Original Research Paper

Selective Susceptibility of Human Bladder Transitional Cell Carcinoma T24 Cells towards NBD Peptide

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Abstract: Bladder cancer is a recurrent disease that often develops chemoresistance and is on the rise in developing countries. After diagnosis surgery followed by immunotherapy and/or chemo- radiation therapy are the available treatments. These treatments do not provide protection against disease progression or recurrence thus highlighting the need to identify and develop drugs that are capable of inhibiting bladder cancer. T24 cell line (human bladder transitional cell carcinoma) was treated with NEMO Binding Domain (NBD) peptide and tested for its cytotoxicity, cell cycle inhibition, apoptosis and migration, *in vitro*. The cytotoxicity of NBD peptide was tested on non-cancerous cells (NIH-3T3-L1, CHO, Vero) and the peptide localization in T24 cells was confirmed using confocal microscopy. The effects of NBD peptide and the standard drugs (cisplatin, gemcitabine) were compared with untreated control. Peptide based chemotherapy for human bladder cells was tested that include combinations of NBD and standard drugs. Statistical significance was assessed between the different treatment groups. The viability of T24 cells after NBD peptide treatment was reduced to 50% at a dose of 18.5 µM after 48 h. The NBD peptide elicited selective cytotoxicity dose-dependently in T24 cells while being non-toxic on normal (non-cancer) cell lines. Sub G0-G1 accumulation of T24 cells was seen where they underwent necrosis and lost the ability to migrate when treated with 100 µM NBD. T24 cell death increased to ~78% when treated with a combination of NBD with cisplatin and gemcitabine (standard drugs) as opposed to ~50% when treated with NBD peptide alone. NBD peptide exhibits specific anti-cancer activity on T24 cells *in vitro* and found to be non-cytotoxic to select normal cell lines. Combining NBD with standard drugs demonstrated excellent inhibition of malignant growth than the individual anti-neoplastic agents.

Keywords: Urinary Bladder Neoplasms, Nemo Binding Domain (NBD), Anti-Tumor Drug Screening, Cisplatin, Gemcitabine

Introduction

Bladder Cancer (BC) is the second most common malignancy of the genitourinary system in industrialized countries (Antoni *et al*., 2017). It is a heterogeneous disease which makes it difficult for accurate molecular analysis of the tumor, identification of the correct biomarkers for assessment and choice of treatment

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modality (Jakobsson *et al.*, 2018). BC incidence is based on genetic predisposition, lifestyle or environmental factors (Burger *et al.*, 2012). BC is predominant in older male patients ≥ 65 years of age at the time of diagnosis. Also, recurrence and chemoresistance are prevalent in BC (Lu *et al.*, 2017; Wang *et al.*, 2018; Woolbright *et al.*, 2018). There are two major types of BC including the superficial disease and the invasive disease. Bacillus Calmette-Guerin (BCG) immunotherapy is the treatment offered after Transurethral Resection of Bladder Tumor (TURBT) for superficial BCs. Many investigations report BCG as effective in preventing BC recurrence (Pettenati and Ingersoll, 2018; Guallar-Garrido and Julián, 2020). However, it is still controversial when considering its ability to prevent BC progression (Kaufman *et al.*, 2009; Buchwald and Efstathiou, 2015). Treatments offered for invasive BC are surgery with neoadjuvant-, adjuvant-, chemo- and radiation therapy which lacks in-depth analysis of the underlying mechanism due to the lack in the number of randomised Control Trials (RCT) performed for BC (Soloway, 2013). Thus it becomes important to identify the inherent heterogeneity and personalize treatment in BC by understanding the key players in its pathogenesis.

Nuclear Factor kappa B (NF-κB) signaling cascade plays an important role in cell survival (Zhang *et al.*, 2017). A number of molecules can block NF-κB pathway and thus the critical node either directly or indirectly through its signaling pathway members to suppress cancer. Drugs that utilize this strategy are effective in reducing tumor but have poor clinical efficacy (Ianaro *et al.*, 2009; Karin *et al.*, 2002). Proteosome inhibitors are the only exception from this trait which is used for the treatment of some haematological malignancies that demonstrate the key role of NF-κB during their pathogenesis (Richardson *et al.*, 2004). There are many reports stating the involvement of NF-κB in BC (Durairajan *et al.*, 2018; Levidou *et al.*, 2008; Mukherjee *et al.*, 2015; Yeh *et al.*, 2010). However, there are limited reports that have considered the NF-κB signaling cascade and BC in a therapeutic setting. This study is the first of its kind to the best of our knowledge to consider NF-κB associated peptide as a treatment option for BC. NF-κB Essential Modulator (NEMO) is a regulatory protein that belongs to the NF-κB pathway. It allows catalytic units (IKKα, IKKβ) to bind in a region called NEMO Binding Domain (NBD) (May *et al.*, 2000). This domain is termed NBD and is of significance since the core peptides are capable of eliciting NF-κB inhibitory effects. NBD has shown anti-inflammatory activity (Baima *et al.*, 2010) in murine models against colitis (Hong *et al.*, 2012), rheumatoid arthritis/osteoclastogenesis (Jimi *et al.*, 2004),

kernicterus (Li *et al.*, 2015) and pancreatitis in rats (Long *et al.*, 2009) and also against canine refractory diffuse large B- cell lymphoma (Gaurnier-Hausser *et al.*, 2011). The inherent heterogeneity of BC and the success rate behind BCG treatment establish the inflammatory milieu prevalence. Thus this preliminary *in vitro* study analyzes the potential held by NBD peptide as an antineoplastic agent in BC.

Materials and Methods

Peptides, Reagents and Materials

NBD peptide (TTLDWSWLQME) according to Dai *et al.* (2004), FITC-NBD (FITC-YGRKKRRQRRR-G- TTLDWSWLQME) and TAT (YGRKKRRQRRR) peptides were procured from Genscript (NJ, USA). The physical and chemical properties of the peptides were characterized before use. Dimethyl Sulfoxide (DMSO), propidium iodide (PI), Cisplatin (C), Gemcitabine (G), Triton X-100, RNase, MTT reagent, sodium pyruvate, lithium lactate, NAD+, acridine orange (AO), DAPI (4', 6- Diamidino-2-phenyl indole) and ethidium bromide (EtBr) were purchased from Sigma-Aldrich (St. Louis, MO, USA). McCoy's 5A Medium, DMEM, Trypsin–EDTA, HEPES (4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid), L-Glutamine and Fetal Bovine Serum (FBS) were procured from Gibco (BRL, CA, USA). A EnzChek® Caspase-3 Assay Kit #2 (Molecular Probes: MP 13184) was purchased from Thermo Scientific (Rockford, IL, USA). All other common reagents were procured from HiMedia Laboratories Pvt. Ltd. (Mumbai, India).

