

## Research Article

### **Gemcitabine and brucine inhibit MDA MB-436 human breast cancer cells**

Mamatha. Serasanambati<sup>1,2</sup>, Shanmuga Reddy. Chilakapati<sup>1,2</sup>, Jagadeeswara Reddy. Kanala<sup>1</sup>, Damodar Reddy. Chilakapati<sup>1\*</sup>

<sup>1</sup>Sugen Life Sciences Pvt Ltd, Andhra Pradesh, India-517 505.

<sup>2</sup>Sri Venkateswara University, Department of Biochemistry, Andhra Pradesh, India-517 502.

#### **\*Corresponding author**

Damodar Reddy. Chilakapati, PhD

Email: [cdr@sugenlife.com](mailto:cdr@sugenlife.com)

---

**Abstract:** Combinations of anticancer agents are likely to be more effective in breast cancer therapy. Hence, in the present study we evaluated the *in vitro* anticancer activity of brucine when combined with gemcitabine in MDA MB-436 human breast cancer cells. The cells were cultured *in vitro* and treated with brucine and gemcitabine combinations. Cell viability was assessed by trypan blue exclusion assay and cell migration was determined by *in vitro* scratch assay. Brucine and gemcitabine treatment showed inhibition of cell proliferation in a dose dependent manner. Combination treatment resulted in additive inhibitory effects. MDA MB-436 cells treated with both drugs showed additive inhibition of cell migration in the *in vitro* cell migration assay.

**Keywords:** MDA MB-436, Trypan blue assay, *In vitro* scratch assay

---

## **INTRODUCTION**

Breast cancer is the most common cancer in women [1] and the second most cause of death [2]. Surgery, radiation and chemotherapeutic approaches are often not enough to improve the survival rate in breast cancer [3]. Thus, alternative interventions such as substances from natural herbal sources can be tested to combine with the existing anticancer agents. Toxicity and drug resistance are the major drawbacks of anticancer chemotherapeutic therapies. The goal of developing newer agents is to overcome such problems and side effects could be less [4]. In this regard, testing the newer agents and their combinations is anticipated to benefit breast cancer drug discovery approaches.

In the current study we investigated whether adding brucine to the gemcitabine will have any additive effects in inhibiting MDA MB-436 cells. Brucine has been isolated from *Strychnos nux-vomica*, a medicinal plant present in many Asian countries. It has been widely used in Chinese folk medicine [5] for the treatment of nervous diseases, analgesic, anti-inflammatory [6] and anti-tumour [7]. Brucine is a plant alkaloid and isolated from the seeds of *Strychnos nux-vomica*. It has anti-proliferative and cytotoxic effects have been reported in HepG2 [7, 8] SMMC-7721 [9] hepatoma cells, and multiple myeloma RPMI 8226 cell lines [10].

Gemcitabine (2',2'-difluorodeoxycytidine, dFdC) is a dCyd analog, which is active against variety

of cancers including non-small cell lung cancer [11], pancreatic carcinoma [12, 13] and breast cancer [14]. Gemcitabine requires phosphorylation by deoxycytidine kinase (dCK) in order to be active, and can be inactivated by deamination by deoxycytidine deaminase (dCDA) to 2',2'-difluorodeoxyuridine (dFdU) [15, 16]. After conversion by kinases to its active form, 2',2'-difluorodeoxycytidine triphosphate (dFdCTP), it can be incorporated into DNA and is a potent inhibitor of DNA-synthesis by inhibition of DNA polymerase [17]. Moreover, gemcitabine, as dFdCTP, is also incorporated into RNA [18].

Thus, we set out to determine if the combination of brucine and gemcitabine is more potent than either agent alone. Our findings suggest that the combination of brucine and gemcitabine are effective in inhibiting the proliferation and migration of MDA-MB 436 cells.

