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Original article

Targeting the anaphase-promoting complex/cyclosome (APC/C) enhanced antiproliferative and apoptotic response in bladder cancer

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ABSTRACT

Improving the chemotherapy sensitivity of bladder cancer is a current clinical challenge. It is critical to seek out effective combination therapies that include low doses of cisplatin due to its dose-limiting toxicity. This study aims to investigate the cytotoxic effects of the combination therapy including proTAME, a small molecule inhibitor, targeting Cdc-20 and to determine the expression levels of several APC/C pathway-related genes that may play a role in the chemotherapy response of RT-4 (bladder cancer) and ARPE-19 (normal epithelial) cells. The IC20 and IC50 values were determined by MTS assay. The expression levels of apoptosis-associated (*Bax* and *Bcl-2)* and APC/C-associated *(Cdc-20*, *Cyclin-B1*, *Securin,* and *Cdh-1)* genes were assessed by qRT-PCR. Cell colonization ability and apoptosis were examined by clonogenic survival experiment and Annexin V/PI staining, respectively. Low-dose combination therapy showed a superior inhibition effect on RT-4 cells by increasing cell death and inhibiting colony formation. Triple-agent combination therapy further increased the percentage of late apoptotic and necrotic cells compared to the doublet-therapy with gemcitabine and cisplatin. ProTAME-containing combination therapies resulted in an elevation in Bax/Bcl-2 ratio in RT-4 cells, while a significant decrease was observed in proTAME-treated ARPE-19 cells. Cdc-20 expression in proTAME combined treatment groups were found to be decreased compared to their control groups. Low-dose triple-agent combination induced cytotoxicity and apoptosis in RT-4 cells effectively. It is essential to evaluate the role of APC/C pathway-associated potential biomarkers as therapeutic targets and define new combination therapy regimens to achieve improved tolerability in bladder cancer patients in the future.

1. Introduction

Bladder cancer is one of the major causes of morbidity and mortality, with approximately 550,000 newly diagnosed patients per year worldwide (Richters et al., 2020). Tumor recurrence is observed in > 50 % of the patients, and drug resistance is one of the main reasons hindering the effect of treatments. Rapid progression in cancer drug development has led to many pre-clinical studies and clinical trials in bladder cancer treatment. Combination chemotherapies containing gemcitabine with cisplatin (GC) or methotrexate, vinblastine, doxorubicin and cisplatin (MVAC) have been used as standard treatments for advanced or metastatic bladder cancer (Milowsky et al., 2016). Many systemic chemotherapies continue to be evaluated in clinical trials to identify the efficient combination for bladder cancer patients. There is no robust prospective or randomized evidence to suggest the use of non-cisplatin-based neoadjuvant therapy in patients according to the National Comprehensive Cancer Network (NCCN) guidelines (Thomas et al., 2020). Split-dose cisplatin is suggested as a safer alternative for patients with borderline/minimal renal dysfunction., although there is still debate about its relative efficacy and optimal schedule (Philippe et al., 2017). Therefore, the current approach to bladder cancer treatment necessitates the development of new and effective therapeutic strategies.

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Anaphase promoting complex/cyclosome (APC/C) is a multisubunit enzyme that ubiquitinates various substrates for ubiquitination and plays a regulatory role in many events including regulation of cell cycle, stability of genome, energy metabolism, apoptosis and carcinogenesis (Zhou et al., 2016). Activation of the APC/C complex is mainly controlled by the Cdh-1 and Cdc-20 proteins. Despite having comparable structural similarities, the APC/C complex is activated by Cdc-20 and Cdh-1 at different stages. Early in mitosis, Cdc-20 binds to the APC/C complex and induces the degradation of many mitotic substrates. Following this, Cdh-1 is replaced by Cdc-20 in the middle of the anaphase and serves until the G1 phase (Zhou et al., 2016).

Spindle assembly checkpoint (SAC) catalyzes the assembly of mitotic checkpoint complex (MCC) containing Mad2/BubR1, Bub3 and Cdc-20 proteins. Inhibition of APC/C activation by MCC is required for SAC and MCC which prevents Cdc-20 from interacting with APC/C so that the cycle does not progress to anaphase in the absence of kinetochore signal (Izawa & Pines, 2015). When the checkpoint signal is deactivated, the MCC complex separates from the APC/C^{Cdc-20} and initiates the metaphase-anaphase transition as well as the degradation of Securin and Cyclin-B1 (Kim & Yu, 2011). The degradation of Securin and Cyclin-B1 activates the Separase that breaks down the Cohesin complex (Wang & Dai, 2005). These reactions are inhibited and the of the cell from metaphase to anaphase is blocked by pharmacological inhibition or siRNA depletion of Cdc-20, as well as genetic damage (Huang et al., 2009; Manchado et al., 2010; Zeng et al., 2010). It is critical for cellular homeostasis to maintain an intracellular balance of Cdc-20 levels. Excessive Cdc-20 expression has been observed in oral squamous carcinomas and breast cancers resulting in premature anaphase and tumor formation associated with aneuploidy (Mondal et al., 2007; Yuan et al., 2006). However, no study has yet investigated the effect of combining proTAME, an inhibitor that inhibits Cdc-20 which is a potential therapeutic target candidate, with agents used in the standard therapy of bladder cancer. The purpose of this study was to investigate the cytotoxic and cell death inducing effects of cisplatin and gemcitabine as single drug treatments and in combination with proTAME in RT-4 and ARPE-19 cells, and whether Cdc-20 and its accompanying proteins can be used as biomarkers associated with therapeutic response in bladder cancer in the presence of proTAME.

2. Materials and Methods

2.1. Culture of cells

ARPE-19 (retinal epithelial normal cell) and the human bladder cancer cell line, RT-4 (ACC 412) (recurrent, differentiated transitional papillary bladder tumor, clinical stage T2, histological grade G1) were cultured using RPMI 1640 medium (Thermo Fisher) supplemented with 10 % FBS (Hyclone, GE Healthcare Life Sciences, Logan, UT, USA) at 37 °C in the presence of 5 % CO_2 in a cell culture incubator. RT-4 and ARPE-19 cells were treated with combinations of cisplatin and gemcitabine (CIS + GEM), cisplatin and proTAME (CIS + PT), gemcitabine and proTAME (GEM + PT), and cisplatin, gemcitabine and proTAME (GEM + CIS + PT) as well as single agent treatments for 48 h.

2.2. Cell growth inhibition assay

Cell seeding was performed in 200 µl culture media per well into 96-well plates by seeding density of $1x10^4$ cells for RT-4, and $2x10^4$ cells for ARPE-19). The cells were incubated overnight prior to addition of cisplatin (Kocak Pharmaceutical, Istanbul, Turkey), gemcitabine (Kocak Pharmaceutical, Istanbul, Turkey) and proTAME (Provided as a 20 mM stock solution in DMSO) (I-440–01 M, Bio-Techne R&D Systems, USA). Varying concentrations of cisplatin (1–30 µM), gemcitabine (50–5000 nM) and proTAME (3–36 µM) were applied to the cells. DMSO controls were prepared along with different concentrations of proTAME. The proliferation of cells was analyzed by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy methoxyphenyl)-2-(4-sulfophenyl)–2H-tetrazolium (MTS) method. MTS chemical (CellTiter 96 Aqueous MTS Reagent, Promega) was prepared by dissolving 4.2 mg/ml and 0.92 mg/ml phenazine methosulphate (PMS, Serva, Heidelberg, Germany) in DPBS without Mg^{+2} and Ca^{+2} (Capricorn Scientific, GmbH, Germany). The media was removed before adding 100 µl of medium/MTS/PMS mixture into each well. After incubation at 37 °C, Multiskan GO microplate reader (Thermo Fisher, USA) was used to measure the absorbance at wavelength of 492 nM. IC50 values were calculated using IC50 toolkit (ic50.tk).

2.3. Examination of cell morphology by Giemsa staining

RT-4 cells were plated in 6-well plates ($3x10^5$ cells/well) and allowed to attach overnight. The morphology of the cells was assessed after 48 h of treatments with agents (cisplatin, gemcitabine, proTAME). After removing the media, the cells were gently washed three times with cold PBS. Cells were stained with Giemsa's azur eosin methylene blue solution (#109203, Merck, Darmstadt, Germany) after being fixed with methanol (Merck, Darmstadt, Germany). Following the washing steps, it was allowed to dry and visualized using an inverted microscope (Leica DM IL LED, Leica Microsystems).

