

Correlation of Nuclear Factor Erythroid 2-Related Factor (NRF2) Expression Level with the Efficacy of Brusatol in Urothelial Cell Carcinoma Cell Lines

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Abstract: *Background & Aim:* Bladder cancer (BC) IS the second most common genitourinary tumour and the fourth most common cancer in men. BC is highly responsive to use the cisplatin based chemotherapy initially, resistance to the platinum based chemotherapy drug develops rapidly which is a major challenge in management of BC facing clinicians. High expression of Nuclear Factor Erythroid 2-related factor (NRF-2) was noticed in different types of cancers. Recently, genetic analyses of human tumours have indicated that NRF2 Overexpression may be cause resistance to chemotherapy. The aim of this study is to determine whether basal Nrf-2 expression correlates with the ability of Brusatol to attenuate Cisplatin resistance. *Methodology:* Four urothelial cell cancer (UCC) cell line cultured, Cisplatin dosing, BCA Assay and Western immunoblot done for all 4 UCC cell lines in order to determine the efficacy of brusatol on Cisplatin cytotoxicity action and to established the basal Nrf-2 expression in different UCC cell lines. *Results:* The results which were obtained prove the hypothesis we were working on. *Conclusion:* there is a significant synergistic relationship between Brusatol and Cisplatin co-administration

Keywords: Nuclear Factor Erythroid 2-related factor (Nrf2) , Brusatol, Urothelial Cell Carcinoma

1. Introduction

Bladder cancer (BC) is the second most common genitourinary tumour and the fourth most common cancer in men [1]. However BC is less than half as common in female population. Making it the fourth most common cancer among men and eighth most common amongst women, with 10.399 people in the UK diagnosed with bladder cancer in 2011, and 5.242 BC related deaths in the UK in 2012 [2].

It occurs mainly beyond the age of fifty five, although it can occur at any age however it is rare under the age of 50 years [3]. BC has many distinguishable types including urothelial bladder cancer which is consists about 90% of bladder cancer and other 10% are either squamous cell carcinoma or adenocarcinoma and some rare types like sarcoma and small cell cancer of the bladder.

The bladder is by far the most frequent site of urothelial cell carcinomas, 50 times more common than TCC of the renal pelvis, and 100 times more common than TCC of the ureter. This may due to the duration and amount of carcinogens presented in urine contacted with the urothelial surface of urinary bladder which is employed as a urine reservoir for a few hours before emptying by urination.

About 80% of bladder cancers are non muscle invasive (NMIBC) and the rest 20% of cases the cancer has invaded the bladder wall (MIBC). NMIBC include tumours confined to the mucosa (Ta), and tumours that have invaded the lamina propria (T1). MIBC includes tumours that have invaded the muscularis (T2), and tumours that invaded perivesical tissue (T3). The tumour which extended to either abdominal or pelvic wall (T4) [4].

Urinary bladder urothelial cell carcinoma starts in the cells lining the bladder and if not treated successfully at an early stage, can spread to nearby organs or other parts of the body.

The standard treatment for muscle invasive bladder cancer is radical or salvages cystectomy and bilateral pelvic lymph-node dissection [5]. Although surgical treatment could be curative, approximately half of patients develop metastases within two years of cystectomy and 46% subsequently die of disease within 5 years [F.1]. Thus systemic Cisplatin- based chemotherapy is considered a corner stone in treatment of muscle invasive, locally advanced macro-metastatic UCC.

The small- molecule platinum based cytotoxic chemotherapeutic drug, Cisplatin [F.2] is known as the “penicillin of cancer” because it is used in treatment of wide range of cancers including: sarcoma, small cell lung cancer, germ cell tumors, lymphoma, and ovarian cancer. Cisplatin is platinum-based and was the first medicine developed in that drug class [6]. Cisplatin is frequently given as part of a combination chemotherapy regimen especially as it is synergistic with other agents such as gemcitabine and a combination is yield most optimal efficacy. Most commonly used regimens are (Gemcitabine and Cisplatin) and the MVAC (Methotrexate, Vinblastine, Adriamycin and Cisplatin) [7].