## **MATERIALS AND METHODS**

### **Cell culture and Reagents**

The human breast cancer cell lines (MDA MB-436) was purchased from American Type Culture Collection (Manassas, VA) and cultured in DMEM (Gibco-BRL, Grand Island, NY) pH 7.4 containing penicillin (100 U/ml), streptomycin (100 µg/ml), and gentamicin (60 µg/ml) and supplemented with 10% Fetal Bovine Serum (FBS- Gibco-BRL, Grand Island, NY) and humidified 5% CO<sub>2</sub> incubator at 37°C. 50 mM brucine and 10 mM gemcitabine (purchased from

Sigma, St. Louis, MO, USA) stocks were prepared in dimethyl sulfoxide (DMSO; Merck, Darmstadt, Germany) and phosphate buffer saline pH 7.2 (PBS) respectively and stored at  $-20^{\circ}\text{C}$  until use. All other chemicals of analytical grade were purchased from Sigma, USA.

### Trypan blue dye exclusion method

To analyse the cytotoxic activity of brucine and/or gemcitabine by trypan blue dye exclusion method adapted to [19]. MDA MB-436 ( $2 \times 10^5$ ) cells were seeded in 6 well plates and allowed to adhere for overnight at  $37^{\circ}\text{C}$ . Then the cells were exposed to different concentrations of the brucine (100, 200 and 300  $\mu\text{M}$ ) or gemcitabine (10, 50, 100 and 150  $\mu\text{M}$ ) or in combination of both drugs. The control well received only maintenance of medium. The plates were incubated at  $37^{\circ}\text{C}$  in a humidified incubator with 5%  $\text{CO}_2$  for 48 h. After drug treatment cells were washed with phosphate buffer saline (PBS), trypsinized and cells were centrifuged at 1200 rpm for 4 min and re-suspended in complete media. Each sample was mixed with an equal volume of 0.4% trypan blue and counted using a hemocytometer (Hausser Scientific, Horsham, PA, USA) under an inverted microscope (Olympus, Germany). At the end of 48 h incubation, cell viability was determined

### In vitro scratch assay

MDA MB-436 ( $5 \times 10^4$ ) cells were seeded in 60 mm culture plates for monolayer formation up to 80% confluence and the cells were subjected to serum starvation for 6 h and treated for 48 h either with brucine (50 and 100  $\mu\text{M}$ ) or gemcitabine (25 and 50  $\mu\text{M}$ ) and their combination. After incubation the drug containing medium was removed, scratch was created by sterile p200 tip after washed with PBS (X2) to remove floating cells and 2% media was added. Photographic images were taken at 0, 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) [20].

## RESULTS

### Viability staining by trypan blue assay

To determine the cell viability of MDA MB-436 cells treated with brucine and/or gemcitabine by trypan blue dye exclusion assay (Figure 1). Individually brucine and gemcitabine exhibited a suppressive effect on cell viability in a dose dependent manner.

Cell viability was significantly decreased to 58.4% at 100  $\mu\text{M}$  brucine ( $p \leq 0.001$ , Figure 1A). Similarly, cell viability was significantly decreased to 60% at 50  $\mu\text{M}$  gemcitabine ( $p \leq 0.001$ , Figure 1B). The  $\text{IC}_{50}$  values were 100 and 30  $\mu\text{M}$  for brucine and gemcitabine respectively.

In contrast cells treated with both the drugs at same time showed highest inhibition (Figure 2) of cell proliferation compared to either brucine or gemcitabine (Figures 1A and B). Combination treatment with both brucine (100  $\mu\text{M}$ ) and gemcitabine (50  $\mu\text{M}$ ) showed significant ( $p \leq 0.001$ ) decrease in cell proliferation (84%) compared to untreated cells.

### Brucine and gemcitabine inhibits the MDA MB-436 cell migration

To investigate the inhibitory effect of brucine and/or gemcitabine on MDA MB-436 cell migration, *in vitro* scratch assay was performed. The % of cells that had migrated into the scratch after 0, 24 and 48 h was calculated and shown in figure 3. There was complete gap closure in 24 to 48 hours in the untreated cells. There was no significant change in cell migration which was treated with brucine (50  $\mu\text{M}$ ) and gemcitabine (25  $\mu\text{M}$ ) compared with control cells. Cell migration was reduced by 25% in cells treated with 100  $\mu\text{M}$  brucine and 36% in cells treated with 50  $\mu\text{M}$  gemcitabine. However, the cell migration was significantly inhibited (83%) when the cells were treated with both brucine (100  $\mu\text{M}$ ) and gemcitabine (50  $\mu\text{M}$ ). These results suggest that in combination treatment shown more additive effect in inhibiting the migration of MDA MB-436 cells compared to individual drugs treated alone.