Cell Culture and Treatment

T24 cell line (Human transitional cell carcinoma of the urinary bladder), NIH-3T3-L1 (mice fibroblasts), CHO (Chinese Hamster Ovary) and Vero (African green monkey kidney) were obtained from the National Centre for Cell Science (NCCS), Pune, India. T24 cells are resistant to cisplatin and were cultured as monolayers in McCoy's 5A medium. NIH-3T3-L1, CHO and Vero were cultured in DMEM. Each of the medium used for maintenance was supplemented with 10% FBS, L-Glutamine and 3 mM sodium bicarbonate/ 20 mM HEPES. A combination of antibiotics (penicillin, streptomycin, kanamycin) and an antimycotic (amphotericin B) was used. The cells were maintained at 37°C with 5% CO_2 and 85-95% humidity. The maintained cultures were subcultured for assays and drug treated at optimal growth (Abdel-Mageed, 2003; Chong *et al.*, 2000).

NBD was prepared in serum free McCoy's 5A medium and used for treatment. TAT is a cell

penetrating peptide (Trans-Activator of Transcription protein derived from human immunodeficiency virus) tagged to NBD peptide to ensure cell entry. It was employed as a vehicle control at a final concentration not exceeding 100 µM in the cell culture experiments. The standard drug, cisplatin was prepared in 0.9% sodium chloride to avoid isomerisation, kept protected from light and used at a final concentration of 50 µM (Tian *et al*., 2008). Gemcitabine was prepared in serum free McCoy's 5A medium and used at a final concentration of 50 nM in the cell culture experiments (Fechner *et al*., 2003). Based on the half maximal inhibitory concentration (IC_{50}) of NBD peptide, all treatments were carried out and incubated for 48 h along with select concentrations: 12.5, 25, 50 and/or 100 µM in every experiment, except for confocal microscopy where 20 µM FITC-NBD was used.

Confocal Microscopy for Localization

T24 cells were seeded on a coverslip placed inside a 35 mm Petri dish at a concentration of 1×10^4 cells. McCoy's 5A medium was added and incubated to attain 80% confluency. FITC tagged NBD peptide (20 µM) was treated for different time periods (10 min, 45 min, 4 h and 24 h). The plates were incubated in the dark at 37°C for the designated durations and counterstained with 10 µL PI (1 mg/mL) for 1 min. The coverslips were briefly rinsed with PBS and mounted onto another coverslip for documentation according to the previous report with slight modifications (Choi *et al*., 2003).

Cell Proliferation and Viability Assessment by MTT Assay

T24, NIH-3T3-L1, CHO and Vero cells were seeded at a concentration of 1×10^4 cells/well on a 96 well plate with 10% medium. At about 80% confluency, different concentrations of the NBD peptide (12.5, 25 and 50 µM), TAT peptide (100 µM) as vehicle control and standard drugs (Cisplatin, 50 µM and Gemcitabine, 50 nM) were treated and the volume was made up to 100 µL. The drug treated plates were incubated for 48 h at 37°C for cell proliferation and viability assessment. Finally, 20 µL of MTT reagent (1 mg/mL) was added and incubated for 4 h at 37°C for quantification. The purple formazan crystals formed were dissolved using 100% DMSO and read at 570 nm in BMG Fluostar Optima multimode reader (Wang *et al*., 2012). The IC_{50} value for NBD peptide on T24 cells was determined from the MTT experiment. Synergistic action of IC_{50} NBD peptide was tested with a combination of standard drugs. The tested combinations are IC_{50} NBD and cisplatin; IC_{50} NBD and gemcitabine; IC_{50} NBD, cisplatin and gemcitabine.

Cell Cycle Analysis Using Flow Cytometry

T24 cells were seeded at a concentration of 1×10^5 cells/ well on a 12 well plate and allowed to attain confluency. Different concentrations of the NBD peptide: 12.5, 18.5 (IC_{50}), 25 µM, TAT and standard drugs were treated. Combinations of NBD peptide along with the standard drugs were also treated. The plates were incubated at 48 h at 37°C, harvested by trypsinization, rinsed with PBS and centrifuged at 1500 rpm for 10 min. Cells were fixed overnight at 4°C in ice-cold alcohol (70%). The fixed cells were washed with PBS and centrifuged at 1500 rpm for 10 min before analysis. Triton X-100 (0.5%) and RNase (0.1 mg/mL) was added and incubated for an hour. Propidium iodide (40 µg/mL) was added to the mix and incubated for 45 min in the dark. Cell cycle analysis was carried out on FACS Calibur (BD Biosciences) (Ma *et al*., 2009; Wang *et al*., 2013).

Assessing Cell Death by Acridine Orange/Ethidium Bromide (AO/ EtBr) Fluorescence Staining Assay

T24 cells were seeded at a concentration of 1×10^5 cells/well on a 6-well plate added with 10% medium and allowed to attain confluency. NBD (12.5, 18.5 and 25 µM), TAT peptide, standard drugs and the combination were treated. The plates were incubated at 48 h at 37°C followed by addition of 10 µL AO/EtBr (1 mg/mL) to each well for morphological documentation (Ishaq *et al*., 2014). The fluorescence screen was applied and the cells were photographed under a microscope (Nikon Eclipse Ti-S, USA).

Caspase-3 Activity

Quantification of caspase-3 release was carried out according to the EnzChek® Caspase-3 Assay Kit #2 (Molecular Probes: MP 13184) protocol. Briefly, T24 cells at 1×10^4 cells per well were resuspended in 50 µL of the 1× cell lysis buffer. Cells were incubated on ice for about 30 min to ensure optimal lysis. After incubation, the cells were centrifuged at 5000 rpm for 5 min in a centrifuge. Then 50 µL of the supernatant from each sample was transferred to individual microplate wells. 1× cell lysis buffer (50 µL) was used as a "no-enzyme control" to determine the background fluorescence. Then 50 µL of 2× substrate working solution was added to each sample (NBD: 100 µM, NBD and standard drug combination) and untreated control. The microplate was covered and incubated at room temperature for 30 min. Fluorescence was measured on a microplate reader (BMG FLUOSTAR OPTIMA multimode reader) using excitation/emission wavelengths of 492/520 nm (Cerón *et al*., 2010).

Cellular Damage Detection Using Lactate Dehydrogenase (LDH) Release

T24 cells were seeded on 48-well plates at a density of 2.5×10^4 cells/well and allowed to become confluent overnight. At >80% confluency, the cells were treated with NBD peptide for 48 h along with standard drugs and vehicle control. After treatment, the conditioned media was used to assess the LDH released by the damaged cells. Buffered substrate (1 mL) and conditioned media (0.1 mL) was added and incubated at 37°C (in a water bath). To this mix, 0.2 mL of NAD+ solution was added, mixed gently and incubated at 37°C for 15 min. DNPH reagent (1 mL) was added and incubated further for 15 min. Finally, 10 mL of sodium hydroxide (0.4 N) was added and the absorbance was read at 440 nm. Standards were also run parallelly and treated as for assays with sodium pyruvate. The amount of color developed was directly proportional to the number of lysed cells (Elumalai *et al.*, 2012).