Cisplatin works by forming a platinum complex inside of a cell which binds to DNA forming cross-links DNA. When DNA is cross-linked in this manner, it causes the cells to undergo apoptosis through cross-linking and as a result the DNA repair mechanisms are activated, and once the repair mechanisms are activated and the cells are found to not be salvageable, the death of those cells is triggered instead [F.3] [8].

Although BC is highly responsive to use the cisplatin-based chemotherapy initially, resistance to the platinum based chemotherapy drug develops rapidly [9]. The major obstacle in cancer treatment is the resistance of cancer cells to chemotherapy [10]. There 2 types of resistance are observed. (I) intrinsic resistance: in which cancer cells are inherently drug resistant so not respond. (ii) Acquired resistance: cancer cells develop resistance to chemotherapy due to initial response, which is result in the recurrence of cancer in many patients following chemotherapy [11]. **Platinum Chemotherapy**

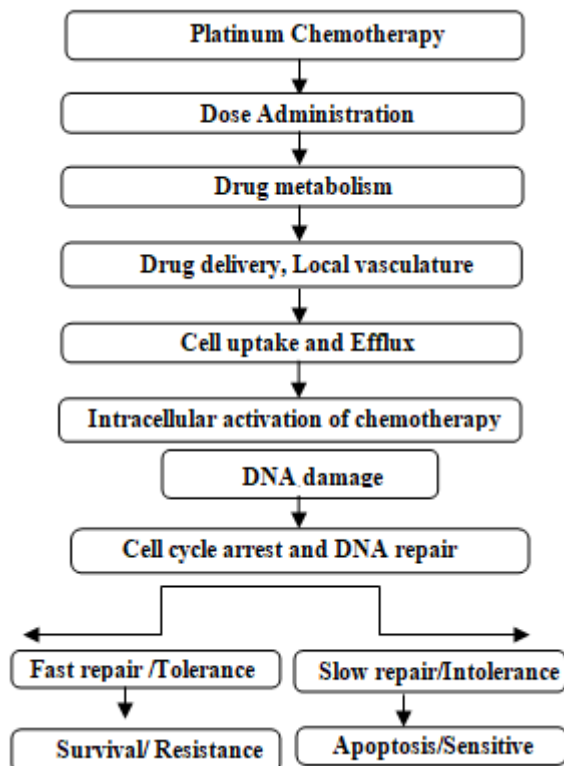


Figure 3: Mechanism of action of Cisplatin

BC is not a unique cancer in which resistance against cisplatin develops. A common example is that of ovarian carcinoma where a 5 years survival rate of 15-20% exists after an initial response rate of 70% as tumours develop resistance. Also patients with small cell lung carcinoma who are treated with cisplatin based chemotherapy tend to relapse at a rate of 95%. Resistance could be explained either by mutation or overexpression of drug target proteins (cisplatin is mainly toxic to DNA, so this would be an unlikely consequence at best), and/or inactivation of drugs by a reduction in uptake or enhanced efflux and intracellular detoxication mechanisms.

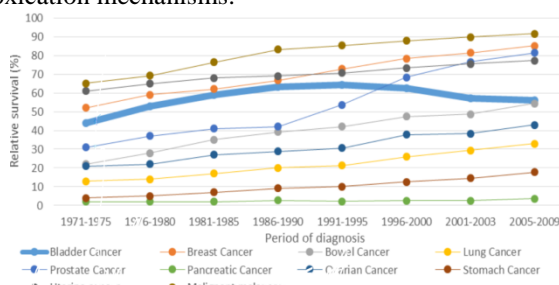


Figure 1: Age Standardised Five –Year Relative Survival Rates, England and Wales 1971-1995,England 1996-2009 Based on Type of Cancer. The graph shows the fall in the 5

year relative survival rate of BC in comparison with other cancers. All cancers illustrates an increase in relative survival, except BC which shows a 12.5% reduction in relative survival between 1991 and 2009

Cisplatin is simple and unique compound, It is the only an inorganic compound used as a chemotherapeutic drug in modern anticancer management.

