cell proliferation than pre-treatment with either brucine or gemcitabine alone. Also pre-treatment with brucine did not show higher inhibition whereas pre-treatment with gemcitabine was more effective. Breast cancer prognosis can be greatly influenced by metastasis. Previous study reported that brucine inhibit the liver cancer cell migration by suppressing the hypoxia inducible factor, a key transcription factor which is responsible for cell migration and invasion [23]. In the present study combination treatment with both brucine and gemcitabine exhibited synergistic attenuation in cell migration than either agent alone. Anchorage independent growth in soft agar assay also showed additive effect. Various reports had suggested that chemotherapeutic drugs which targeted the NF-kB activity would be helpful in cancer therapy [14]. NF-kB plays a key role both in inflammation, cancer cell progression, proliferation, invasion, angiogenesis and metastasis [5]. Earlier it was shown that brucine suppresses NF-kB activity by inhibiting phosphorylation of Ik-B as well as the nuclear translocation of p65 [20]. Our results have shown that, cells treated with brucine significantly inhibited expression of p65 protein, whereas, gemcitabine did not show any effect on p65 expression.

Conclusion

Overall, the experimental evidence showed that combination treatment of brucine and gemcitabine exhibits additive effect in inhibiting MDA MB-231 cells. Pre-treatment with brucine and gemcitabine or vice versa and alone did not show greater advantage when compared to the combination treatment. Further,

experimental evidence from this study revealed that alteration of cell proliferation, cell survival, migration, and nuclear factor kB subunit (p65) protein expression levels were observed after the treatment. We consider combined treatment of brucine-gemcitabine could be a potential anti-proliferative agent for breast cancer cells.

Acknowledgments

Authors are thankful to Sugen Life Sciences Pvt Ltd for financial support and thank Dr. Suresh Kumar Royala, Rohan Prasad Surabi, Jayadev Mavuluri, Shilpa Tentu, Praveen and Anuj Kaushik, Department of Biotechnology, IIT, Madras for their help with cell culture work.

References

[1]. Parkin DM, Pisani P, Ferlay J. Global cancer statistics. CA Cancer J Clin. 1999; 49(1): 33- 64.

[2]. Coughlin S S, Ekwueme D U. Breast cancer as a global health concern. Cancer Epidemiology 2009;33(5): 315-318.

[3]. Heinemann V. Gemcitabine in metastatic breast cancer. Exp. Rev. Anticancer Ther 2005;5(3):429-443.

[4]. Cragg GM, Newman DJ. Plants as a source of anticancer agents. J Ethopharmacol 2005;100(1-2):72-79.

[5]. Berkovich L, Ron I, Earon G, Abu-Ghanem S, Rimmon A, Lev-Ari S. The role of medicinal herbs with anti-inflammatory properties in prevention and treatment of cancer. Harefuah 2012;151(11): 629-632.

[6]. Behpour M, Ghoreishi SM, Khayatkashani M, Motaghedifard M. A new method for the simultaneous analysis of strychnine and brucine in *Strychnos nux-vomica* unprocessed and processed seeds using a carbon-paste electrodemodified with multi-walled carbon nanotubes. Phytochem Anal 2012;23(2):95-102.

[7]. Agrawal SS, Saraswati S, Mathur R, Pandey M. Cytotoxic and antitumor effects of brucine on ehrlich ascites tumor and human cell line. Life Sci 2011;89(5-6):147-58.

[8]. Cai BC, Wang TS, Kurokawa M, Shiraki K, Hattori M. Cytotoxicities of alkaloids from processed and unprocessed seeds of *Strychnos nux-vomica*, Acta Pharm Sinic 1998;19(5):425-28.

[9]. Deng XK, Yin W, Li WD, Yin FZ, Lu XY, Zhang XC, Hua ZC, Cai BC.The anti-tumor effects of alkaloids from the seeds of *Strychnos nux vomica* on HepG2 cells and its possible mechanism, J Ethnopharmacol 2006;106(2):179-86.

[10]. Yin W, Deng XK, Yin FZ, Zhang XC, Cai BC. The cytotoxicity induced by brucine from the seed of *Strychnos nux-vomica* proceeds via apoptosis and is mediated by cyclooxygenase-2 and Caspase-3 in SMMC 7721 cells. Food Chem Toxicol 2007; 45(9):1700-08.