Detection of Apoptosis by DAPI Staining

T24 cells were seeded on 12-well plates at a density of 5×10^4 cells/well and allowed to become confluent overnight. For the nuclear changes, the monolayer of cells was washed with PBS, fixed with 3% paraformaldehyde (10 min) and were permeabilized with 0.2% Triton X-100 in PBS (10 min) at ambient temperature. The cells were finally incubated with 0.5 µg/mL of DAPI for 5 min to identify the apoptotic nuclei which were intensely stained or fragmented or had condensed chromatin. The changes were observed and recorded under a fluorescent microscope (ex 359 nm; em 461 nm) (Tajmohammadi *et al.*, 2019).

Wound Healing Assay

T24 cells were seeded at a concentration of 1×10^5 cells/well on a 6-well plate and allowed to attain confluency before treatment. Confluent monolayers were wounded by physical shearing across the well using a sterile micropipette tip. The diameter of the wound inflicted was measured immediately using a phase contrast microscope (Nikon Eclipse Ti-S) with ImagePro software. NBD (18.5 and 100 µM), TAT peptide, standard drugs and the combination were treated. The plates were incubated for 60 h at 37°C, observed for wound closure and documented (Gao *et al.*, 2006).

Statistical Analysis

All the experiments were repeated thrice for accuracy and each sample was assessed in triplicates. The data are presented as the mean ± SD. Statistical analysis was performed with IBM SPSS Statistics 23v and differences between groups were examined using one-way Analysis of Variance (ANOVA) followed by Tukey's multiple comparison test wherever applicable to prove statistical significance. *P* value of $\leq$ 0.05 was considered statistically significant.

Results

NBD Peptide Localization and Selective Inhibition of T24 Cancer Cells

The ability of the NBD peptide to gain entry into the T24 cells and their stability inside the cells was determined using confocal microscopy after incubation of cells with the peptide for 10 min, 45 min, 4 h and 24 h. The NBD peptide tagged with FITC fluorochrome emits fluorescence upon intracellular localization and is not affected by pH changes in the endolysosomal compartments. The minimum time taken for NBD peptide cell penetration was $\leq$ 10 min where it was able to localize the cytosol and nucleus with detectable fluorescence. At 45 min of treatment, there were prominent signals from the NBD peptide in the cytoplasm and nucleus retaining cell morphology until 4 h of treatment. The T24 cells started to show aggregation at 24 h of treatment, however, the fluorescence signal was stable with detectable emissions from the peptide. Figure 1 shows that the NBD peptide was stable for up to 24 h of treatment and was capable of acting on the cell's internal machinery. The cytotoxicity was tested on NIH-3T3-L1, CHO and Vero (non-cancerous) cells along with T24 cancer cell line. NIH-3T3-L1, CHO and Vero cells were resistant to NBD peptide treatment ($P\leq$ 0.05). However, T24 BC cells were susceptible to NBD peptide treatment (25, 50, 100 µM) at 48 h duration *($P\leq$* 0.05). The susceptibility was compared with the untreated control cells and standard drugs like cisplatin and gemcitabine (Fig. 2). The inhibition of T24 cell growth in MTT assay allowed us to determine the IC_{50} value as 18.5 µM and this concentration was used to test the adjuvant capability of NBD peptide. A combination of NBD peptide along with the standard chemotherapeutic drugs was tested on T24 cells. The results showed higher susceptibility of T24 cells to the synergistic action of NBD: Cisplatin: Gemcitabine combination at 48 h *($P\leq$* 0.05) than the individual agents (Fig. 3).

NBD Peptide Elicits Cell Cycle Inhibition

Flow cytometry was performed on T24 cells treated for 48 h with NBD peptide (12.5, 18.5 and 25 µM), standard chemotherapeutic drugs and the combination of NBD with standard chemotherapeutic drugs. Control represents the normal cell cycle progression in T24 cells. Treatment with the NBD peptide increases the accumulation of cells in the sub G0- G1 phase with an overall reduction in the number of events recorded. The

standard drugs cisplatin shows an increase in the cells at sub G0- G1 and disruption of G2- M phase; gemcitabine shows an increase in the cells at sub G0- G1 phase; dual action of cisplatin and gemcitabine shows accumulation of cells in the S phase indicating a disruption of G2- M phase. The combination of NBD along with standard drugs indicates the effects of the individual agents combined. The NBD & gemcitabine combination shows G2- M phase disruption which was absent in their individual effect and has increased the accumulation of cells in sub G0- G1 phase. The NBD & standard drugs combination panel shows an overall reduction in the number of events and an increase of cells in sub G0- G1 phase. The histograms of flow cytometry (Fig. 4) and the number of events after gating are tabulated (Table 1).
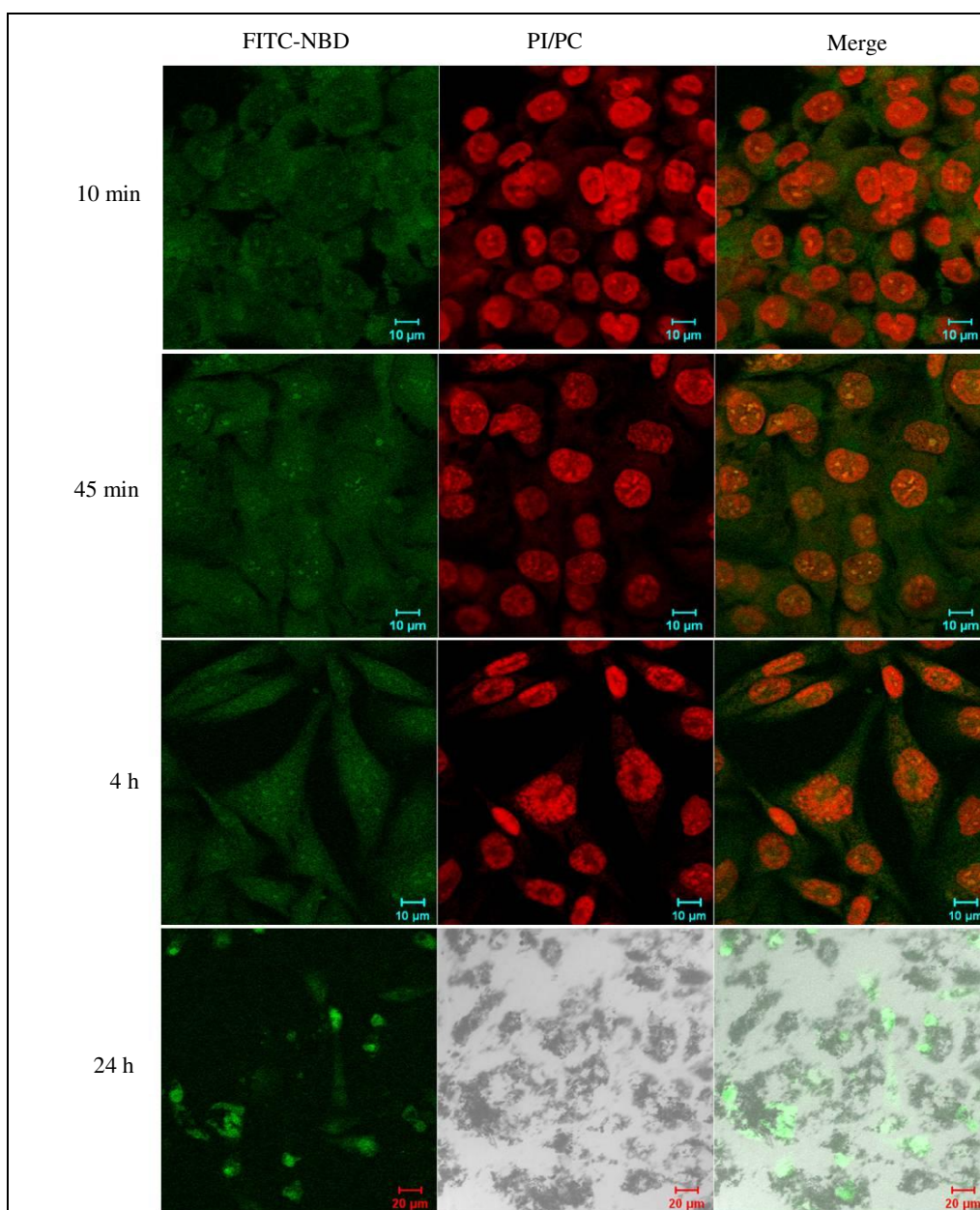