2.4. Apoptosis staining

RT-4 and ARPE-19 cells were grown on round coverslips placed in 12-well plates and allowed to adhere for 16 h. The cells were incubated for 48 h either untreated or with chemotherapeutic agents with or without proTAME. The cell culture media was carefully removed, and the cells were washed three times with cold PBS. Annexin V-FITC Kit (Abcam, cat no: ab14085) was used to stain the cells according to the manufacturer's protocol. A camera (DFC425, Leica) attached fluorescence microscope (Leica LM 4000B) was then used to examine the cells.

2.5. Clonogenic survival assay

RT-4 cells (250 cells/well) and ARPE-19 cells (500 cells/well) were seeded in a 6-well plate in 3 ml RPMI medium supplemented with 10 % FBS to assess clonogenic survival. After 24 h, IC20, IC50 and combination doses of cisplatin, gemcitabine, proTAME were added. Incubation was continued for RT-4 (10 days) and ARPE-19 (14 days) cells. Methanol fixed colonies were stained with Giemsa solution (#109203), and destained in distilled water. Image J software, cell counter tool (National Institute of Health, Bethesda USA) was used to count colonies (>50 cells).

2.6. Quantitative Real-Time polymerase chain reaction (qRT-PCR)

Total RNA was isolated using a kit (Macharey-Nagel, Germany). Total RNA concentration was determined using µdrop plates on Thermo Multiskan Go (Thermo Fisher Scientific, USA). High-Capacity cDNA Synthesis Kit was used to generate cDNA (Applied Biosystems, USA) from total RNA (1000 ng RNA/20 µl reaction). qRT-PCR was performed in a 96-well optical plate using LightCycler 480 Probes Master in Roche Lightcycler 480 II and diluted using nuclease free water. 30 ng of cDNA was used in each qRT-PCR reaction (20 µl) included in 1 µl. The following primers and probes were purchased from Roche (Mannheim, Germany);

Cdc-20 (CDC20, cat:102870), Cyclin-B1 (CCNB1, cat:101373), Cdh-1 (FZR1, cat:127122), Securin (PTTG1, cat:111161), Bax (BCL2L4, cat:142318), Bcl-2 (BCL2, cat:100083). Details of the *GAPDH* gene are as follows; forward primer (5- GAAGGTGAAGGTCGGAGTC-3′, reverse primer 5′-GAAGATGGTGATGGGATTTC-3′, GAPDH YAK (YAK-CAAGCTTCCCGTTCTCAGCCT-BBQ) (TIB MOLBIOL, Germany). *GAPDH* gene expression was used as a reference to normalize results. The $2^{-\Delta\Delta Ct}$ method was used to calculate relative gene expression.

2.7. Statistical analysis

To determine the significance of the difference between the two groups, a two-tailed Student's *t*-test was used. The one-way analysis of variance (ANOVA) is used to determine whether there are any statistically significant differences between the means of three or more groups. Heat-map was created using GraphPad Prism software (San Diego, USA) was used for all analyses. $p < 0.05$ was considered significant.

3. Results

3.1. ProTAME causes a decrease in cell viability in a dose-dependent manner

MTS assay was used to assess the cytotoxic effects of gemcitabine, cisplatin, and proTAME on RT-4 and ARPE-19 cells after 24 and 48 h. Since 50 % growth inhibition in RT-4 cells was reached at the tested doses of gemcitabine (50–5000 nM), cisplatin (1–30 μM) and proTAME (3–36 μM) at very high doses after 24 h incubation, the incubation was continued for 48 h (24 h data not shown). At the end of 48 h incubation, high doses of gemcitabine (Fig. 1.A) and cisplatin (Fig. 1.B) resulted in significant cytotoxicity on both RT-4 and ARPE-19 cells. Gemcitabine and cisplatin treatments decreased the viability of cells more effectively in RT-4 cells than ARPE-19 cells. While ARPE-19 cells showed a more sensitive profile than RT-4 cells to proTAME at low doses (3–12 μM), similar levels of cytotoxicity results were obtained in both cells at high proTAME doses (24–36 μM) (Fig. 1.C). Cell cytotoxicity was observed in groups treated with proTAME and the chemotherapy agents dose-dependently. IC20 doses were determined to be 42 nM, 2.13 μM and 12 μM for gemcitabine, cisplatin and proTAME, respectively (Fig. 1.D). IC50 values for gemcitabine cisplatin and proTAME were 165 nM, 7.5 μM and 22 μM, respectively (Fig. 1.D). Apoptosis-related features such as cytoplasmic shrinkage, nuclear condensation, nuclear fragmentation, and apoptotic body formation were observed after treatments with increasing doses of gemcitabine, cisplatin and proTAME (Fig. 1.E).

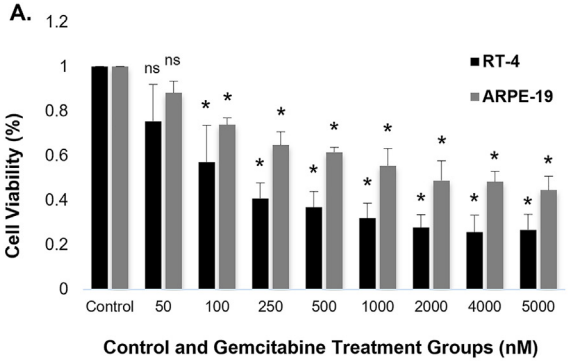
3.2. ProTAME-combined treatments exhibit potent inhibition in colony formation similar to chemotherapy doublet

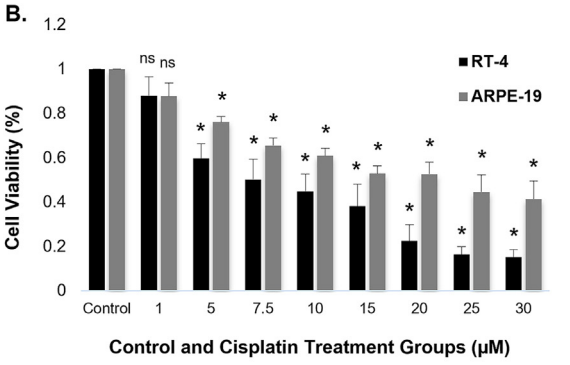
The proliferation and clonogenic survival ability of RT-4 and ARPE-19 cells after treatments was examined using a clonogenic survival experiment. Normal cell morphology and multiple colonies were seen in untreated and the DMSO control groups in RT-4 cells (Fig. 2.A). The number of colonies that survived was reduced in the DMSO control groups in ARPE-19 cells compared to the untreated control (Fig. 2.B). No significant number of survived colonies was observed in the groups treated with cisplatin, gemcitabine and proTAME individually at IC50 doses or in combination at IC20 doses, in both RT-4 and ARPE-19 cells. While there are a few viable cell populations in the cisplatin treatment group, significantly decreased number of cell colonies were observed in the proTAME-treated group and no colony formation was observed in the gemcitabine group at IC20 doses in RT-4 cells. The IC20 doses of gemcitabine and cisplatin determined for RT-4 cells inhibited cell proliferation and clonogenic survival more effectively than the proTAME IC20 dose. ProTAME and other chemotherapeutic agents significantly inhibited cell proliferation and colony formation at IC50 doses. It was also observed that cisplatin and gemcitabine therapy had a higher cytotoxic effect on ARPE-19 cells at IC20 doses than proTAME at IC20 doses. Doublet- and triple-agent combination therapies exhibited potent inhibition of cell colony formation.

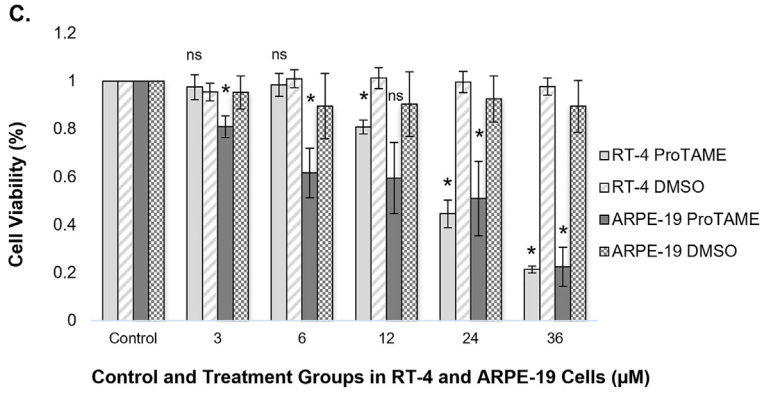
3.3. In RT-4 cells, ProTAME induced cell death in alone and combined therapy groups

