INTRODUCTION

Despite the advancement in cancer therapy, bladder cancer remains one of the major challenges leading to worldwide cancer-related death [1]. The initial treatment of non-muscle-invasive transitional cell carcinoma of the bladder generally is a complete cystoscopic resection of all visible tumour followed by adjuvant intravesical therapy [2]. Mitomycin C (MMC) is one of the most commonly used intravesical chemotherapeutic agents although its clinical effectiveness is often limited by high rate of recurrence due to the emergence of drug-resistant tumour cells [3-4]. So, multiple approaches have been tried to improve efficacy and decrease recurrence including increasing the drug concentration and extending the dwell time [5].

Sufficient body of evidence has recognized the association between bladder cancer, as well as resistance to chemotherapy, and the production of abnormal signal transducer Ras protein [6]. This association has been early reported in T24 bladder cancer cell line where the first human oncogene was identified [7]. Mutations in RAS genes can direct the expression of constitutively active Ras proteins which promote the activation of the immediate effector Raf kinase family. Once activated, Raf initiates the sequential downstream...
phosphorylation of MEK and ERK which finally interact with transcription factors responsible for cell cycle progression and proliferation. Locked in the GTP-bound mode, the abnormally activated Ras protein triggers incessant activation of the Raf/MEK/ERK cascade leading to uninterrupted cell division and carcinogenesis [8-9]. Overexpression of at least 1 of the 3 canonical Ras proteins was found in 77% of analyzed bladder tumours [10] while even a single nucleotide polymorphism in the H-Ras locus was associated with development of more aggressive types of bladder cancer [11]. Ras inhibition was employed as a treatment strategy to control cell proliferation, to induce apoptosis and to increase the sensitivity of tumour cells to different tumour cell-killing agents. The inhibition was targeted at many levels of the Ras/MAPK signaling circuit [12-13].

Curcumin (CUR) is the principal polyphenolic compound (Fig 1) isolated from the powdered rhizome of turmeric (Curcuma longa L.), a member of the ginger family. Although several research point at curcumin as having wide range of pharmacological activities but clinical studies have yielded frustrating results due to its chemical instability and poor oral bioavailability [14], however, recent reports demonstrated promising anti-tumour effects of curcumin against variable types of cancer cells in vitro [15]. The precise target(s) of curcumin action have remained elusive with some reports described its interaction with several signal transduction pathways including NF-kB, AKT, Ras/MAPK, p53, JAK/STAT and AMPK [16]. Recent observation has also shown that modulation of cell signaling pathways through the pleiotropic effects of curcumin likely activate cell death signals and induce apoptosis predominantly in Ras-activated tumour cells [17]. On the other hand, we have recently shown that silencing the RAS oncogene by small interfering RNA (siRNA) reversed the resistance of T24 bladder cancer cells to MMC and induced apoptosis when exposed to small doses of MMC (0.3 to 10 µg/ml) [18]. Based on this finding and other related experiments demonstrating that inhibition of Ras decreased resistance and invasiveness of cancer cells in vitro, if curcumin can disrupt the Ras/Raf/MEK/ERK signaling pathway, it should reverse Ras-dependent resistance of cancer cells to chemotherapeutic agents. Therefore, we aimed at investigating the combined effect of curcumin with small dose of MMC on the Ras/MAPK-dependent apoptosis in T24 bladder cancer cells which were reported earlier to be naturally resistant to MMC [19]. We hypothesise that curcumin may induce synergistic tumor cell killing with MMC comparable to that previously observed with Ras siRNA, consequently, curcumin might be elected as a safe and available intravesical adjuvant with MMC in clinical trials.

**MATERIALS AND METHODS**

**Cell culture**

Human bladder cancer cell line T24, obtained from the European Collection of Authenticated Cell Cultures (ECACC), were grown in confluent monolayers with McCoy’s 5A medium (Sigma-Aldrich Co. Ltd., Poole, Dorset, England) supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin and 100 µg/ml streptomycin, and maintained at 37 °C with 5% CO2. Under these conditions the doubling time was 9–10 h and cell density of 60–70% confluency on the day of experiments. Individual cell suspensions were obtained by trypsinization (0.25% trypsin-EDTA) of adherent monolayer.