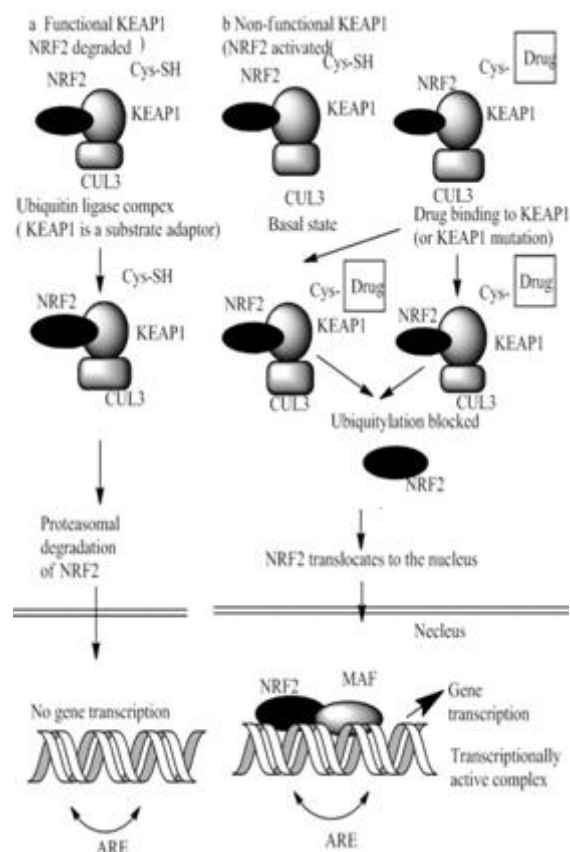


Figure 4: Nrf2 Ubiquitylation expressed low constitutive levels in response to Keap1 regulation, in presence of substrate adaptor protein for a Cullin3-based E3 ubiquitin ligase.

Nrf2 is an important transcription factor that regulates the antioxidant response via inducing the expression of genes bearing an antioxidant response element (ARE) in their regulatory regions [12]. Nrf2 is ubiquitously expressed in all human organs at low constitutive levels due to tight regulation by Keap1, a substrate adaptor protein for a Cullin3-based E3 ubiquitin ligase. Under oxidative stress, Nrf2 induces the transcription of cellular protective genes to combat carcinogenic reactive intermediates [F.4].

High expression of Nrf2 was noticed in urinary bladder cancer (BC) as well as different types of cancers. Recently, genetic analyses of human tumours have indicated that NRF2 overexpression may be cause resistance to chemotherapy [13].

From theoretical view an agent, which can inhibit the unwanted activity of Nrf-2, may illustrate clinical benefit if used in combination with Cisplatin. Brusatol [Fig 5] is a natural product originally isolated from *Brucea javanica*. It was found to show potent anticancer activity. Furthermore, it was reported that brusatol could effectively enhance the efficacy of chemotherapy by inhibiting the Nrf2-mediated defence mechanism.

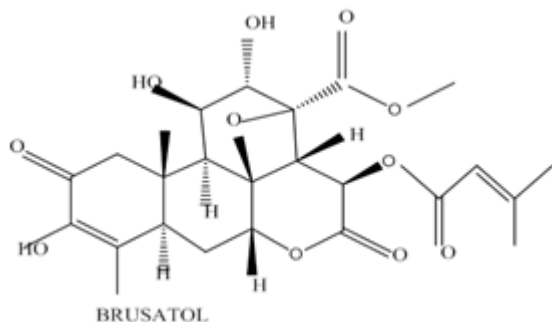


Figure 5: Brusatol Structure

The aim of this study is to determine whether basal Nrf-2 expression correlates with the ability of Brusatol to attenuate Cisplatin resistance.