Fluorescence microscopy was used to detect early apoptotic, late apoptotic and necrotic cells in the control group and different treatment groups in both RT-4 (Fig. 3) and ARPE-19 (Fig. 4) cells. A few positive cells were found for Annexin V and PI in the untreated control, DMSO-IC20, and DMSO-IC50 control groups in RT-4 cells. Significantly higher percentage of early apoptotic cells (Annexin V+/PI- cells) was observed in CIS-20, GEM-20, GEM-50, GEM + CIS, GEM + PT groups. The percentage of late apoptotic cells (Annexin V+/PI + cells) was found to be significantly lower in all groups compared to the percentage of both early apoptotic and necrotic cells (Annexin V-/PI + cells). The presence of necrotic cells was observed in all groups except the DMSO-20 control group. In RT-4 cells, treatments with IC20 and IC50 doses of gemcitabine, cisplatin and proTAME showed a decreased percentage of early apoptotic cells and an increase in necrotic cell percentage dose-dependently. The CIS + PT and GEM + CIS + PT groups had a higher percentage of late apoptotic and necrotic cells than the GEM + CIS combination. Similarly, in ARPE-19 cells, early apoptotic cell proportion was decreased. However, in combination therapy groups and with increasing doses of monotherapies, the percentages of late apoptotic and necrotic cells increased. When the results for RT-4 cells and ARPE-19 cells were compared, the number of early apoptotic cells in the GEM + CIS group in ARPE-19 cells was found to be low, but the number of necrotic cells was significantly higher. In RT-4 cells, an increase in necrotic cell proportion was observed in CIS + PT and GEM + CIS + PT groups; interestingly, it was found to be decreased in ARPE-19 cells.

The expression levels of Bax and Bcl-2, as well as the Bax/Bcl-2 ratio, were investigated to determine changes in the intrinsic apoptotic pathway in cells following treatments. Untreated ARPE-19 cells, had 3.3-fold ($p < 0.05$) higher Bax expression and 2.5-fold ($p > 0.05$) higher Bcl-2 expression compared to RT-4 cells (Supplementary Fig. 1.A). The expression level of *Bax*, a proapoptotic gene, in RT-4 cells increased significantly in the GEM + CIS and GEM + CIS + PT therapy groups. Furthermore, the levels of the anti-apoptotic gene *Bcl-2* increased in the CIS-50, GEM-50, GEM + CIS, and GEM + CIS + PT groups (Fig. 3.C). When the Bax/Bcl-2 ratio was examined, a significant level of induction of apoptosis was found in the GEM + CIS, GEM + CIS + PT and PT-50 groups compared to the control groups. The Bax/Bcl-2 ratio was found to be significantly lower in the CIS-50 and GEM-50 groups when compared to the control group. While there was no significant increase in Bax levels in any group of ARPE-19 cells, Bcl-2 levels increased 1.3–3-fold, especially in groups treated with proTAME alone and in combination compared to the control groups (Fig. 4.C). In ARPE-19 cells, Bax/Bcl-2 ratios were significantly increased in CIS-20, CIS-50 and GEM-20 compared to the control group, while PT-20, GEM + PT, GEM + CIS + PT and PT-50 groups showed a significant decrease in Bax/Bcl-2 ratios in comparisons with their control groups.

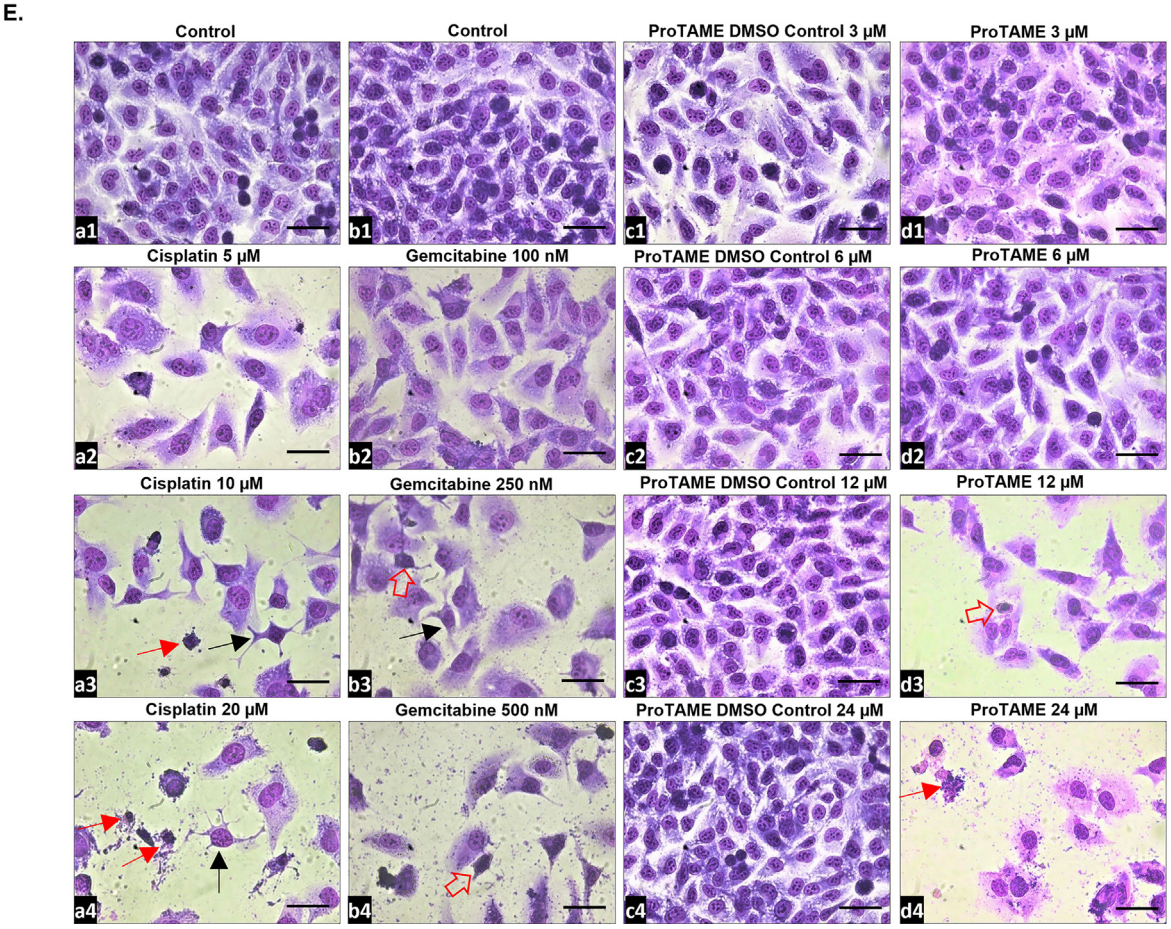






D.

Drugs and Inhibitor	IC_{20}	C_{50}
Gemcitabine	42 ± 4.1 nM	165 ± 14.2 nM
Cisplatin	2.13 ± 0.08 μM	7.5 ± 0.6 μM
ProTAME	12 ± 0.4 μM	22 ± 1.9 μM