Fig. 1: NBD peptide localization in T24 cancer cells. Localization of NBD peptide in T24 cells under 40 and 20 × objectives. FITC-NBD tagged peptide (20 µM) fluoresces upon localization (green). Nucleus was counterstained by propidium iodide (PI, red). The localization of FITC-NBD peptide in the nucleus along with PI, fluorescence emission is shown as a merge in yellow. At 24 h there was high cell aggregation after NBD treatment and PI nuclear staining was uneven and hence phase contrast (PC) images with FITC-NBD were shown
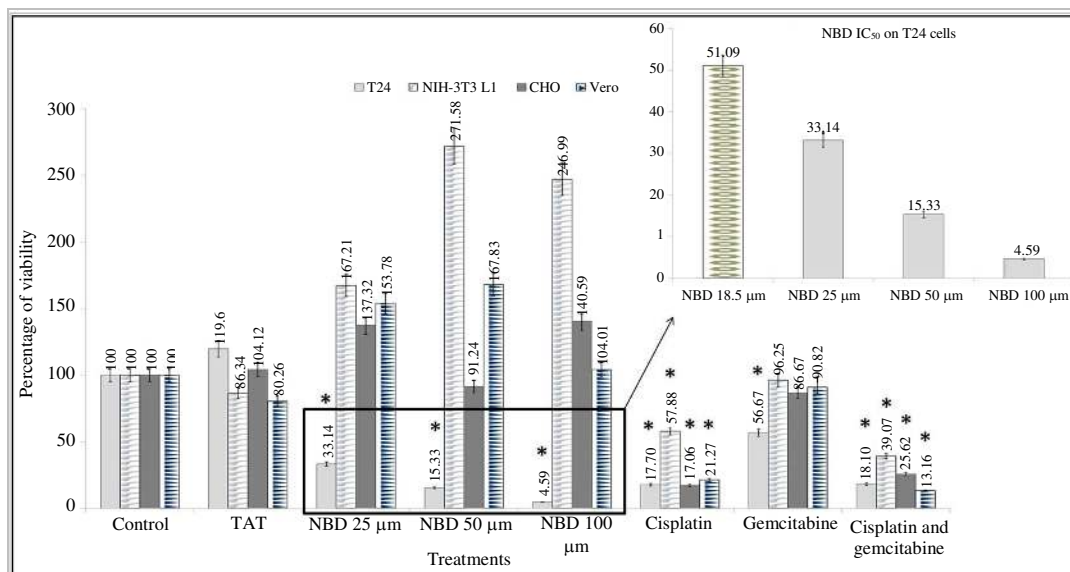
Fig. 2: Cytotoxicity of NBD peptide and standard drugs on T24, NIH-3T3-L1, CHO and Vero cells. T24 cells have been inhibited by NBD peptide and the standard drugs when compared to the non-cancerous cells (NIH-3T3-L1, CHO and Vero). Data are presented as Mean ± SD of three independent experiments. The statistical significance is indicated by * ($P \leq 0.05$). The dose response of the NBD peptide at IC_{50} has been shown as figure insert
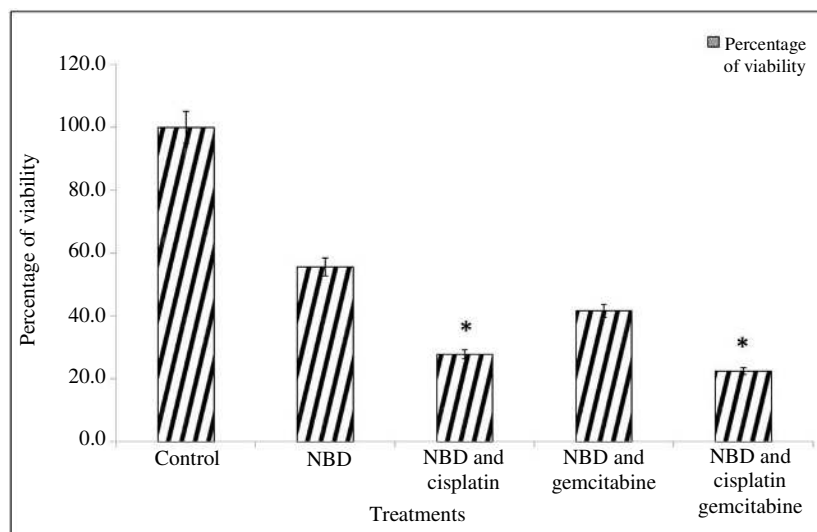

Fig. 3: Cytotoxicity of IC_{50} NBD peptide and a combination with standard drugs on T24 cells. The IC_{50} NBD peptide showed about 50%, NBD and Cisplatin showed about 27%, NBD and Gemcitabine showed about 41% and NBD and Cisplatin and Gemcitabine showed about 22% cell viability. Data are presented as Mean ± SD of three independent experiments. The statistical significance is indicated by * ($P \leq 0.05$)