2. Experimental Procedures

Cell Culture

4 urothelial cancer cell lines were re-covered from liquid nitrogen, namely EJ, 253-J, RT122 and RT112-CP. Cryogenic vials were removed from liquid nitrogen and warmed in a water bath at 37 °C. Vials were centrifuged with appropriate media at 1300 rpm for 5 minutes. Supernatant was then discarded in 2% Virkon, with the remnant re-suspended in media and transferred into the appropriate sized culture vessel.

EJ Cells were cultured in DMEM supplemented with 10% Fetal Bovine Serum (FBS, and 1% Antibiotic Antimycotic x100 solution (100U/ml penicillin and 100µ/ml streptomycin). 253-J cells were cultured in 1:1 DMEM: RPMI-1640 supplemented with 5% FBS) and 1% Antibiotic Antimycotic x100 solution (100U/ml penicillin and 100µ/ml streptomycin). RT112 Cells were cultured in RPMI-1640 supplemented with 10% FBS and 1% Antibiotic Antimycotic x100 solution (100U/ml penicillin and 100µ/ml streptomycin). RT112-CP cells were cultured in RPMI-1640 supplemented with 10% FBS and 1% Antibiotic Antimycotic x100 solution (100U/ml penicillin and 100µ/ml streptomycin) and 0.3µm cisplatin. Cells were regularly washed using Phosphate Buffered Saline (PBS) and split at 90% confluence using of trypsin-EDTA solution in order to detach the cell monolayer from cell flask during passages. Cells were grown in appropriate sized vented flask (T75) (Corning incorporated, USA) at 37C° in a 5% CO₂ atmosphere.

Table 1: The all 4 UCC cell lines involved in experiment, its origin and appropriate media for cultured

Cell line	Origin	Culture Conditions
EJ (T24, MGH-U1,82J)	G3 T3 TCC, primary bladder tumour, Established from a high grade TCC in 1973, Female, age 81, Caucasian	DMEM-10% FBS
253-J	G4 T4 UCC-Elliott et al 1974 In 1972, a retroperitoneal lymph node was removed from a 53 year old white male (253J) who had a 2 year history of multiple invasive TCC neoplasm of bilateral renal pelvis and ureters and the urinary bladder High grade T4 origin (Journal of the NCRI ,v53 , n5 , Nov 1974)	DMEM:RPMI (1:1) – 5% FBS , 1x LG
RT112	Primary urinary bladder carcinoma ,TCC , G2 , Primary bladder papillary not treated	RPMI , 10% FBS
RT112-CP	Cisplatin resistant sub-line of RT112 .	RPMI, 10% FBS (continuous culture with cisplatin)

Cisplatin Dosing

Cell lines from each flask were washed with PBS, split with trypsin and centrifuged. The resulting supernatant was discarded to harvest cells for eventual 96 well plating. Cells were re-suspended with appropriate volume of media. The number of cells/ml was then estimated using the TC20™ Automated Cell Counter (BioRad, UK)

All cells were plated in a volume of 200µl /well at a density of 3x10³ cells /well in a 96 well plate. The plate was then cultured for 24 hours at 37 C° 5% CO₂.

Cis-Diamineplatinum (II) dichloride was dissolved in N,N-Dimethylformamide (DMF) (Sigma-Aldrich , Dorest, UK) and serial dilutions were used to make up stock solutions of cisplatin in 0.1 M PBS at 30m (300µM final concentration), 5m (50µM), 0.1m (1µM), 0.05m (0.5µ), 0.01m (0.1µM) and 0m (a DMF control). Dilutions were prepared in sterile Eppendorf tubes, which were mixed throughout.

Once stock solution was prepared each stock was mixed with appropriate media at 1:1000. Each lane was then labelled and 200µl of the corresponding solution of cisplatin added.