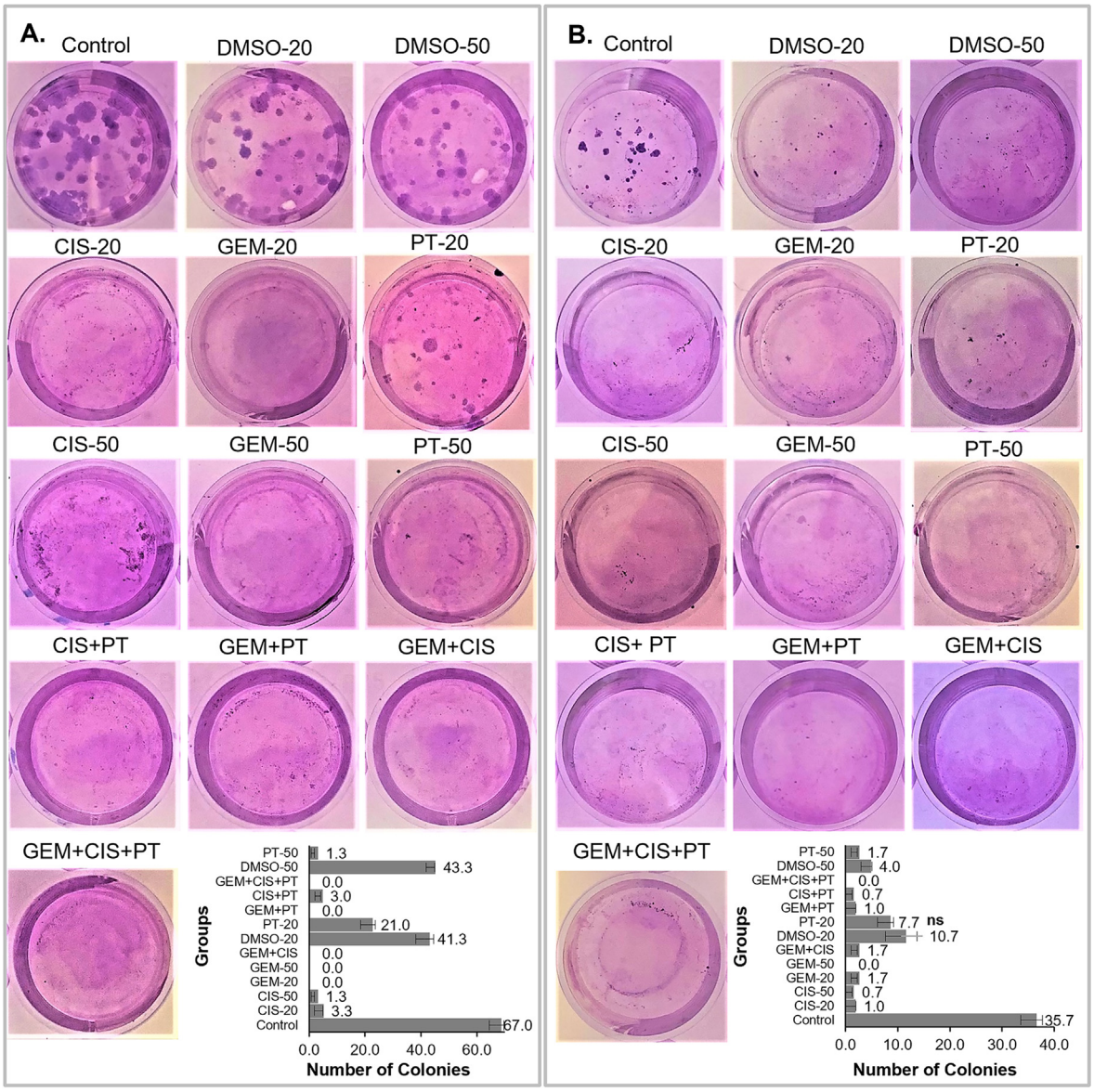
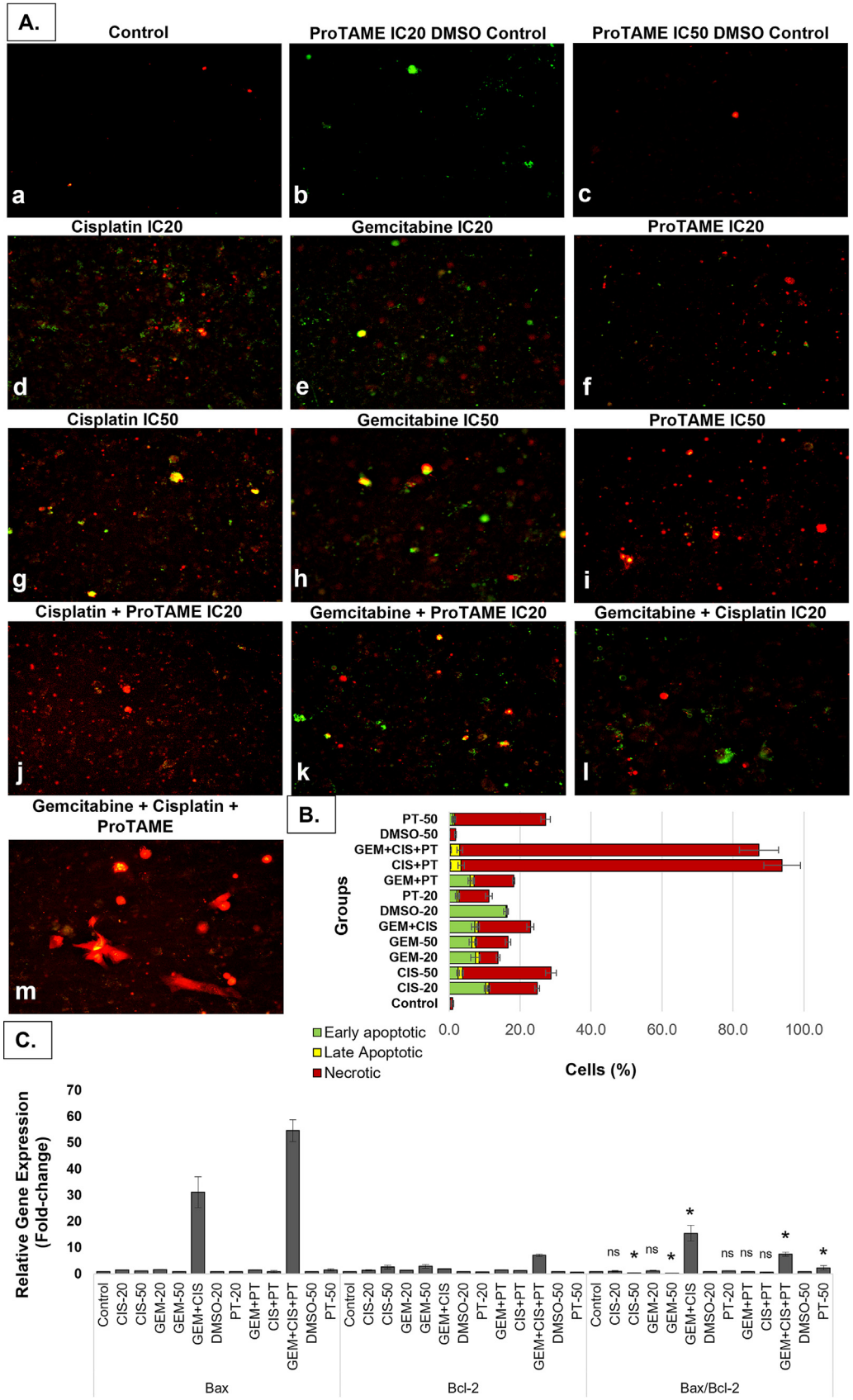


Fig. 2. Clonogenic assay in RT-4 (A.) and ARPE-19 (B.) cells treated with IC20 and IC50 concentrations of cisplatin, gemcitabine, proTAME. Cells were seeded onto 12-well plates at an initial plating density of 250 cells/well and 500 cells/well, for RT-4 and ARPE-19 cells, respectively. The drugs and proTAME doses were added to the wells at 24 h after cell seeding. The cells in the groups treated with or without drug and proTAME were washed with PBS after incubation (RT-4; 10 days and ARPE-19; 14 days), then stained with Giemsa solution following methanol fixation. Combination therapies included the IC20 doses of each drug/inhibitor tested. CIS: cisplatin, DMSO: Dimethyl sulfoxide, GEM: gemcitabine, PT: proTAME.

3.4. ProTAME caused changes in the expression of proteins associated with the APC/C complex

Changes in the expression of the APC/C pathway genes *Cdc-20*, *Cdh-1*, *Cyclin-B1*, and *Securin* were examined after 48 h of incubation in the control and different treatment groups (Fig. 5). When the untreated groups were compared, it was found that expression of Cdc-20 and Securin was considerably higher in ARPE-19 cells compared to RT-4 cells, while Cyclin-B1 was significantly lower and Cdh-1 levels did not differ between the two cell lines (Supplementary Fig. 1.B). Significantly decreased Cdc-20 levels were detected in RT-4 cells in both the PT-20 and PT-50 treatment

Fig. 1. Cytotoxic effects of gemcitabine, cisplatin and proTAME on RT-4 and ARPE-19 cells. (A.) Assessment of cell viability by MTS assay in RT-4 and ARPE-19 cells in the control group and groups treated with different doses of gemcitabine. **(B.)** Cell viability was determined by MTS assay in RT-4 and ARPE-19 cells in control groups and in groups treated with different cisplatin doses. **(C.)** Determination of cell viability by MTS assay in RT-4 and ARPE-19 cells in control, DMSO control groups and groups treated with different doses of proTAME. Statistical analyzes for proTAME were performed and each group was compared with its own DMSO control group. **(D.)** IC50 doses determined for drugs and proTAME tested for their effect on viability of RT-4 cells. **(E.)** Morphological changes were observed with Giemsa staining in RT-4 cells after 48 h of incubation. Untreated control groups (a1, b1), cisplatin doses (a2, a3, a4), gemcitabine doses (b2, b3, b4), DMSO-treated groups corresponding to DMSO concentrations in which proTAME dissolves in (c1, c2, c3, c4) and groups treated with different doses of proTAME (d1, d2, d3, d4). Black arrow: cytoplasmic shrinkage (a3, a4, b3), red arrow (hollow): nuclear condensation (b3, b4, d3), red arrow (solid): nuclear fragmentation and formation of apoptotic bodies (a3, a4, b4). Scale bar: 30 μm. *<0.05, DMSO: Dimethyl sulfoxide, IC20: Inhibitory concentration 20, IC50: Inhibitory concentration 50, ns: non-significant.