**Treatment protocols**

Human pan-Ras siRNA targeting all isoforms of Ras and the non-targeting negative control siRNA were made up according to manufacturer’s instructions (siIMPORTER transfection kit, Upstate Biotechnology Inc., VA, USA). Curcumin (Sigma–Aldrich, St. Louis, MO, USA) was prepared by dissolving the pure powder (≥ 80% purity) with dimethyl sulfoxide (DMSO) then with 100% ethanol. Subsequently, a series of diluted concentrations was made up from 0 to 20 µM. T24 cells were divided into 5 groups according to treatment protocol – group 1 cells were left as blank control (without any drug treatment). Group 2 were cells exposed only to 10 µg/ml MMC (Kyowa Hakko UK Ltd, England) for 1 h then washed and incubated with drug free media for 72 h. Group 3 were cells cultured in media containing different concentrations of curcumin (5, 10, 20 µM). Group 4 were cells exposed to 10 µg/ml MMC for 1h then washed and further transfected with 50 nM of pan-Ras siRNA and incubated in
serum free medium for 4h at 37 °C then FCS was added and cells were incubated for 72h. Group 5 were cells exposed to MMC for 1 h then washed and incubated for 72 h with new media containing the previous concentrations of curcumin. A full set of controls were used to detect false negative and false positive results. The final concentration of DMSO for all experiments was maintained at less than 0.1%. These concentrations were previously reported to be non-cytotoxic for 72 h [17].

**Cell proliferation assay**

The anti-proliferative potential of treatments was assessed by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction test according to the manufacturer’s instructions (Abnova, USA). Cells were seeded at 5 × 10³ cell density in a 96-well tissue culture plate and left to attach for 24 h at 37 °C with 5% CO2 then fresh media containing different drug treatments were added and incubated for 72 h. Four h prior to the end of the 72 h incubation period with treatments, 20 µl of MTT solution (5 mg/ml PBS) was added to each well and incubated at 37 °C to the end of the 72 h. After 4 h, all media were removed and the insoluble purple formazan crystals were dissolved by adding 200 µl DMSO to each well and incubated in the dark for additional 30 min. The absorbance was read at 590 nm using a spectrophotometric plate reader. The relative percentage of viable cells was calculated from triplicate measurements using the background-corrected absorbance as follows [20]: % viable cells=(Mean absorbance of test samples)/(Mean absorbance of negative controls) x 100

**Assessment of apoptosis and cell cycle analysis**

Tumour cells were fixed in 70% alcohol for 1h and stained with Giemsa and H&E. Architectural features described the overall arrangement of the cell sheets and the extent of cell loss, mitotic activity, apoptotic count, and the most prevalent apoptotic stage were assessed morphologically on a set of three slides using the X40, X100 and X400 objectives on a Leica DM2500 microscope (Leica, Germany) equipped with high resolution DC300 Leica camera. Progression of cells through the cell cycle and cell apoptosis were measured by propidium iodide (PI)/fluorescence-activated cell analysis of subG0/G1 DNA content as follows – after fixation in 70% alcohol for 1h, cells were washed and suspended in 1 ml of fluorescent probe solution containing PBS, 1% Triton X-100, 50 µg/ml PI and 0.5 mg/ml RNase for 30 min in the dark at room temperature. Ten thousands events were acquired using a Coulter EPICS Elite flow cytometer (Beckman Coulter, FL, USA) and cells were discriminated according to levels of red fluorescence collected via 610 nm long band pass filter. DNA histograms were analyzed using WinCycle version 3.0 (Phoenix Flow Systems, San Diego, USA).

**Immunocytochemistry (ICC)**

Alcohol-fixed cells were washed prior to immunostaining using 5 µg/ml of anti-pan-Ras (clone Ras10, Upstate, VA, USA) and 1 µg/ml of mouse polyclonal ERK which reacts with both ERK1/2 (Santa-Cruz Biotechnology, Santa-Cruz, CA) or matched isotypes IgG2a (Ras) and IgG1 (ERK) at equivalent concentrations diluted in PBS with 0.04% Tween 80 (PBST). Immunoreactivity was visualised using HRP Mouse (DAB+) EnVision Kit (Dako). Computer-aided quantification of ICC staining was performed based on the principle described before [21] with slight modification [22]. Briefly, digital images were acquired at X100 using Leica digital camera and analyzed with VideoTesT-Morphology software (Russia). Color sampling representing Ras and ERK1/2 staining was selected and the number of selected pixels was read from the histogram of colours and their percentage per section was calculated. Ten random images from each slide were analyzed and averaged.