Cytotoxicity measurement

After 24 hours of incubation, the 96 well plates were ready to be assessed for cell viability. Following removed of all media, MTT ((3-(4, 5-Dimethyl thiazol-2-yl)-2, 5-diphenyltetrazolium bromide) (2mg/ml) was added along with appropriate media 1:10 to each well, mixing the components thoroughly prior to administration. The plate was then incubated for 4 hours. All media was once again carefully removed and plate was left to dry for 10 minutes. Once dried, 150µl of Dimethyl Sulfoxide (DMSO) was added to each well and the plate sealed for MTT analysis. Absorbance was measured at 560nm using a microplate reader for all 4 cell lines.

Protein extraction

RIPA buffer was used to extract protein lysate for subsequent quantification and protein analysis. Cells were plated in 60mm petri-dishes, and incubated over-night. Cell from each were each washed with ice cold PBS, this was then removed, and fresh ice-cold PBS was then transferred to a 1.7ml Eppendorf. The solution was then centrifuged at 13000 RPM for 5 minutes at 4C°, with the supernatant removed and transferred to an Eppendorf tube. 150 ul of RIPA buffer along with 5% protease inhibitor were added. The solution was mixed using vortex mixer every 10 minutes for 30minutes, in between which the Eppendorf tubes were store d on ice. The supernatant (protein lysate) was then removed with care not to remove any cell debris and transferred into a new Eppendorf tube. This was then stored at -20 C°.

BCA (bicinchoninic acid assay)

Is a detergent-compatible formulation based on bicinchoninic acid (BCA) for the colorimetric detection and quantitation of total protein.

Diluted Bovine Serum Albumin (BSA) standard prepared by dilute the contents of one Bovine Serum Albumin standard (BSA) ampoule provided by(Pierce™ BCA protein Assay Kit, Thermo scientific, USA) into several 1.7ml Microfuge tube.

Table 2: Dilution Scheme for Standard Protocol Microplate Procedure (Working Range = 20-2,000µg/mL)

Vial	Volume of Diluent (µL)	Volume and Source of BSA (µL)	Final BSA Concentration (µg/mL)
A	0	300 of stock	2000
B	125	375 of stock	1500
C	325	325 of stock	1000
D	175	175 of vial B dilution	750
E	325	325 of vial C dilution	500
F	325	325 of vial E dilution	250
G	325	325 of vial F dilution	125
H	400	325 of vial G dilution	25
I	400	0	0= Blank

And then BCA Working Reagent volume prepared using the formula:

$(\# \text{ Standards} + \# \text{ unknowns}) \times (\# \text{ replicates}) \times (\text{volume of WR pre sample}) = \text{total volume WR required}$

To prepare WR 50 parts of BCA reagent A mixed with 1 part of BCA reagent B.

Then microplate procedure done as follows, 10 µL of each standard or unknown sample replicate into microplate well (working range = 125-200µg/ML).

Then 200µL of the working reagent was added to each well and mix plate thoroughly using a plate shaker for 30 seconds. Plate then covered and incubated at 37 C° for 30 minutes. After incubation plate was cooled to room temperature and absorbance of unknown samples was determined using: $y=mx+c$

Western Immunoblot

Protein from cell lysate samples were diluted to 30µg/well protein, PBS and Lamelli buffer and heated at 95c° for 5

minutes and separated on a 10% SDS-polyacrylamide gel. These were then transferred onto a Polyvinylidene Difluoride (PVDF) membrane and 10% milk in Tris-Buffered Saline Tween (TBST) (0.1% v/v) was then used for blocking overnight at 4C°. Incubation with primary antibody (Nrf-2 1:400 (68 kDa) and β – Actin 1:10000 (42 kDa)) for 4 hours was subsequently performed . Membranes were then washed in TBST for 1 hour, changing the TBST every 15 minutes. Membranes were incubated with secondary antibodies for 1 hour (Horseradish Peroxide (HRP) conjugated anti-rabbit and anti-mouse 1:20000) at room temperature. The membranes were the washed once again as per primary incubation washing procedure and an additional 4 times using TBS after that. A laboratory rocker was used throughout incubation and washings to ensure adequate membrane cover. Proteins were identified through Enhanced Chemi Luminescence (ECL) Prime Western Blot detection reagent and Hyperfilm ECL (GE Healthcare Life Science, Buckinghamshire, UK). Image J was used to quantify blots.