A.
Control
ProTAME IC20 DMSO Control
ProTAME IC50 DMSO Control
a
b
c
Cisplatin IC20
Gemcitabine IC20
ProTAME IC20
d
e
f
Cisplatin IC50
Gemcitabine IC50
ProTAME IC50
g
h
i
Cisplatin + ProTAME IC20
Gemcitabine + ProTAME IC20
Gemcitabine + Cisplatin IC20
j
k
l
Gemcitabine + Cisplatin + ProTAME
m
B.
PT-50
DMSO-50
GEM+CIS+PT
CIS+PT
GEM+PT
PT-20
DMSO-20
GEM+CIS
GEM-50
GEM-20
CIS-50
CIS-20
Control
Groups
Early apoptotic
Late Apoptotic
Necrotic
0.0
20.0
40.0
60.0
80.0
100.0
Cells (%)
C.
Relative Gene Expression (Fold-change)
70
60
50
40
30
20
10
0
Control
CIS-20
CIS-50
GEM-20
GEM-50
GEM+CIS
DMSO-20
PT-20
GEM+PT
CIS+PT
GEM+CIS+PT
DMSO-50
PT-50
Bax
Bcl-2
Bax/Bcl-2
ns
*

groups compared to the DMSO controls (Fig. 5.A). However, the Cdc-20 level increased significantly in the CIS-20 and GEM-20 groups. While Cdc-20 levels increased dose-dependently in the cisplatin therapy groups, they did not increase dose-dependently in the gemcitabine therapy groups. While GEM + CIS from the combined treatment groups showed a dramatic rise in Cdc-20 expression, a decrease in Cdc-20 levels was observed in the groups combined with proTAME. The addition of proTAME to the triple combination therapy significantly reduced the high Cdc-20 expression found in the GEM + CIS combination. In ARPE-19 cells, however, there was no significant change in Cdc-20 expression levels. Cdh-1 expression levels in RT-4 cells increased significantly in all groups except CIS-20 and GEM-50 when compared to the control of each group (Fig. 5.B). No significant change was observed in Cdh-1 expression in ARPE-19 cells in the groups treated with proTAME alone and GEM + CIS compared to the control group. However, an increase in Cdh-1 levels was noted in the proTAME-containing combination groups. Cyclin-B1 expression levels in RT-4 cells increased significantly in all therapy groups except GEM-20 group. CIS-50, PT-20, GEM + CIS, CIS + PT and GEM + CI S + PT groups showed approximately 1.5–3 fold higher Cyclin-B1 expression compared to the controls of each treatment group (Fig. 5.C). No considerable increase in Cyclin-B1 expression fold-change was observed between groups in ARPE-19 cells. Securin expression levels showed a partial decrease in proTAME treatment groups compared to the control groups in RT-4 cells (Fig. 5.D). The expression of Securin was increased 25-fold in the GEM + CIS and 15-fold in the GEM + CIS + PT group compared to the control group.

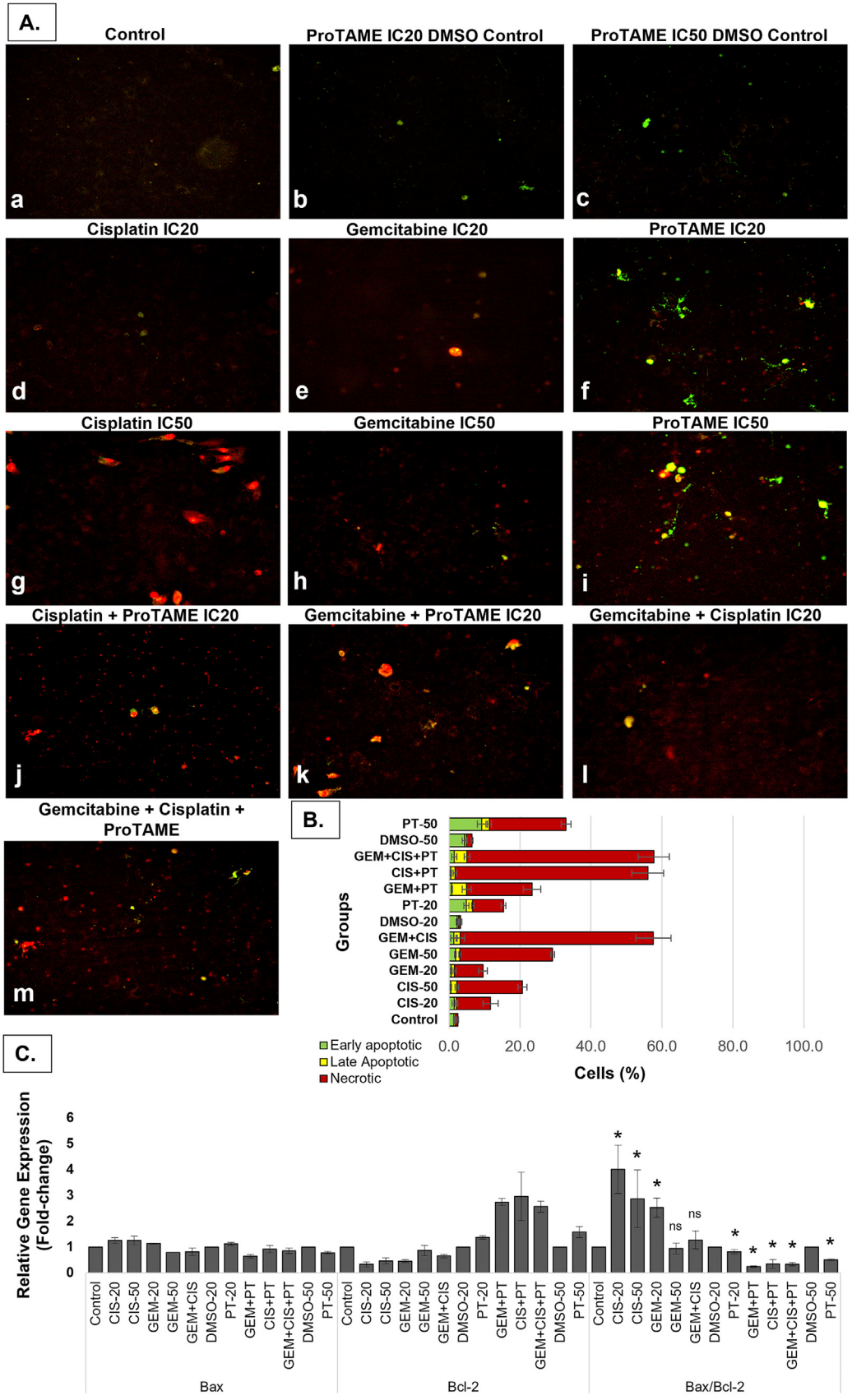
4. Discussion

Cancer chemotherapy does not always result in success due to the complex response mechanisms to drugs. These complex mechanisms cause tumor populations to give refractory responses to chemotherapy regimens and eventually, the tumor relapse. Therefore, a better understanding of the mechanisms affecting chemotherapy response is of clinical importance. In this study, the effect of proTAME on cell cytotoxicity and death in addition to chemotherapeutic agents used in the standard treatment regimen of bladder cancer was investigated. Expressions of several genes functionally related to APC/C pathway were also evaluated to assess the response of bladder cancer cells against combination chemotherapy involving proTAME. Although the normal cell line used in the present study was not originated from a normal human bladder tissue, an immortalized normal retinal epithelium cell line was used to better evaluate the results obtained using a human bladder cancer cell line, RT-4 which has an epithelial morphology as well.