**Western blotting**

After trypsinization and centrifugation, washed cell pellets were lysed for 15 min on ice using 300 µl of Celllytic-M mammalian reagent (Sigma-Aldrich, UK) followed by centrifugation at 15,000 x g for 20 min at 4 °C. Soluble protein concentration in the supernatant was determined using Pierce BCA Protein Assay kit and instructions (Thermo Scientific, USA). Aliquots of 15 µg of denaturated and reduced protein were resolved on NuPAGE Novex 4–12% Bis-Tris Gels (Invitrogen, USA) by SDS-PAGE prior to transfer onto nitrocellulose membrane (Bio-Rad Lab., USA). After incubation in blocking buffer (5% non-fat milk in TBST) for 2 h at room temperature, membranes were incubated overnight at 4 °C with mouse monoclonal antibodies (Santa Cruz Bio-tech., Santa Cruz, CA, USA) against pan-Ras, pERK1/2,
and β-actin followed by incubation with horseradish peroxidase-conjugated secondary anti-mouse IgG (1:1000; Santa-Cruz) for 1 h at room temperature. After three washes in PBS with 0.1% Tween-20, Images were digitized using ViewPix 700 gel scanner and analyzed by TotalLab software (TotalLab Ltd. UK).

Statistical analysis
The statistical analysis was performed using SPSS 15.0 (SPSS Inc., Chicago, IL, USA). Data were quantified from triplicate measurements and were represented as mean ± SD. Multiple comparison between means was performed using the one-way analysis of variance (ANOVA) followed by Tukey post hoc test. A P-value of less than 0.05 was considered statistically significant.

RESULTS
T24 cells sensitivity after single MMC or curcumin treatment
Untreated T24 cells grew in irregularly dispersed sheets containing cells of varying size and shape. As expected, MMC treatment caused significant architectural disruption, increase in apoptotic bodies, and reduction of mitotic indices observed after 24 h and reached the maximum after 72 h. There was no significant changes in cells grew in 5 µM of curcumin while there were progressive architectural disruption, increase in apoptotic bodies, and reduction of mitotic indices started to show with 10 and 20 µM curcumin after 72 h as compared with non-treated cells (P<0.05 and P<0.01 respectively, Fig 2). Cell proliferation assay revealed 38% reduction of cell viability after exposure to 10 µg/ml MMC. There was also dose-dependent reduction of cell viability with 10 and 20 µM curcumin alone (P<0.05, Fig 3).

Flow cytometric analysis revealed 4.0 fold increase in the mean subGo/G1 events after 72 h in MMC-treated cells as compared with blank controls (32.1 ± 5.12 compared with 7.84 ± 2.15 in non-treated cells, P < 0.01, Fig 4). There was 1.4 fold increase in the mean subGo/G1 events after 72 h in 20 µM curcumin-treated cells (15.32 ± 3.52 compared with 7.84 ± 2.15 in non-treated cells, P < 0.05, Fig 4).

As analyzed with computer image analysis, the intensity fraction of Ras and ERK1/2 immunostaining decreased progressively after 72 h from 26.78% ± 4.17 and 22.38% ± 3.65 in non-treated cells to 17.49% ± 3.42 and 14.81% ± 3.02 respectively after MMC treatment (P < 0.01 and P<0.05 respectively). Likewise, both Ras and ERK1/2 immunostaining were significantly reduced with 10 and 20 µM, but not with 7.84 ± 2.15 in non-treated cells, P < 0.05, Fig 4).
up to 5 µM, of curcumin (P < 0.05, Fig 5 and 6). Western blot analysis revealed significant reduction of Ras and phosphorylated ERK1/2 expression in MMC and curcumin (10 and 20 µM)-treated cells as compared with non-treated controls (Fig 7).

**T24 cells sensitivity after combined treatments**

Morphological examination of Ras siRNA transfected T24 cells were compared to other control slides for better characterization of the effect of Ras inhibition on cells phenotype. Due to the significant cell death induced in T24 cells following the individual use of high concentrations of Ras siRNA observed in our previous study [18], low dose of Ras siRNA (50 nM) was used with MMC in the synergy experiment. As shown in Fig 2, at the end of 72 h culture time, the morphological picture of T24 cells treated with 10 µM of MMC in synergy with 50 nM Ras siRNA showed greater architectural disruption, increased apoptotic bodies, and reduced mitotic indices than those exposed to MMC alone. The overall morphological picture of T24 cells treated with combined MMC and 20 µM of curcumin, including significant architectural

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**Fig 3.** Cell viability percentage as assessed by MTT assay after 72 h culture with different treatments. The maximum inhibition was observed with combined MMC 10 µg/ml and 50 nM Ras siRNA. A dose-dependent inhibition of cell viability was observed with CUR treatments. Combination of MMC plus CUR exerted significant inhibition as compared with MMC alone. Significance levels: *P<0.05; **P<0.01; ***P<0.001 vs untreated control. #P<0.05 vs MMC alone (ANOVA of triplicate samples).