3. Results**Determination of cisplatin mediated cytotoxicity in cancer cell lines**

MTT (Colorimetric) Assay was used to find out the level of cisplatin mediated cytotoxicity in 4 cell lines by measuring cell viability percentage via absorbance reader at 650 nm . As this assay amid to measure the reduction of yellow (3-94, 5- Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. As reduction of MTT can establish only via metabolically active cells, the cell viability could be estimated.

Cisplatin reduced cell viability in dose response manner in all 4 cell lines when it administrated alone and its cytotoxicity action was improved when dosing with Brusatol while dosing of Brusatol alone showed a low response of cell viability.

The linear graph below shows the efficacy of Cisplatin and Brusatol co-administration on EJ UCC Cell line cell viability percentage.

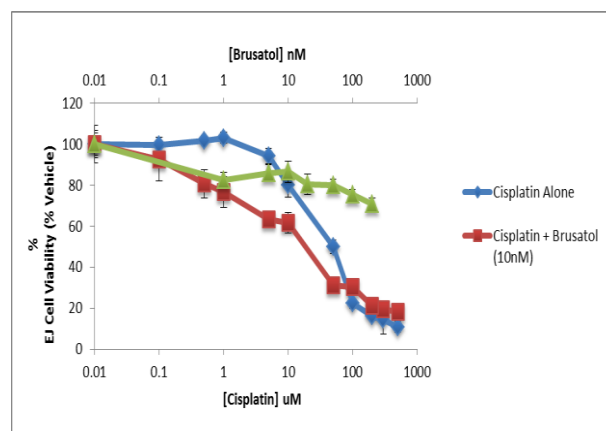


Figure 9: Linear graph shows cytotoxic efficacy of Cisplatin alone, brusatol alone and Cisplatin and brusatol co-administration.

Determination of protein concentration of cancer cell lines

Protein concentrations were accomplished by using BCA Assay. The micro plate based BCA assay was done and absorbance was measured at 560 nm. The results illustrated protein concentration variation in all 4 cell lines post Brusatol dosing as established by BCA. The EJ UCC cell lysate highest protein concentration was detected on 2 hr and 24 hr by 2600µg/ml and approximately 2800µg/ml respectively [7A]. 253-J UCC cell line lysate protein concentration reach maximum range at 24 hr around 1600µg/ml [7B].

RT112 UCC Cell lyaste peak protein concentration notice at 24 hr closing to 1700µg/ml [7C]. In RT112-CP cell line the highest reading was seen at 24 hr with value more than 2800µ/ml [7D]. BCA standard curves for UCC cell lines were established and the four cancer cell lines [8 A and B].

The BCA Assay was followed by Western immunoblot.

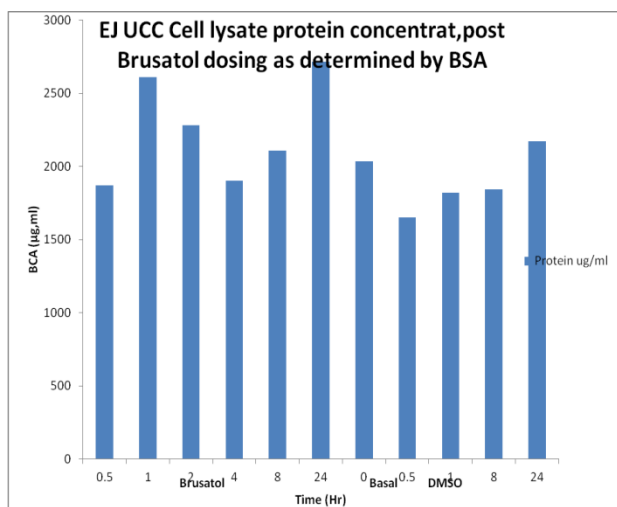


Figure 7 A: lyaste protein concentration of EJ UCC cell line among time course divided into half hour,1,2,4,8,24 hr post Brusatol and half hour, 1 ,8 and 24hr after treated with DMSO show variable protein concentration.