NBD Peptide Induces Caspase-3 Independent Cell Death of T24 Cells and Inhibits Cell Migration

The accumulation of sub G0-G1 population in cell cycle after NBD treatment led us to use acridine orange and ethidium bromide staining to reveal the mode of death induced by NBD peptide. The early or late apoptosis marked by the orange-yellow coloration was scanty in the standard drug panel and negligible in the combination treatment panel (Fig. 5). To confirm that there was no

caspase activity we quantified the cysteine-aspartate proteases that are released when the cells undergo apoptosis. We tested the highest concentration of NBD peptide (100 µM) on T24 cells for 48 h. Negligible effect was observed with the combination of NBD and standard chemotherapeutic drugs when compared with untreated cells (Fig. 6). Similarly, the LDH release was quantified to assess cellular damage as it is a stable cytosolic enzyme, released into the culture medium upon cell lysis. Oxidation of lactate to pyruvate (reversible) is catalysed by LDH,

forming NAD+ from NADH and thus the determination of LDH is based on the detection of NADH in the reaction. Thus Fig. 7 indicates T24 cell death by necrosis (resultant of ruptured plasma membrane) upon treatment with NBD and its combination with cisplatin and gemcitabine. Further

confirmation for the non-apoptotic mode of death was by DAPI staining where there was no chromatin condensation at the nuclear membrane periphery except in combinations that contained cisplatin: NBD and cisplatin; NBD, cisplatin and gemcitabine (Fig. 8).

Table 1: Total number of recorded events/ cells after gating for cell cycle analysis by Flow cytometry

| Treatments | % Total | Events (%) Cell cycle phases | | | |
		Sub G0-G1	G0-G1	S	G2-M
Control	91.32	1.58	72.62	9.23	8.07
NBD (12.5 µM)	84.97	9.9	55.07	9.13	10.52
IC_{50} NBD (18.5 µM)	71.72	6.8	49.54	11.95	3.95
NBD (25 µM)	82.85	19.36	45.44	10.13	8.04
Cisplatin	85.60	14.5	51.66	12.22	7.53
Gemcitabine	84.19	9.83	62.31	4.78	7.51
Cisplatin and Gemcitabine	93.86	10.16	42.78	17.17	4.11
IC_{50} NBD: Cisplatin	92.51	18.76	38.76	22.86	12.1
IC_{50} NBD: Gemcitabine	93.41	25.69	41.82	19.59	6.58
IC_{50} NBD: Cisplatin: Gemcitabine	76.57	17.26	30.35	21.5	7.44
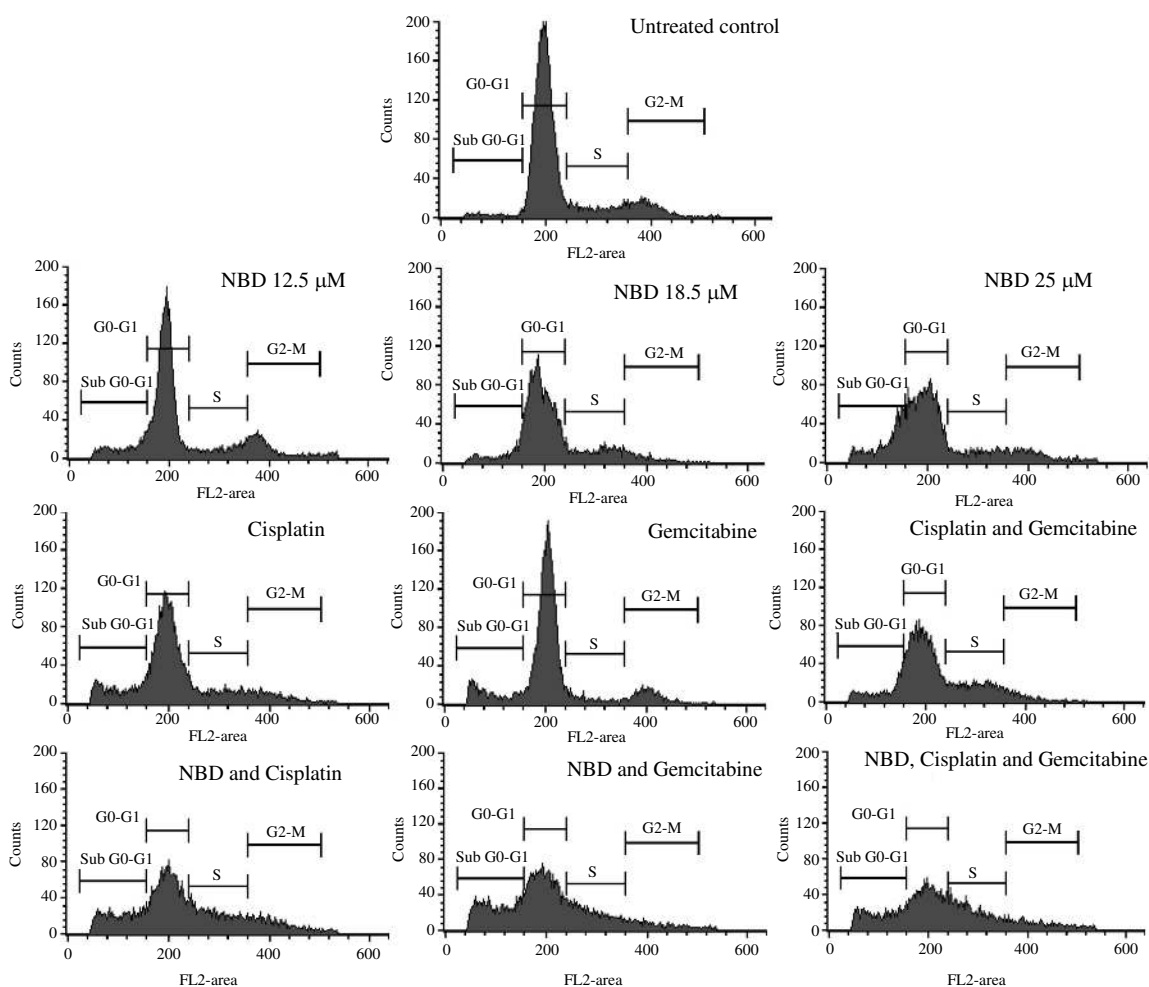
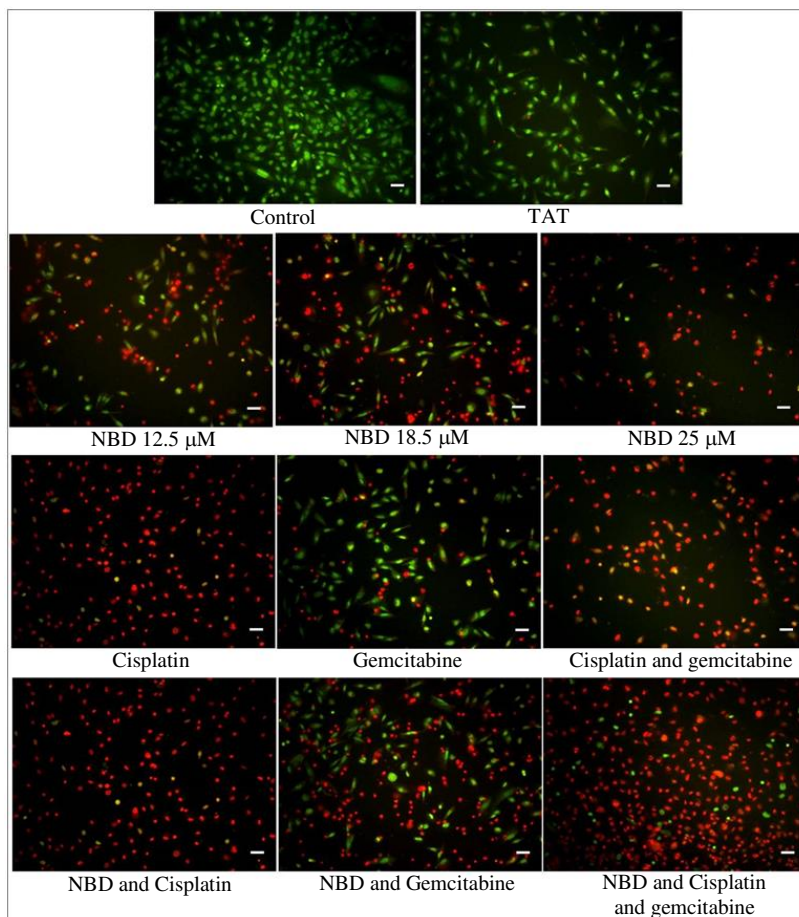**Fig. 4:** NBD peptide effect on the cell cycle of T24 cells