Cisplatin-based combination therapy includes classical MVAC, ddMVAC (dense dose MVAC), CMV, and GC. Although no randomized trials have been conducted to compare different neoadjuvant regimens, retrospective evidence indicates that there is no significant difference in pathologic complete response between the various regimens (Galsky et al., 2015). Furthermore, treatment-related mortality has been reported to be 3 % for MVAC and 1 % for GC (Rizzo et al., 2021). It has also been reported that GC therapy showed similar efficacy but less toxicity compared to MVAC (Sonpavde et al., 2012). A recent retrospective study stated that GC therapy is comparable to MVAC and MVAC therapy is not superior to GC (Okabe et al., 2018). The standard treatment protocol approach for metastatic bladder cancer shows shift towards GC therapy. The side effects of cisplatin treatment, on the other hand, limit its efficacy. Split cisplatin dose protocols demonstrated better clinical efficacy than Gemcitabine/Carboplatin in cisplatin-ineligible advanced or metastatic urothelial carcinoma patients (Izumi et al., 2019). Thus, in this study, the effects of cytotoxic gemcitabine, cisplatin therapies and their combinations at IC20 doses, as well as a Cdc-20 inhibitor, proTAME, were assessed to investigate as an alternative novel standard therapy regimen. Our present cytotoxicity data revealed an IC50 of 7.5 μM for cisplatin in RT-4 cells. In a previous study, the IC50 value for cisplatin was reported to be 13.8 μM for RT-4 cells (Xylinas et al., 2016). In addition, the dose of gemcitabine used in the combined therapies of GC with mTOR inhibitor in bladder cancer cells was 100 nM, while the dose of cisplatin was 2.5 μM (Pinto-Leite et al., 2014). In line with the findings of this study, the IC20 doses for gemcitabine and cisplatin were 42 nM and 2.13 μM respectively. Gemcitabine, cisplatin and proTAME inhibited both cell viability and colony-forming ability of cells in a dose-dependent manner. MTS assay showed consistent results with clonogenic survival assay. In the MTS assay, proTAME produced slightly greater cytotoxicity against normal cells than cisplatin and gemcitabine. However, in the clonogenic survival assay, cisplatin and gemcitabine were found to have a greater cytotoxic effect on ARPE-19 cells at IC20 doses than proTAME. To date, the cytotoxic effect of proTAME on non-cancerous cells was investigated only in one study, which reports that high dose proTAME (73.6 μM) induced cytotoxicity and cell death on healthy peripheral blood mononuclear cells (Lub et al., 2016). Zeng et al. reported that proTAME was synthesized as a precursor form due to the cell-impermeability of TAME and transformed into active TAME by intracellular esterases after its entry into the cell (Zeng et al., 2010). In the same study, it was stated that active proTAME was effectively activated in ovarian cancer, HeLa cells, but not in non-tumorigenic epithelial MCF10A cells (Zeng et al., 2010). In the present study, the cytotoxic effect of proTAME on ARPE-19 cells showed that the prodrug was activated. A potential contribution to the differences observed between MTS and colony-forming assay results may arise from the specific time periods of these assays. In comparison to a short-term MTS assay used to evaluate the mitochondrial activity of viable cells, colony formation assay is a long-term assay especially useful to assess proliferative and clonogenic growth of the cells.

Morphological changes such as cell shrinkage, bubble formation in the membrane, chromatin condensation and nuclear fragmentation are observed during the apoptosis process in cells (Monier & Suzanne, 2021). In the morphology of RT-4 cells stained with

Fig. 3. Evaluation of early, late apoptotic and necrotic cells after gemcitabine, cisplatin and proTAME treatments in RT-4 cells, expression fold changes of *Bax*, *Bcl-2* genes and Bax/Bcl-2 ratio. **A.** Annexin V/PI double-staining assay**.** RT-4 cells ($1x10^5$/well) were seeded on sterilized round 10 mm coverslips placed in the bottom of 12-well plates. After 24 h of incubation, DMSO was added to the DMSO control groups in the amount of DMSO included in the IC20 and IC50 doses of proTAME (A.b, -c). For drug and/or inhibitor, the cells were treated with IC20 (A.d, -e, -f), IC50 (A.g, -h, -i) and combined doses (A.j, -k, -l, -m). No drug or inhibitor was added to the control group (A.a). IC20 concentrations of each inhibitor or drug(s) were used as combination therapies. After 48 h of incubation, the cells were stained as detailed in the Methods section and photographed at 20X magnification. Annexin V+/PI- cells (green), Annexin V-/PI + cells (red) and Annexin V+/PI + cells (yellow) are early apoptotic, necrotic and late apoptotic, respectively. **B.** Quantitative representation of early-late apoptotic and necrotic cells. **C**. Relative mRNA expression of *Bax* and *Bcl-2* genes and Bax/Bcl-2 ratio measured by qRT-PCR after incubation with single IC20 and IC50 doses and combined doses of cisplatin, gemcitabine and proTAME in RT-4 cells. Data (mean +/-SEM) was obtained by using the *GAPDH* gene as a reference from three independent experiments. The fold-changes of the GEM, CIS, GEM + CIS groups were normalized to the untreated control group and the fold-changes of the groups containing proTAME were normalized to the DMSO control groups. Data (mean +/-SEM) results from three independent experiments were obtained using the *GAPDH* gene as a reference. Combination therapies included the IC20 doses of each drug/inhibitor tested. CIS: cisplatin, DMSO: Dimethyl sulfoxide, GEM: gemcitabine, PT: proTAME.


A.
Control
ProTAME IC20 DMSO Control
ProTAME IC50 DMSO Control
a
b
c
Cisplatin IC20
Gemcitabine IC20
ProTAME IC20
d
e
f
Cisplatin IC50
Gemcitabine IC50
ProTAME IC50
g
h
i
Cisplatin + ProTAME IC20
Gemcitabine + ProTAME IC20
Gemcitabine + Cisplatin IC20
j
k
l
Gemcitabine + Cisplatin + ProTAME
m
B.
PT-50
DMSO-50
GEM+CIS+PT
CIS+PT
GEM+PT
PT-20
DMSO-20
GEM+CIS
GEM-50
GEM-20
CIS-50
CIS-20
Control
Groups
Early apoptotic
Late Apoptotic
Necrotic
0.0
20.0
40.0
60.0
80.0
100.0
Cells (%)
C.
Relative Gene Expression (Fold-change)
0
1
2
3
4
5
6
Control
CIS-20
CIS-50
GEM-20
GEM-50
GEM+CIS
DMSO-20
PT-20
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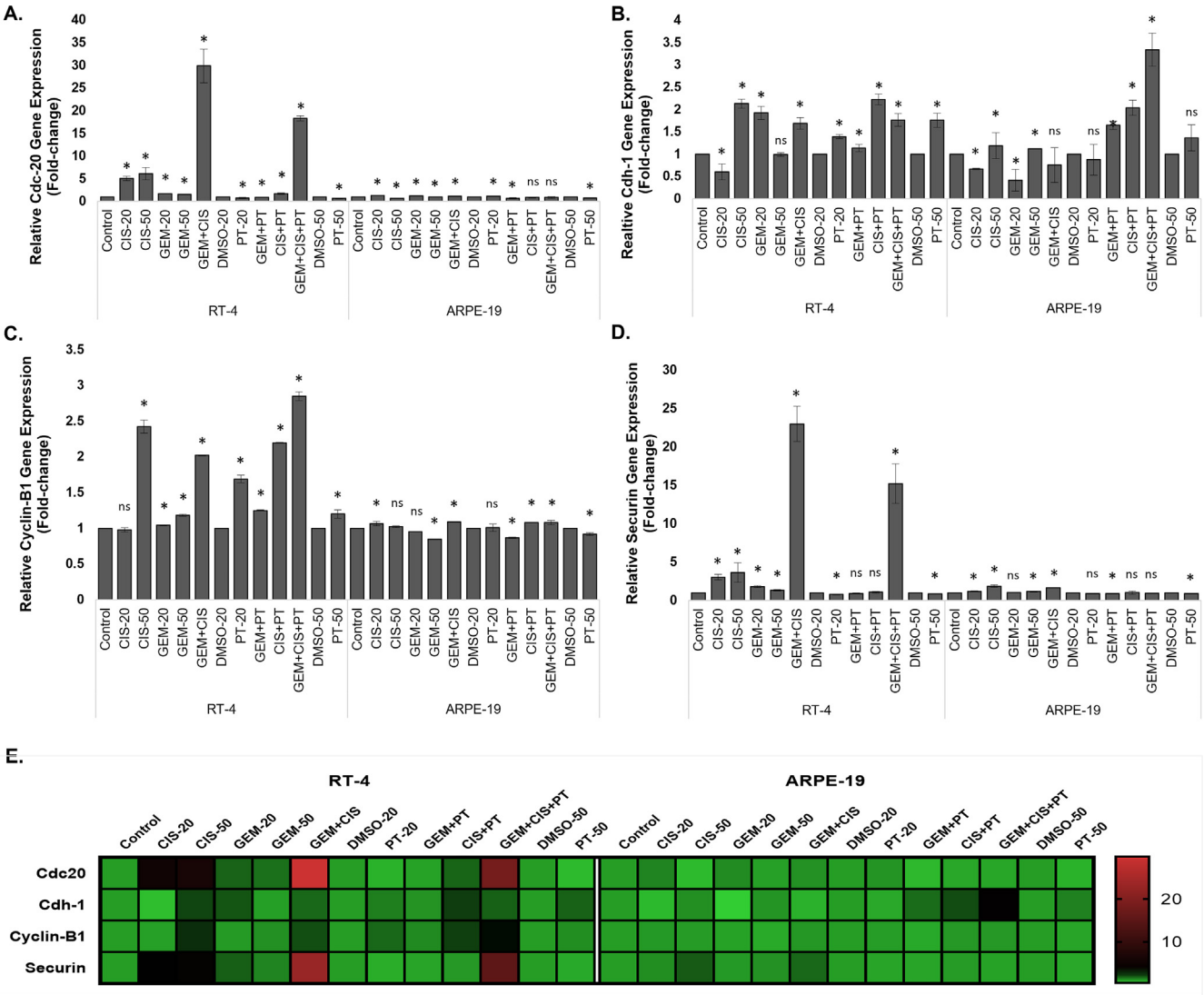