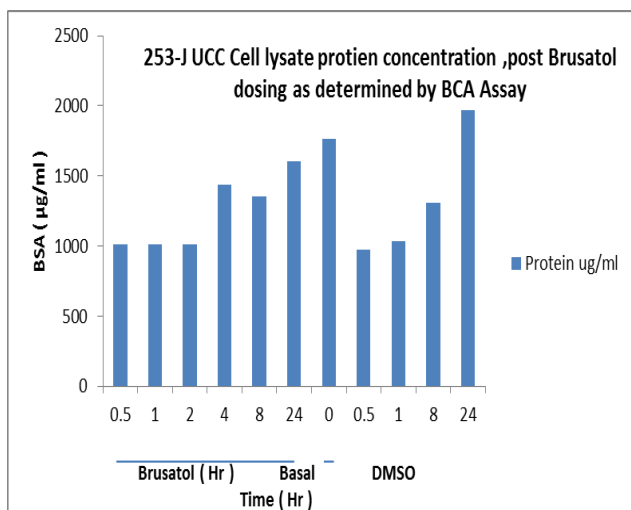


Figure 7 B: lyaste protein concentration of 253-J UCC cell line among time course divided into half hour,1,2,4,8,24 hr post Brusatol and half hour, 1 ,8 and 24hr after treated with DMSO show variable protein concentration.

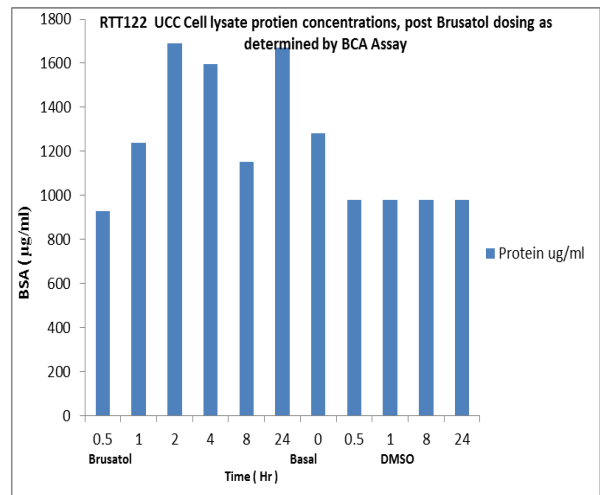


Figure 7 C: lysate protein concentration of RT112 UCC cell line among time course divided into half hour,1,2,4,8,24 hr post Brusatol and half hour, 1 ,8 and 24hr after treated with DMSO show variable protein concentration.

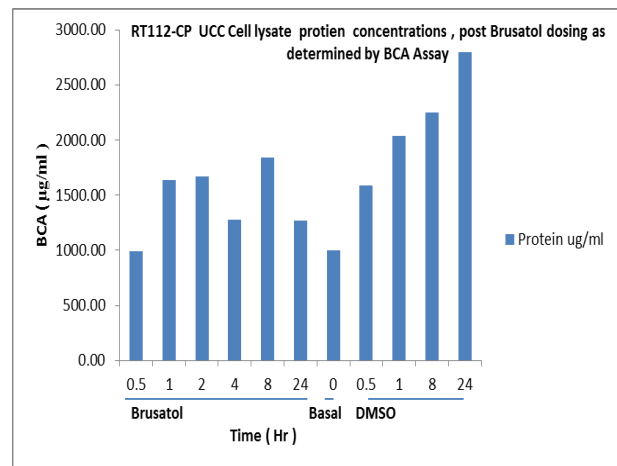


Figure 7D: lysate protein concentration of 253-J UCC cell line among time course divided into half hour, 1, 2, 4,8,24 hr post Brusatol and half hour, 1, 8 and 24hr after treated with DMSO show variable protein concentration.