Fig. 5: NBD peptide imparts necrotic mode of cell death in BC. Acridine orange and ethidium bromide staining of the IC_{50} NBD peptide treated T24 cells shows 50% viable cells (green) and 50% necrotic cells (red) under 10 × objectives. Scale bar indicates 20 µM. The cells that show orange-yellow color indicate early or late apoptotic cells. The combination of cisplatin and gemcitabine had a mix of apoptotic and necrotic cells as shown by the cell color. Treatment with IC_{50} NBD peptide and the standard drugs shows red colored cells indicating cell necrosis

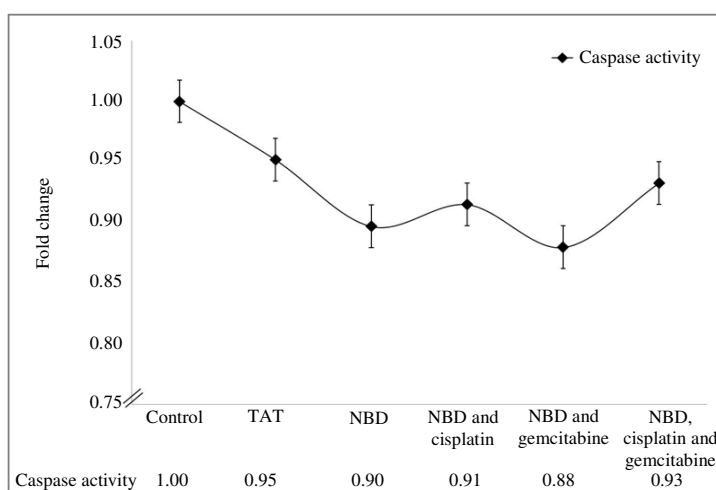|	Control	TAT	NBD	NBD and cisplatin	NBD and gemcitabine	NBD, cisplatin and gemcitabine
Caspase activity	1.00	0.95	0.90	0.91	0.88	0.93

Fig. 6: Apoptosis detection using caspases. Caspase enzyme activity (arbitrary units) tested at the highest concentration of NBD peptide on T24 cells and the combination. Data are presented as Mean ± SD of three independent experiments. None of the groups showed statistical significance ($P \geq 0.05$)
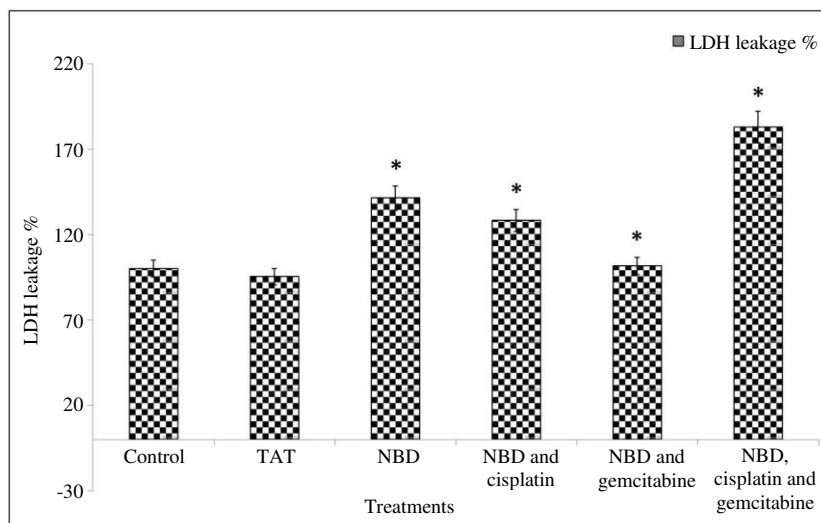
Fig. 7: Assessing necrosis using LDH release. The percentage release of LDH increases when compared to the control group as the plasma membrane integrity and permeability is compromised upon treatment with NBD peptide and in combination with standard drugs. Data are presented as Mean ± SD of three independent experiments. The statistical significance is indicated by * ($P \leq 0.05$)
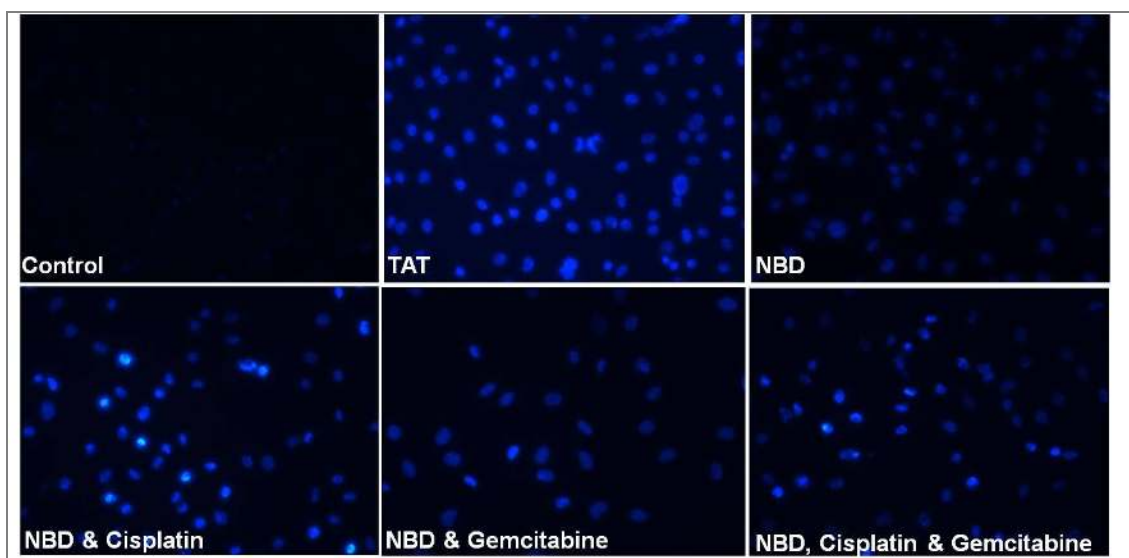