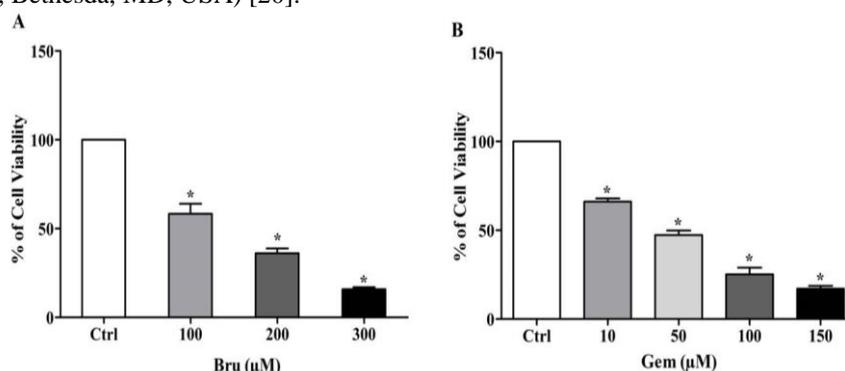


Figure 1: Brucine and gemcitabine suppresses the cell viability of MDA MB-436 cells. A. Cells (20,000 cells/well) were incubated with the indicated concentrations of brucine at  $37^{\circ}\text{C}$  for 48 h. B. Cells (20,000 cells/well) were incubated with the indicated concentrations of gemcitabine at  $37^{\circ}\text{C}$  for 48 h. Cell viability was assayed using 0.4% trypan blue dye exclusion method. Data represented as Mean  $\pm$  SD. \* $p \leq 0.001$ , treated cells were compared with untreated cells. (Bru-Brucine; Gem-Gemcitabine).

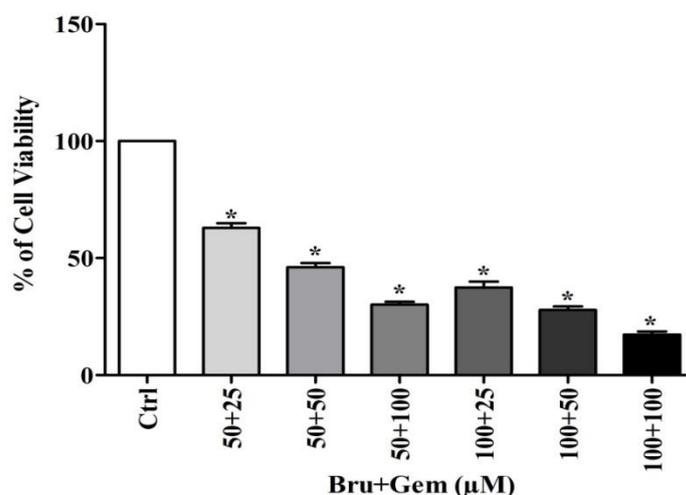


Figure 2: Effect of brucine or gemcitabine treatment on cell viability of MDA MB-436 cells. Cells were treated with brucine (50 or 100 µM) in combination with gemcitabine (25 or 50 or 100 µM). After 48 h treatment, to evaluate the cell viability using trypan blue assay. Data represented as Mean ± SD.\* $p \leq 0.001$ , treated cells were compared with untreated cells.