**Fig 4.** Flow cytometric illustrations of SubG0/G1 events in T24 cells after 72 h culture with different treatments. (A) Untreated T24 cells; (B) Cells treated with 10 µg/ml MMC; (C, D, E) Cells treated with 5, 10, and 20 µM CUR respectively; (E, F, G) Cells treated with 10 µg/ml MMC for 1 h followed by culture with 5, 10, and 20 µM CUR respectively for 72 h. The DNA histograms illustrate an increase in percentage of subG1 events after MMC treatment. There is also dose-dependent increase of subG1 events in response to solitary CUR treatment. The apoptotic events are increased dose-dependently in cells treated with binary MMC and CUR treatment which are significantly higher than with MMC alone. Error bars are the 95% confidence limits of triplicate results. Significance levels: *P<0.05; **P<0.01; ***P<0.001 vs untreated control.
disruption, number of apoptotic bodies and reduction of mitotic index, was comparable to this seen in cells treated with MMC and further knocked down with 50 nM Ras siRNA (Fig 2). Slight, but significant, changes were observed when 10 µM of curcumin was used, while lower concentrations of curcumin (5 µM) did not show synergistic effects.

Cell viability/cytotoxicity assessed with MTT assay showed greater reduction of cell proliferation/viability (68%, P<0.001) after transfection with 50 nM Ras siRNA following MMC exposure. Significant inhibition was also observed in cells treated with combined MMC and 20 µM curcumin as compared with MMC alone (58%, P<0.001 vs control; P<0.05 vs MMC alone, Fig 3). In addition, the conjoined treatment with 10 µg/ml MMC and 50 nM Ras siRNA provoked significant increase of subG/G1 events when compared to the number induced by single treatment with MMC alone (49.6 ± 5.23 compared with 32.1 ± 5.12, P<0.05). There was significant dose-dependent increase in subG/G1 events with 5, 10 and 20 µM curcumin when combined with MMC as compared with MMC alone (P<0.05, P<0.01, P<0.001 respectively, Fig 4).

The decrease of the intensity of Ras and ERK1/2 immunoreactivity recognized by ICC image analysis after combined MMC and Ras siRNA treatment was significantly greater than that observed after MMC treatment alone (Fig 5 and 6). Ras and ERK1/2 immunostaining fraction decreased progressively after 72 h from 17.49% ± 3.42 and 14.81% ± 3.02 in MMC-treated cells to 9.64 ± 2.37 and 7.44 ± 2.41 respectively (P<0.05 for both) in cells treated with combined MMC and 50 nM Ras siRNA. Likewise, the decrease of the intensity of Ras and ERK immunostaining recognized by IHC image analysis after combined MMC and curcumin (10 and 20 µM) treatments were significantly greater than that observed after MMC treatment alone (Fig 5 and 6). Ras and ERK1/2 immunostaining showed progressive decrease with the maximum inhibition seen with 20 µM dose of curcumin (10.31 ± 2.42 and 8.37 ± 2.11 respectively in MMC plus curcumin compared to 17.49% ± 3.42 and 14.81% ± 3.02 in MMC alone, P<0.05 for both). Western blot studies also confirmed these results (Fig 7).

DISCUSSION

Transurethral resection followed by intravesical installation of chemotherapy remains the preferred choice in the management of non-muscle invasive bladder cancer [2]. MMC is an anticancer drug that is used intravesically in the management of bladder cancer. However, tumor cell resistance to this agent remains the main obstacle in successful cancer therapy, therefore, novel therapeutic combination treatments are required to improve chemoresponsiveness. The tumorigenic role of Ras/MAPK pathway in T24 cells can afford key information to our understanding as to why 30-60% of bladder tumors expressing mutated Ras protein have...
tendency to develop tumor invasiveness and resistance [23]. It is now established that activated Ras-GTP triggers mitogenic signaling and ultimately leading to activation of transcriptional ERKs [24]. The activated Ras/Raf/ERK-signaling pathway creates a robust proliferative stimulus that leads to uncontrolled cell division [25-26]. In a previous study [18], we have shown that knocking down the Ras oncogene resulted in enhanced MMC-induced chemosensitivity and apoptosis in T24 cells which has been previously reported to be MMC-resistant [19].

Conclusion of synergism between drug combinations should be investigated carefully. Ideal synergism should involve positive interaction between the two drugs on one common target by exerting different mechanisms of action.