Fig. 8: Detecting the apoptotic nuclei by DAPI staining. Apoptotic nuclei have characteristic features of condensed or fragmented chromatin that are intensely stained by DAPI. Control T24 cells, TAT, NBD, NBD and gemcitabine show negligible amounts of DAPI fluorescence indicating necrotic mode of cell death. Intense chromatin staining is observed in NBD and cisplatin and NBD, cisplatin and gemcitabine treated T24 cells showing a mixed population of apoptotic and necrotic cells

Cancer metastasis is mimicked by the process of wound healing on the monolayer of cells as they require the same phenomenon of proliferation and migration. In our study, the ability of T24 cells to convalesce to their viable state after NBD treatment was demonstrated using cell migration technique. The T24 cells after treatment with NBD peptide, standard drugs and the combination showed significant cell migration inhibition in wound closure when compared to the untreated cells. NBD peptide, when used at the IC$_{50}$ concentrations of 18.5 µM was unable to restrict the migration of T24 cells and hence the highest concentration of NBD peptide (100 µM) was used in the wound closure assay. The highest concentration of NBD peptide combination along with standard drugs had better inhibition of cell migration than the individual agents (Fig. 9).
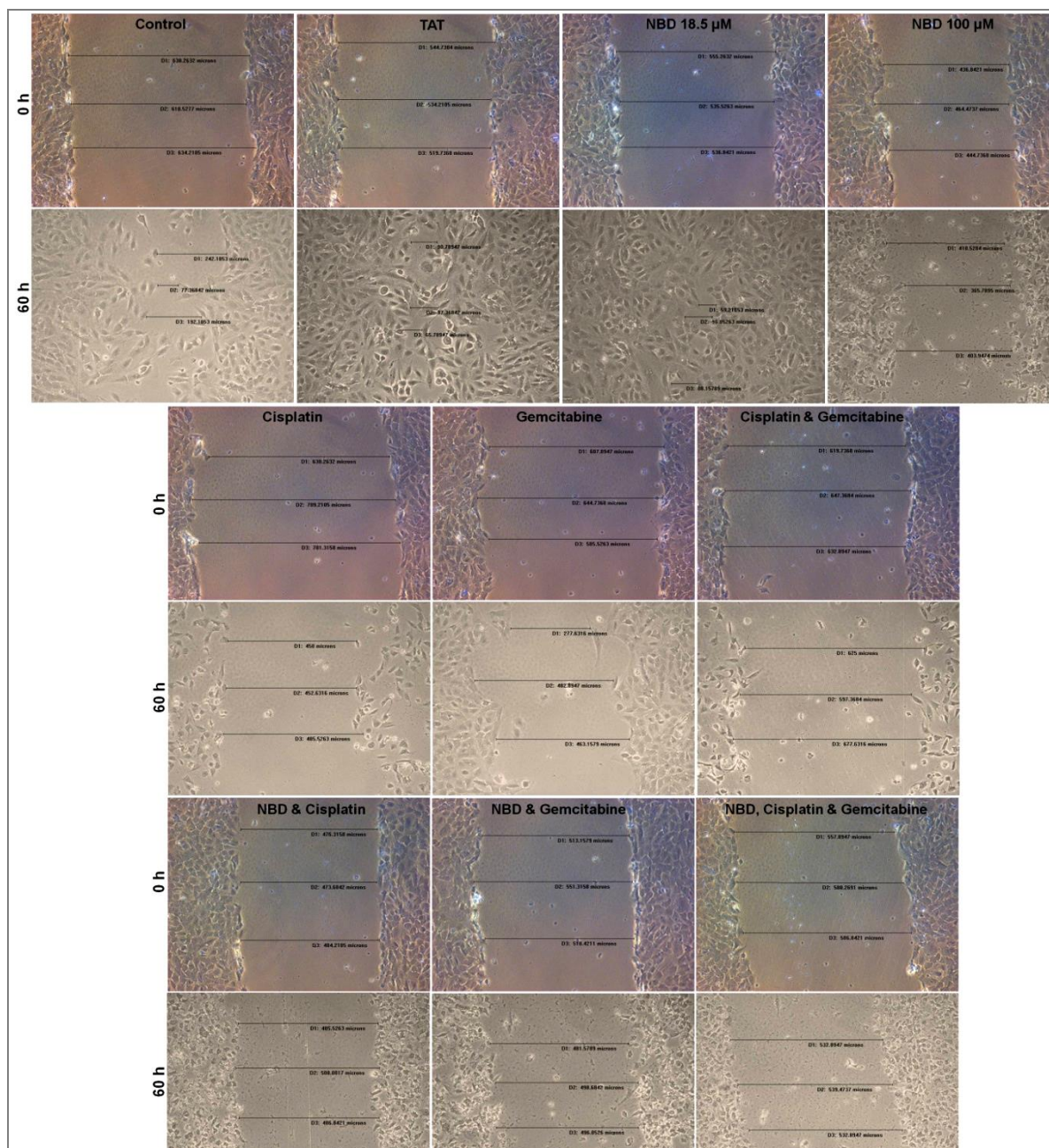
Fig. 9: NBD peptide, standard drugs and combination effect on T24 cell migration. T24 cells after treatment with NBD peptide (100 µM) and standard drugs showed minimal recovery from the inflicted wound when compared to the untreated control cells

Discussion

BC is a recurrent disease and lacks thorough investigations (Mukherjee *et al*., 2015). The tissue microenvironment is a niche that comprises various signaling factors that are involved in several regulatory functions of a cell. BC has highly active NF-κB along with other transcription factors (Degoricija, 2014). During BC initiation presence of aberrant NF-κB in the tumor milieu has been reported (Levidou *et al*., 2008;

Zhu *et al*., 2017; Mukherjee *et al*., 2017). Targeted therapy is sought after in the treatment of BC (Jones *et al*., 2016) overexpression of NF-κB heterodimers can be used to differentiate cancer cells from normal cells. Previously, we were able to show that the NF-κB RelA preferably binds to other dimers of the family other than p50 during BC progression (Durairajan *et al*., 2018). Since the involvement of the NF-κB heterodimers in BC progression was evident, we utilized this to explore the treatment potential by targeting this pathway.