Fig. 5. Relative gene expression in RT-4 and ARPE-19 cells treated with IC20 and IC50 doses and combination of cisplatin, gemcitabine and proTAME. **A.** *Cdc-20*, **B.** *Cdh-1*, **C.** *Cyclin-B1*, **D.** *Securin* and **E.** Heat map for the differential gene expression profiles. mRNA levels were assessed by qRT-PCR using *GAPDH* as a reference gene. The fold-changes of the CIS, GEM, GEM + CIS groups were normalized to the untreated control group and the fold-changes of the groups containing proTAME were normalized to the DMSO control groups. Results (mean +/-SEM) from three independent experiments are shown. Combination therapies included the IC20 doses of each drug/inhibitor tested. CIS: cisplatin, DMSO: Dimethyl sulfoxide, GEM: gemcitabine, PT: proTAME.

Giemsa solution, we observed an increase in cytoplasmic shrinkage, nuclear condensation, fragmentation and apoptotic body formation with increasing doses of cisplatin, gemcitabine and proTAME. Similarly, in a study using cisplatin and gemcitabine in RT-4 cells, an increase in cytotoxicity and apoptosis-related features was observed in a dose-dependent manner (da Silva et al., 2018). Cytotoxicity and apoptosis-related morphological characteristics increased in direct proportion to the dose.

Since the effects of cisplatin, gemcitabine, and proTAME combination therapy are not yet known in bladder cancer, we sought to investigate cell death using the Annexin V/PI staining method to detect early apoptotic, late apoptotic and necrotic cells. In the

Fig. 4. Evaluation of early, late apoptotic and necrotic cells after gemcitabine, cisplatin and proTAME treatments in ARPE-19 cells, expression fold changes of *Bax*, *Bcl-2* genes and Bax/Bcl-2 ratio. **A.** Annexin V/PI double-staining assay**.** ARPE-19 cells ($1.25x10^5$/well) were seeded on sterilized round 10 mm coverslips placed in the bottom of 12-well plates. After 24 h, DMSO was added to the DMSO control groups in the amount of DMSO included in the IC20 and IC50 doses of proTAME (A.b, -c). For drug and/or inhibitor, the cells were treated with IC20 (A.d, -e, -f), IC50 (A.g, -h, -i) and combined doses (A.j, -k, -l, -m). No drug or inhibitor was added to the control group (A.a). IC20 concentrations of each inhibitor and/or drug(s) were used as combination therapies. After 48 h of incubation, the cells were stained as detailed in the Methods section and photographed at 20X magnification. Annexin V+/PI- cells (green), Annexin V-/PI + cells (red) and Annexin V+/PI + cells (yellow) are early apoptotic, necrotic and late apoptotic, respectively. **B.** Quantitative representation of early-late apoptotic and necrotic cells. **C**. Relative mRNA expression of *Bax* and *Bcl-2* genes and Bax/Bcl-2 ratio measured by qRT-PCR after incubation with single IC20 and IC50 doses and combined doses of cisplatin, gemcitabine and proTAME in ARPE-19 cells. Data (mean +/-SEM) was obtained by using the *GAPDH* gene as a reference from three independent experiments. The fold-changes of the GEM, CIS, GEM + CIS groups were normalized to the untreated control group and the fold-changes of the groups containing proTAME were normalized to the DMSO control groups. Data (mean +/-SEM) results from three independent experiments were obtained using the *GAPDH* gene as a reference. Combination therapies included the IC20 doses of each drug/inhibitor tested. CIS: cisplatin, DMSO: Dimethyl sulfoxide, GEM: gemcitabine, PT: proTAME.

GEM + CIS + PT and CIS + PT groups, the percentage of late apoptotic and necrotic cells was higher than in the GEM + CIS combination. Similarly, in ARPE-19 cells, a decrease in early apoptotic but an increase in late apoptotic and necrotic cell percentage were observed in combination therapy treated groups. It was observed that the number of early apoptotic cells decreased critically in the CIS + PT and GEM + CIS + PT groups in RT-4 cells, but there were significantly increased numbers of apoptotic and necrotic cells. The number of early apoptotic cells was low, but the number of necrotic cells was significantly higher in the GEM + CIS treated ARPE-19 cells. ProTAME sensitized RT-4 cells to the cytotoxic effects of cisplatin and gemcitabine. While cisplatin and gemcitabine alone produced a certain level of lethal effect in cells, a collaborative effect was observed in cell death, especially in RT-4 cells combined with proTAME. Cells inhibited APC/C activity undergo mitotic catastrophe over time and die similar to microtubule inhibited cells (Zeng et al., 2010). However, molecules that produce APC/C inhibition are more advantageous than microtubule inhibitory agents that produce many off-target effects during chemotherapy (Huang et al., 2009; Manchado et al., 2010). Although no study reported the effects of proTAME on bladder cancer cells, only one study documented that the combination of cisplatin and proTAME sensitized HeLa and MCF-7 cells to cisplatin treatment. (Eguren et al., 2014). In addition, another study found that proTAME combined with paclitaxel increased paclitaxel sensitivity of ovarian carcinoma cells, SKOV-3 and OVCAR-3 (Raab et al., 2019). In a study examining the effect of TAME, on non-small cell lung carcinoma cells, it was reported that TAME treatment sensitized the cells to 5-FU therapy (Dai et al., 2016). The combination of proTAME with adriamycin and cisplatin increased the cytotoxic effects of these chemotherapeutic agents in osteosarcoma cells in vitro (Hu et al., 2014). The sensitivity of multiple myeloma cells has been increased by therapy modalities, including proTAME with etoposide, doxorubicin and vincristine (Crawford et al., 2016). Collectively, these previous studies suggest that proTAME increased the sensitivity of tumor cells to several chemotherapeutic agents. Therefore, the protein–protein interaction, destroyed by pharmacological interference with APC/C, may exert a potent anti-tumor activity. Thus, pharmacological modulation of the APC/C substrates can retard tumor growth, and an advantage over cancer can be achieved by specifically targeting interactions specific to cancer types (Huang et al., 2009; Wan et al., 2017).

Generally, apoptosis is controlled by pro- or anti-apoptotic signals. Bax overexpression increases cell death in response to apoptotic stimuli. Bcl-2 functions in the regulation of apoptosis and forms heterodimers with Bax (Dewson et al., 2012). In this study, the expression levels of *Bax*, (pro-apoptotic) in the intrinsic pathway, and *Bcl-2* (anti-apoptotic) gene were determined, and the rise in the Bax/Bcl-2 ratio was interpreted as evidence of apoptosis. In the groups where no therapy was applied, ARPE-19 cells expressed 3.3 times more Bax and 2.5 times more Bcl-2 than RT-4 cells. Consistent with these results, ARPE-19 cells were reported to be a potent Bcl-2 expresser in a previous study (Bhattacharya et al., 2011). Bax/Bcl-2 ratio in RT-4 cells increased significantly in GEM + CIS, GEM + CIS + PT and PT-50 groups compared to the control groups, indicating that apoptosis was induced with these treatments. In the CIS-50 and GEM-50 groups, a significant decrease was found in the Bax/Bcl-2 ratio compared to the control group. It was concluded that cisplatin and gemcitabine, administered at IC50 doses as monotherapies, produce a partial anti-apoptotic effect, especially when applied alone in RT-4 cells. In a previous study, findings showed that cisplatin resistance can be mediated by Bcl-2 and that cisplatin therapy induces chemoresistance by increasing Bcl-2 mRNA expression in cisplatin-resistant endometrial cancer cell lines (Rouette et al., 2012). In a study, Bcl-2 mRNA levels were shown to be higher in T24R1 and T24R2 (bladder cancer cell lines resistant to cisplatin) than parent T24 cells (Hong et al., 2002). Our results showed that combined therapies increased the ratio of Bax/Bcl-2 in RT-4 cells. Interestingly, in Annexin V/PI staining, there was an increase in necrotic cell counts in RT-4 cells in the groups treated with proTAME combinations, while a decrease in necrotic cell count was observed in ARPE-19 cells. This may be due to the higher Bcl-2 expression in ARPE-19 cells compared to RT-4 cells, and in addition to this, especially in ARPE-19 cells, an increase in Bcl-2 expression was observed in the groups combined with proTAME. Our findings showed that the inclusion of proTAME in the combination of two chemotherapy drugs acting through different mechanisms could reduce the dose of cisplatin used as monotherapy, and this therapy induced Bax expression and consequently apoptosis in RT-4 cells more significantly than ARPE-19 cells.