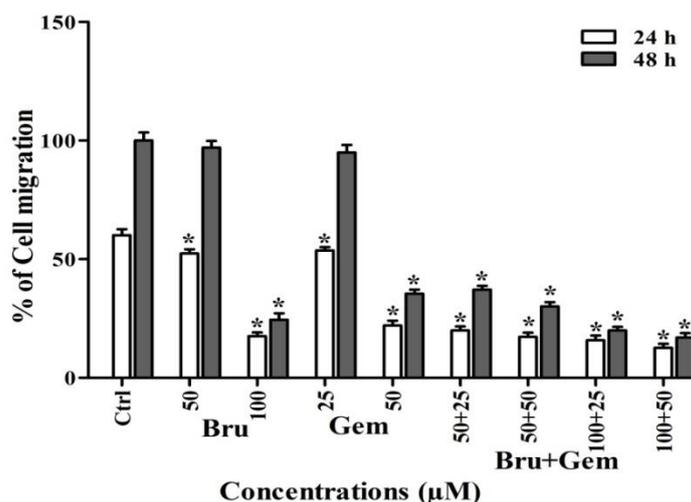


Figure 3: Effect of brucine and gemcitabine on cell migration. Cells were plated to obtain 80% confluence in 60 mm culture plate. A scratch was created and treated the cells for 48 h with either brucine or gemcitabine or both. After drug treatment the migration of cells to the gap region created was monitored and photographed at 0, 24 and 48 h and the cell migration was analyzed and represented as Mean ± SD obtained from three independent experiments.\* $p \leq 0.001$ , when treated samples are compared with respective untreated cells (Bru-Brucine; Gem-Gemcitabine).

## DISCUSSION

Current cancer treatment strategies favour combination therapies which would offer low toxicities to the cancer patients [4]. Natural products have proven to be the most reliable source of new and effective anticancer agents [21, 22]. The most important rationale is the realization that individual chemotherapeutic agents for the majority of tumours have not increased cure rates in the treatment of cancer [14]. Hence, the present study focussed on combinational therapy of brucine and gemcitabine in breast cancer cells. Gemcitabine an analogue of deoxycytidine is active against several types of solid tumors, including breast cancer [14] and pancreatic carcinoma [12]. In addition, brucine has been used as an anticancer agent in various

types of cancers including lung cancer [23] and hepatoma cancer [24]. Previous reports have investigated the mechanisms of brucine in HepG2 cells [7]. In the present study, brucine as well as gemcitabine exhibited a suppressive effect on MDA MB-436 cell viability in a dose dependent manner (Figure 1). Such an inhibitory activity of cell viability was enhanced significantly when the MDA MB-436 cells were exposed to combination with brucine and gemcitabine (Figure 2). Recent studies showed that combination of gemcitabine and curcumin is effective in pancreatic [25] and bladder cancers [26]. Results of the present study revealed that combination exposure of both drugs at same time point exhibited highest inhibition in cell viability than either brucine or gemcitabine alone.

These observations may be of value while carefully considering the combination therapies in a clinical setting. In addition, cell migration was significantly inhibited (47%) with 100  $\mu$ M brucine and 50  $\mu$ M gemcitabine combination (Figure 3). The combined results may conclude that the combinational therapy with brucine and gemcitabine would be more effective and beneficial in breast cancer therapy. Our results suggest that concurrent treatment of brucine and gemcitabine show additive effect in human breast cancer (MDA MB-436) cells and these drugs may have future clinical utility for treating breast cancer.

#### Acknowledgments

The authors are grateful to the Sugan Life Sciences Pvt. Ltd. for financial support and providing laboratory facilities to perform the research work.