Alkylation of DNA is the most favoured mechanism of action for MMC, but other modes of action, such as inhibition of rRNA and redox cycle interference, may also contribute to the biological action of the drug [27]. The understanding of the appropriate dose and duration of MMC exposure was acquired from our previous study which proved that MMC treatment using low concentrations over 72 h was the best combination regimen with Ras siRNA. This pre-rationale assessment strategy meant that the siRNA treatment protocol was based on a substantial background and information of how the proteins worked in response to individual agents. This was particularly important because the “hit” of the second therapy could be scheduled to coincide with the lowest expression of the target. Data presented herein have shown that T24 cells express more Ras, supporting previous reports that Ras expression was associated with tumor cell resistance [28]. We observed that both Ras and ERK1/2 immunoreactivity in T24 cells was decreased 72h after treatment with MMC, and this could be perceived in light of depletion of the protein synthesis capacity in T24 cells after 72h. These data suggest that Ras siRNA would be most effective 72h after treatment with low concentrations of MMC.

In our previous study [18], we used MMC in low and high concentrations (up to 100 μg/ml) in combination with two different concentrations of Ras siRNA, and we found that Ras siRNA sensitized T24 cells to apoptosis induced by...
MMC at concentration ranges from 0.3 to 10 μg/ml which proved previously to be insufficient to induce significant cytotoxicity. In this study, we used only the 10 μg/ml as a standard concentration reported to possess the median cytotoxic effect. We used Ras siRNA to break the resistance of T24 cells to MMC through inhibition of Ras-dependent resistance. Combination between MMC and Ras siRNA was employed as a reference synergy experiment.

Several studies have pointed at the proficiency of the natural compound curcumin to have antitumor activity and enhance chemosensitivity of cancer cells to cytotoxic compounds. For instance, curcumin reverses cisplatin resistance in cervical cancer cells [29], breast cancer cells [30], and reduces multidrug resistance in human colon cancer cells [31]. However, the exact mechanism and/or molecular targets of curcumin remain elusive. So, we hypothesized that curcumin might act through the inhibition of the Ras/MAPK pathway, and accordingly, could be matched with Ras siRNA as combination strategy with MMC on T24 cells. The data presented herein demonstrate that curcumin at concentrations of 10 and 20 μM, alone, exerted antitumor activity against T24 cells as shown from decreased cellularity, inhibited proliferation and increased apoptotic signals in a dose-dependent manner. Likewise, curcumin reversed resistance of these cells to 10 μg/ml MMC as did specific knocking down the Ras oncogene with Ras siRNA. Curcumin-treated cells showed reduced Ras protein expression along with reduced both ERK1/2 and its phosphorylated form, the two canonical proteins of the MAPK pathway. This reduction of crucial MAPK proteins coincided with significant morphological architectural disintegration and increased frequency of subG0/G1 apoptotic events, so, at this point we can confirm that curcumin-related cytotoxicity is, at least partially, dependent on inhibition of the Ras/MAPK signaling pathway.

ERK is the straight downstream MAPK effector responsible for cell resistance to apoptosis, and therefore acts as indicator of Ras activation and expression levels [32]. To further study the effects of combined MMC and Ras siRNA knockdown, a set of experiments employing antibodies against the two isozymes ERK1/2 were considered. Several studies have shown that Ras activates a number of signal transduction pathways, including the Raf/MEK kinase pathway [33]. In the present study, 10 and 20 micromolar concentrations of curcumin, either alone or in combination with MMC decreased immunoreactivity of ERK1/2 and their phosphorylated forms together with increased apoptosis as did combination of 50 nM Ras siRNA with MMC. Meanwhile, MMC alone was associated with only minor apoptosis. Hence the combination of MMC with curcumin was synergistic and achieved the utmost inhibition of Ras synthesis and ERK expression. The combination of DNA alkylation by MMC and withdrawal of proliferation factors by curcumin achieved synergistic effect through targeting different pathways of apoptotic resistance. This finding highlights the necessity to investigate combination therapies that interfere with two pathways or intended to hit one pathway and its linking feedback loop.

In conclusion, this study indicates that resistance of T24 cells to cytotoxic effect of MMC is dependent, at least partially, on Ras/ERK activation. We have demonstrated that curcumin at concentrations of 10 and 20 μM in combination with low dose MMC induced toxic synergism in T24 cells. Clinical translation of this experimental study may be reasonable in light of wide safety margin and availability of curcumin which elect it as a safe and valuable intravesical adjuvant with MMC in management of superficial bladder cancers.

Conflict of interest

The authors declare that they have no conflict of interest.

Funding

This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

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