Bcl-2-like proteins function as an important apoptosis barrier. This might be accomplished by binding Bcl-2-like proteins to BH3-exposed conformers of Bax and Bak, thus, preventing these important cell death mediators from being fully activated (Roufayel et al., 2022). Increased Bax expression observed in the present study can lower the threshold of drug-induced cell death, the possibility exists that there are targets in cell death signaling that might affect apoptotic threshold and may modulate drug-induced cell death.

Targeting the ongoing processes of RT-4 cells associated with cell proliferation and replication with the combination of a DNA alkylating agent cisplatin, a nucleoside analogue gemcitabine, and the Cdc-20 inhibitor proTAME, resulted in stronger apoptotic effects than in normal epithelial, ARPE-19 cells. This may be due to a better triggering of cytotoxic and pro-apoptotic pathways in cancer cells with higher proliferation rates.

Two co-activators are required for APC/C regulation. Cdc-20 controls APC/C regulation during the progress of the cell from metaphase to anaphase, and Cdh-1 controls APC/C regulation during the mitotic exit-G1 phase. This complex recruits substrates for proteasomal degradation by binding the co-activator to APC/C. The targets of APC/C^{cdc20} are Cyclin-B1 and Securin. The decrease in their levels by proteasomal degradation means the beginning of anaphase and mitotic exit. ProTAME, which suppresses Cdc-20 activity, causes a displacement of the C-terminal of the isoleucine-arginine (IR) tail of Cdc-20 (Zeng et al., 2010). The treatments with cisplatin, gemcitabine and proTAME caused changes in *Cdc-20*, *Cdh-1*, *Cyclin-B1* and *Securin* gene expressions. Significantly decreased Cdc-20 levels were detected in RT-4 cells in both the PT-20 and PT-50 treatment groups compared to the DMSO controls. However, the Cdc-20 level increased significantly in the CIS-20 and GEM-20 groups. While the Cdc-20 level increased in a dose-dependent manner in the cisplatin therapy groups, the increase in the gemcitabine therapy groups did not occur in a dose-dependent manner. Cdc-20 expression was dramatically increased in the GEM + CIS combined treatment group, whereas it was significantly decreased in the proTAME groups. The high Cdc-20 expression found in the GEM + CIS combination was significantly decreased with the addition of proTAME in the triple combination therapy. The possible effect of proTAME for delaying the transition of cells to anaphase may require that cells progress to mitotic exit more slowly with low Cdc-20 expression, unlike gemcitabine and cisplatin treatments. In one study, it is stated that, despite the proTAME treatment, a certain level of Cdc-20 can bind to APC regardless of the IR-tail. (Zeng et al., 2010). A study in leukemia cell, HL-60, reported a decrease in Cdc-20 expression levels after cisplatin therapy, contrary to our results (Velma et al., 2016). In another study investigating the effect of cisplatin and gemcitabine combination on RT-4 cells, it was reported that Cdc-20 levels in RT-4 cells decreased after therapy. However, the concentration of gemcitabine (1.56 μM) used in this study is approximately 10 times

higher than the doses that we used in the present study (Velma et al., 2016). This may help to understand the differences between the findings of this study and previous studies. It has been proposed that in cancer therapy, targeting Cdc-20 directly rather than SAC could be a more effective alternative (Huang et al., 2009; Manchado et al., 2010). Complete regression of the tumor has been reported with genetic silencing of Cdc-20 in the aggressive fibrosarcoma model (Manchado et al., 2010). In bladder cancer, the Cdc-20 was first included in the whole genome microarray study in 2011 as one of the genes with 17 different expressions in 10 patients (Zaravinos et al., 2011). Clinical studies have linked Cdc-20 overexpression to a poor prognosis in bladder cancer and some solid cancers (Chang et al., 2012; Choi et al., 2013; Kato et al., 2012).

TAME separates Cdc-20 from APC/C by stimulating the auto-ubiquitination of Cdc-20, thus stabilizing Cyclin-B1 (Zeng & King, 2012). Cyclin-B1 levels in RT-4 cells increased approximately two-fold in the CIS-50, PT-20, GEM + CIS, CIS + PT, and GEM + CIS + PT groups. Decreased Cyclin-B1 levels are required for cells to enter the anaphase because Cyclin-B1/Cdk1 inhibits the separation of sister chromatids. In general, the decrease in Cdc-20 levels after proTAME therapy may explain the stable state or increased levels of, APC/C^{cdc20} substrate, Cyclin-B1 in our results. It has been reported in a study that the translation of APC/C substrates, Cyclin-B1 and BubR1 continues during mitotic arrest. (Zeng et al., 2010). The partial decrease in Securin levels in the GEM + CIS + P T group when proTAME was administered alone or compared with GEM + CIS therapy in RT-4 cells can be explained by the residual activity of APC/C and the partial degradation of the APC/C substrate Securin prior to the onset of anaphase. It can also be explained by that the degradation of the target substrates such as Cyclin-B1 and Aurora B are achieved not only by an APC/C complex-mediated mechanism but also via Parkin, a ubiquitin ligase, independent of the APC/C complex (Lee et al., 2015). It has been reported that Securin and Cyclin-A are intensely ubiquitinated in the presence of increasing concentrations of Cdh-1 even at low levels (Rape et al., 2006). As we observed in our study, the increase in Cdh-1 levels in the proTAME therapy groups may help explain the results for Securin. In RT-4 cells, an approximately twofold increase in the expression level of Cdh-1 was observed in all combination therapy groups and CIS-50, GEM-20, PT-20 and PT-50 groups compared to the control of each group. An increase in Cdh-1 expression levels was significantly induced in the proTAME combination groups. It has been previously suggested that while Cdh-1 functions as a tumor suppressor in many cancers during tumorigenesis, Cdc-20 acts as an oncogene (Wang et al., 2013). In ARPE-19 cells, no significant considerable level of Cdc-20 and Securin expression was observed. The significantly higher expression of Securin was observed in GEM + CIS (23-fold) and GEM + CIS + PT (15-fold) groups. It has been shown that although mitotic arrest is triggered, APC/C is not completely inhibited by pharmacological inhibitors and Cdc-20 might still be partially active under these conditions (Zeng et al., 2010).

Overall, we examined the effects of proTAME in combination with gemcitabine and cisplatin on cytotoxicity, cell proliferation and cell death. Combination treatment-related changes in the expression of selected genes associated with the APC/C pathway were also evaluated. Since bladder cancers are heterogeneous at the molecular level, it is unlikely that a single biomarker candidate can precisely characterize the potential malignant behavior of cancer to lead to reliable treatment conclusions (Soria et al., 2019). Although monotherapies and combination therapies have been studied in many experimental groups in this study, future studies are needed to assess the effect of the suggested triple-combination therapy on a more aggressive bladder cancer cell line and maybe in primary cells may strengthen the data. In addition, comprehensive studies can be planned to examine dose combinations in experimental animal tumor models.

5. Conclusion

The additive effects of gemcitabine, cisplatin and proTAME used in combination in this study can provide an alternative preclinical approach. The high tolerability of gemcitabine and potential superior effect on inhibiting DNA repair after combination with DNA-damaging agents such as cisplatin made it reasonable to study this combination. This study proposes a novel and effective multi-therapeutic modality, in which the cisplatin and gemcitabine doses were reduced in the combination therapy for bladder cancer cells. To our knowledge, this is the first study examining the effect of a GC in combination with proTAME on bladder cancer cells. Identifying changes in the expression of APC/C-associated genes after gemcitabine and cisplatin treatments, as well as proTAME combination therapy, provides insight into anti-cancer mechanisms and these expression profiles of genes can be used to predict response to therapy.

Data and materials availability

Data available on request from the authors.

CRediT authorship contribution statement

Hatice Sevim Nalkiran: Conceptualization, Methodology, Writing – original draft, Funding acquisition. **Dilara Akcora Yildiz:** Writing – review & editing. **Faruk Saydam:** Writing – review & editing. **Ali Irfan Guzel:** Writing – review & editing. **Ihsan Nalkiran:** Writing – original draft, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.sjbs.2023.103564.

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