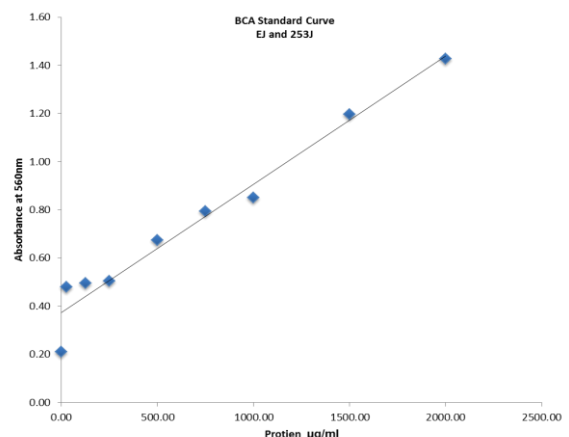


Figure 8 A: BCA Standard Curve shows total protein concentration in EJ and 253 J Cell Lines according their amples absorbance in spectrophotometer at 560 nm.

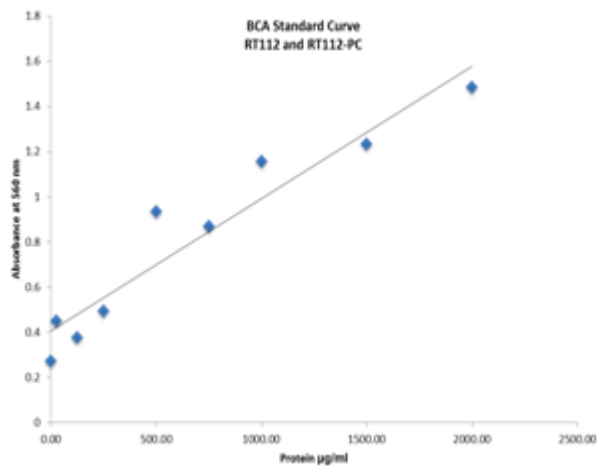


Figure 8 B: BCA Standard Curve shows total protein concentration in RT112 and RT112-CP Cell Lines according their samples absorbance in spectrophotometer at 560 nm.

Determination of Nrf-2 expression in various cancer cell lines

Western immunoblot was tried to determined Nrf-2 expression. Although all steps of western blot procedure were done following the protocol step by step with high concentration but we couldn't reach the expected result [10].



Figure 10: Ponceau S Staining Buffer used to detect protein transfer, no protein transfer occurs but marker protein dose.

4. Discussion

When Cisplatin first introduced in treatment of MIBC and locally metastatic BC, it was a promising invention but resistance to Cisplatin based chemotherapy rapidly developed resulting in reduce in relative survival rate in patients with BC. This fact represents a major obstacle in management of BC for clinicians.

Understanding the role of Nrf2 high expression during chemical and oxidative stress in develop a chemotherapy resistance and explore the Brusatol as an inhibiting factor to Nrf2 gives hope to enhance Cisplatin cytotoxic action and reverse its resistance potential.

Here we tried to demonstrate the brusatol efficacy on Cisplatin cytotoxici action by different doses in 4 UCC cell lines involved in MTT assay.

MTT assay result shows a significant reduction in cell viability on dose 10nM of Cisplatin and Brusatol when dosing together as compared to dosing of Cisplatin alone and Brusatol alone. This result may indicate that the Brusatol may enhance the cytotoxic effect of Cisplatin and help to overcoming the resistance of UCC to Cisplatin.

5. Conclusion

It was clear that there is a significant synergistic relationship between Brusatol and Cisplatin co-administration. Although the results obtained by BCA Assay support our hypothesis, it is not highly specific. The western immunoblot is required to strength and confirm our results which indicate that the induction of Brusatol with Cisplatin had a significant statistically efficacy on UCC Cell Lines than Cisplatin only dosing. This experiment still need more work including western immunoblot and may use more UCC Cell Line may MGH-U3.

6. Future Work

May it will be more helpful if this project become more extended by study the effect of Brusatol (an NRF-2 inhibitor) using more UCC Cell Lines

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