#### REFERENCES

1. DeSantis C, Siegel R, Bandi P, Jemal A; Breast cancer statistics, 2011. *Cancer Journal for Clinicians* 2011; 61:409-18.
2. Merrill RP, Weed DL; Measuring the public health burden of cancer in the United States through lifetime and age-condition rich estimates. *Annals of Epidemiology*, 2001;11(8):547-53..
3. Cooper GM; *Elements of Human Cancer*. Jones and Bartlett Publishers, Boston 1992.
4. Cheah YH, Nordin FJ, Sarip R, Tee TT, Azimahtol HL, Sirat HM, et al; Combined xanthorrhizol-curcumin exhibits synergistic growth inhibitory activity via apoptosis induction in human breast cancer cells MDA-MB-231. *Cancer Cell International*, 2009;2:9-1.
5. Bensky D, Gamble A; *Chinese Herbal Medicine*. Eastland Press, Seattle, 1986; p. 646.
6. Yin W, Wang TS, Yin FZ, Cai BC; Analgesic and anti-inflammatory properties of brucine and brucine-N-oxide extracted from seeds of *Strychnos nux vomica*. *Journal of Ethnopharmacology*, 2003; 88(2-3):205-14.
7. Deng XK, Yin W, Li WD, Yin FZ, Lu XY, Zhang XC et al; The anti-tumor effects of alkaloids from the seeds of *Strychnos nux vomica* on HepG2 cells and its possible mechanism. *Journal of Ethnopharmacology*, 2006; 106(2):179-86
8. Agrawal SS, Saraswati S, Mathur R, Pandey M; Cytotoxic and antitumor effects of brucine on ehrlich ascites tumor and human cell line. *Life sciences*, 2011; 89(5-6):147-58.
9. 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 and Chemical Toxicology*, 2007;45(9):1700-8.
10. 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 and Chemical Toxicology*, 2009; 47(2):283-8.
11. 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.
12. Wolff RA; Chemotherapy for pancreatic cancer: from metastatic disease to adjuvant therapy. *Cancer Journal*, 2007; 13(3):175-84.
13. Abbruzzese JL; New applications of gemcitabine and future directions in the management of pancreatic cancer. *Cancer*, 2002;95(4 Suppl):941-5.
14. Heinemann V; Gemcitabine in metastatic breast cancer. *Expert Review of Anticancer Therapy*, 2005; 5(3):429-43.
15. Heinemann V, Hertel LW, Grindey GB, Plunkett W; Comparison of the cellular pharmacokinetics and toxicity of 2',2'-difluorodeoxycytidine and 1-beta-D-arabinofuranosyl cytosine. *Cancer Research*,1988; 48(14):4024-31.
16. Carmichael J, Possinger K, Phillip P, Beykirch M, Kerr H, Walling J et al; Advanced breast cancer: a phase II trial with gemcitabine. *Journal of Clinical Oncology*, 1995; 13(11):2731-6.
17. Ross DD, Cuddy DP; Molecular effects of 2',2'-difluorodeoxycytidine (Gemcitabine) on DNA replication in intact HL-60 cells. *Biochemical Pharmacology*, 1994; 48(8):1619-30.
18. Ruiz van Haperen VW, Veerman G, Vermorcken JB, Peters GJ; 2,2'- Difluorodeoxycytidine (gemcitabine) incorporation into RNA and DNA of tumour cell lines. *Biochemical Pharmacology*, 1993; 46(4):762-6.
19. Vinodhini Jeyaraman and Sudha Sellappa. 2011. *In-vitro* anticancer activity of organic germanium on human breast cancer cell line (MCF-7). *Journal of Current Pharmaceutical Research* 5:39-41.
20. Cao XC, Zhang WR, Cao WF, Liu BW, Zhang F, Zhao HM, et al; Aquaporin3 is required for FGF-2-induced migration of human breast cancers. *PLoS ONE*, 2013;8(2):e56735.
21. Newman DJ, Cragg GM; Natural products as sources of new drugs over the last 25 years. *Journal of Natural Products*, 2007; 70(3):461-77.

22. Kingston, D.G. 2009. Tubulin-interactive natural products as anticancer agents. *Journal of Natural Products* 72(3):507-15.
23. Xu XY, Cai BC, Pan Y, Wang TS; Pharmacokinetics of the alkaloids from the processed seeds of *Strychnos nux-vomica* in rats. *Yao Xue Xue Bao*, 2003; 38(6):458-61.
24. 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 Ca<sup>2+</sup> involved mitochondrial pathway. *Toxicological Sciences*, 2006; 91(1):59-69.
25. 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- $\kappa$ B-regulated gene products. *Cancer Research*, 2007; 67(8):3853-3861.
26. 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. *Biochemical Pharmacology*, 2010; 79(2):218-228.