[11]. Rao PS, Ramanadham M, Prasad MN. Anti-proliferative and cytotoxic effects of *Strychnos nux-vomica* root extract on human multiple myeloma cell line-RPMI 8226. Food Chem Toxicol 2009;47(2):283-88.

[12]. Zheng L, Wang X, Luo W, Zhan Y, Zhang Y. Brucine, an effective natural compound derived from nux-vomica, induces G1 phase arrest and apoptosis in LoVo cells. Food and Chem Toxicolo 2013;58:332-39.

[13]. Chen J, Wang X, Qu YG, Chen ZP, Cai H, Liu X, Xu F, Lu TL, Cai BC. Analgesic and anti-inflammatory activity and pharmacokinetics of alkaloids from seeds of *Strychnos nux-vomica* after trans dermal administration: effect of changes in alkaloid composition. Journal of Ethno pharmacolo 2012;139(1):181-88.

[14]. Baud V, Karin M. Is NF-kappaB a good target for cancer therapy? Hopes and pitfalls. Nat Rev Drug Discov 2009;8(1):33-40.

[15]. Ramachandran C, Resek A, Esclon E, Aviram A, Melmick S. Potention of gemcitabine by tumeric force in pancreatic cancer cell lines. Oncology Reports 2010; 23(6):1529-1535.

[16]. Ramalingam S, Belani C. Systemic chemotherapy for advanced non-small cell lung cancer: recent advances and future directions. Oncologist 2008;13 (suppl 1):5-13.

[17]. Kunnumakkara AB, Guha S, Krishnan S, Diagaradjane P, Gelovani J, Aggarwal BB. Curcumin potentiates antitumor activity of gemcitabine in an orthotopic model of pancreatic cancer through suppression of proliferation, angiogenesis, and inhibition of nuclear factor- kB-regulated gene products. Cancer Res 2007; 67:3853-61.

[18]. Tharakan ST, Inamoto T, Sung B, Aggarwal BB, Kamat AM. Curcumin potentiates the antitumor effects of gemcitabine in an orthotopic model of human bladder cancer through suppression of proliferative and angiogenic biomarkers. Bioc Pharmacol 2010; 79(2): 218-28.

[19]. Biswas DK, Shi Q, Baily S, Strickland I, Ghosh S, Pardee AB, Iglehart JD. NF-kappa B activation in human breast cancer specimens and its role in cell proliferation and apoptosis. Proc Natl Acad Sci U S A 2004; 101(27):10137-42.

[20]. Zhu G, Yin F, Deng X. Effect of NF-kappaB on inhibition of non-small cell lung cancer cell cyclooxygenase-2 by brucine. Zhongguo Zhong Yao Za Zhi 2012; 37(9):1269-73.

[21]. Zheng L, He X, Ma W, Dai B, Zhan Y, Zhang Y. Ta1722, an antiangiogenesis inhibitor targeted on VEGFR-2 against human hepatoma.

Biomed Pharmacother 2011; 66(7):499-05.

[22]. Keane MM, Ettenberg SA, Nau MM, Russell EK, Lipkowitz S. Chemotherapy augments TRAIL-induced apoptosis in breast cell lines. Cancer Res 1999;59:734-41.

[23]. Shu G, Mi X, Cai J, Zhang X, Yin W, Yang X, Li Y, Chen L, Deng X. Brucine, an alkaloid from seeds of *Strychnos nux-vomica* Linn, represses hepatocellular carcinoma cell migration and metastasis: The role of hypoxia inducible factor 1 pathway. Toxicol Lett 2013; 222(2): 91-101.

[24]. Lai HW, Chien SY, Kuo SJ, Tseng LM, Lin HY, Chi CW, Chen DR. The Potential utility of curcumin in the treatment of HER-2-Overexpressed breast cancer: An *in vitro* and *in vivo* comparison study with Herceptin. Evid Based Complement Alternat Med 2012;10:486568.

[25]. Cheah YH, Nordin FJ, Sarip R, Tee TT, Azimahtol HL, Sirat HM, Rashid BA, Abdullah NR, Ismail Z. Combined xanthorrhizol-curcumin exhibits synergistic growth inhibitory activity via apoptosis induction in human breast cancer cells MDA-MB-231. Cancer Cell Int 2009; 2:9-1.

[26]. Deng X, Yin F, Lu X, Cai B, Yin W. The apoptotic effect of brucine from the seed of *Strychnos nux-vomica* on human hepatoma cells is mediated via Bcl-2 and Ca2+ involved mitochondrial pathway. Tox Sci 2006; 91(1):59-69.