NBD peptide was identified to have anti-inflammatory properties by acting as indirect NF-κB inhibitor (Gilmore, 2006; Gilmore and Garbati, 2011). NF-κB inhibition either directly or indirectly will affect the basal NF-κB activity required for cell survival and upon its inhibition will lead to toxicity (Davé *et al.*, 2007). Few studies investigating NBD peptide have eradicated this concern where prolonged NBD peptide administration ameliorated inflammatory responses in inflammatory animal models effectively without side effects (Jimi *et al.*, 2004; di Meglio *et al.*, 2005) while retaining the basal level activity of the NF-κB heterodimer to perform the regulatory functions (Jana *et al.*, 2017; May *et al.*, 2000). NBD peptide was able to reduce skeletal muscle atrophy and tumor induced muscle wasting dose-dependently with the ability to decrease the tumor burden (Madonna *et al.*, 2012; Wysong *et al.*, 2011).

NBD peptide core amino acids determine its activity and any changes in the core region disrupt its anti-inflammatory activity, rendering it inactive (Dai *et al.*, 2004). Analysis of binding energy hot spots between those on NEMO (Nuclear Factor kappa B (NF-κB) essential modulator) and IKKβ (IκB kinase subunit β) interacting interface identified an NBD (NEMO Binding Domain) region on IKKβ to contain the highest concentration of hot-spot residues (W739, W741 and L742). The residing region of these residues creates a druggable binding site on NEMO (Golden *et al.*, 2013). In the present study, NBD peptide was fused with HIV coat protein fragment, TAT to achieve cell penetration. This peptide fragment, NBD inhibits the growth of T24 BC cells. According to our hypothesis, we observed dose-dependent inhibition of the T24 cells with half maximal inhibition at 48 h, while the non-cancerous (NIH-3T3-L1 cells, CHO and Vero) cells did not show any cytotoxicity (Fig. 2). The specificity of NBD peptide towards cancer cells makes it evident that it targets a specific molecule in T24 BC cells that is uncommon in the non-cancerous cell lines. The viability assay confirmed dose-dependent cell death and the NBD peptide's ability to interrupt cellular processes to bring about cell death. The unaffected growth of the non-cancerous cells after NBD treatment was high when compared with the untreated control group. The statistical significance of the T24 cancer cell line ($P \leq 0.05$) is due to the decrease in the number of viable cells that are compared with the untreated control group.

NBD peptide localization in the nucleus was suggestive of the interference to cell cycle. Accumulation of the cells in Sub G0-G1phase indicated necrosis of cells caused by NBD peptide treatment. Combining NBD peptide with standard drugs showed decrease in the overall number of events in all cell cycle phases. Further, differential staining of T24 cells with acridine orange and ethidium bromide revealed majority of cells emitting red fluorescence post NBD peptide treatment suggesting necrosis. Ethidium bromide is known to shift its fluorescence upon binding to intracellular DNA after entering cells with damaged membranes in necrotic cells (Liu *et al.*, 2015; Vethakanraj *et al.*, 2015). This was confirmed by the negligible caspase activity at the highest concentration of the NBD peptide tested compared to the untreated control cells (Fig. 6). Thus acridine orange/ethidium bromide staining, caspase activity, LDH release, DAPI staining confirmed that there was no apoptosis in T24 cells after NBD peptide treatment and also inhibited cell migration *in vitro*. To the best of our knowledge, this is the first report on NBD peptide to have necrotic activity in T24 bladder cancer cell line. Our observations in this study was in accordance with other studies that state different cell lines do respond differently to a treatment based on their cancer phenotype (McConnell *et al.*, 2015); the factors that cause a peptide to act differently in a tumor microenvironment (Pan *et al.*, 2014); the amino acid difference from the originally reported NBD peptide (May *et al.*, 2000; Dai *et al.*, 2004; May *et al.*, 2002) and the different cell penetrating peptide tags that allow them to act unlike their original counterparts (Shaheen *et al.*, 2018).

Currently, peptide based trials have been predicted to be clinically successful in urological cancers (Obara *et al.*, 2018) and the ability of NBD peptide to sensitize cancer cells to chemotherapy have been reported in pancreatic cancer (Holcomb *et al.*, 2008). Our study was in conformity with another study that combined NBD peptide with other chemotherapeutics like cisplatin and gemcitabine producing higher rates of proliferation inhibition than the peptide or drug alone (Ndikuyeze *et al.*, 2014). Definitive use of NBD peptide determines its function to act as a cell protective agent with minimal adverse effects (Rehman *et al.*, 2003) or to act as an anti-inflammatory/anti-cancer agent. This dynamic change in action for NBD could be due to more than just inhibition of activated NF-κB (Cerón *et al.*, 2010). Our study suggests that the mechanism of NBD peptide action varied from each cell type analyzed. This only emphasizes the need for more mechanistic studies that allows characterization of the physiological and immunological responses with NBD treatment in BC.

Conclusion

We investigated NBD peptide in BC since the available chemotherapeutics lack specificity with adverse side effects. In our study, we have shown that NBD peptide selectively reduces the proliferation of BC cells *in vitro* by necrosis after localization. NBD peptide lacked cytotoxicity towards non-cancerous cell lines across all tested doses. The combination treatment of

NBD peptide along with cisplatin and gemcitabine showed stronger inhibition than single drug treatment. Though we need further investigations to understand NBD peptide's mechanism of action, its use as an anti-cancer agent in the recurrent and often chemoresistant BC seems promising.

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Author's Contributions

Sankari Durairajan and Charles Emmanuel Jebaraj W: Conceived, designed and performed the experiments, analyzed the data, contributed reagents/materials/analysis tools, prepared figures and/or tables, performed and interpreted formal analysis, authored or reviewed drafts of the paper and approved the final draft.

Kalaiselvi Periyandavan, **George Priya Doss C**, **Dicky John Davis G**, **Hannah Rachel Vasanthi A**, **Gayathri Thiruvengadam and Thanka Jhonson:** Analyzed the data, contributed reagents/materials/analysis tools, performed and interpreted formal analysis, reviewed drafts of the paper and approved the final draft.

Ethics

All authors have read and approved the final version of this manuscript. There are no ethical issues to declare that could arise after the publication of this manuscript.

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