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ORIGINAL ARTICLE

PARP inhibitor Olaparib Enhances the Apoptotic Potentiality of Curcumin by Increasing the DNA Damage in Oral Cancer Cells through Inhibition of BER Cascade

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Abstract

Although Olaparib (Ola, a PARP-inhibitor), in combination with other chemotherapeutic agents, was clinically approved to treat prostate cancer, but cytotoxicity, off-target effects of DNA damaging agents limit its applications in clinic. To improve the anti-cancer activity and to study the detailed mechanism of anti-cancer action, here we have used bioactive compound curcumin (Cur) in combination with Ola. Incubation of Ola in Cur pre-treated cells synergistically increased the death of oral cancer cells at much lower concentrations than individual optimum dose and inhibited the topoisomerase activity. Short exposure of Cur caused DNA damage in cells, but more increased DNA damage was noticed when Ola has incubated in Cur pre-treated cells. This combination did not alter the major components of homologous recombination (HR) and non-homologous end-joining (NHEJ) pathways but significantly altered both short patch (SP) and long patch (LP) base excision repair (BER) components in cancer cells. Significant reduction in relative luciferase activity, expression of BER components and PARylation after Cur and Ola treatment confirmed this combination inhibit the BER activity in cells. Reduction of PARylation, decreased expression of BER components, decreased tumor volume and induction of apoptosis were also noticed in Cur + Ola treated Xenograft mice model. The combination treatment of Cur and Ola also helped in recovering the body weight of tumor-bearing mice. Thus, Cur + Ola combination increased the oral cancer cells death by not only causing the DNA damage but also blocking the induction of BER activity.

Keywords Oral cancer · Olaparib · Curcumin · PARP inhibitor · PARylation

Introduction

Oral squamous cell carcinoma (OSCC; a subtype of head and neck squamous cell carcinoma (HNSCC)) is a fatal ailment of mankind worldwide [1]. Although several traditional therapies (e.g. surgery, chemo and radiation) are available in the treatment of oral cancers but acquired resistance to these therapies is a major challenge [2]. The chemotherapeutic agents are not able to cure cancer completely due to their off-target effects, toxicity, and hence need higher permissible concentrations [3]. Currently, combined drug therapies against various cancers using two or more small molecules have shown a promising therapeutic effect over traditional treatment options [4].

Curcumin (Cur, a bioactive and biocompatible, relatively nontoxic to normal cells) was studied for its anti-malarial and anti-cancer property since a long time [5, 6]. Reports suggested that Cur has anti-inflammatory, anti-oxidant and chemo-preventive property in different cancer model systems [7–9]. Evidence has shown that Cur caused anti-cancer activity via induction of DNA damage, chromatin condensation, and have a role in reducing angiogenesis and tumor metastasis [10, 11]. It is also reported that Cur induced cell death and cell cycle arrest, DNA damage and inhibition of DNA damage repair associated proteins in different cancer cell lines [12, 13]. It is well documented that Cur induced DNA damage through down-regulation of RAD51 dependent homologous

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recombination (HR) [14]. Cur can also suppress two major DNA double strand break (DSB) repair pathways, NHEJ and HR [15]. Cur is a DNA intercalator and it interacts with DNA to form DNA adduct [16] and this kind of DNA adducts can be mainly repaired by BER pathway [17].

The modulation of DNA repair pathways for cancer therapy is now possible with the development of poly (ADP-ribose) polymerase inhibitors (PARPi) [18]. PARP-1 was the first among 17 known PARP super families to be discovered and its function in the maintenance of genomic integrity has been well documented [15]. The activity of PARP is also essential in the repair of ssDNA breaks via BER cascade [19, 20]. Depending upon the involvement of enzymes and repair gap size, BER pathway can either be utilized Short Patch (SP) or Long Patch (LP) to repair apurinic/Apyrimidinic (AP) sites [21, 22]. Repairing the damaged DNA is initiated by DNA glycosylase which makes an AP site. Then AP site is cut by AP endonucleases-1 (APE-1) creating a 3′ hydroxyl group and a 5′ deoxyribose phosphate (dRP) end. In SP-BER, POL-β removed the dRP moiety by its dRP lyase activity and incorporates a single nucleotide to 3′ end and leaves a nick, and after that DNA ligase seals the nick. When AP sites are either oxidized or reduced, they become resistant to β-elimination and cannot be excised by the dRP lyase activity of POL- β. In these cases, the modified AP-site is repaired via LP-BER pathway in which POL-β, δ or ε incorporates 2-15 nucleotides displacing the strand containing the modified AP-site. The DNA flap structure is cleaved by FEN-1, and the nick is sealed by a DNA ligase [22, 23]. PARP inhibition sensitizes the tumor cells to the cytotoxic agents by the induction of DNA damage that would normally be repaired by BER cascade [24]. Hence, inhibition of PARP could be a better therapeutic approach in order to target the specific DNA repair pathway in cancer. Various types of PARP inhibitors in combination with other DNA damaging drugs are in a clinical trial as well as in investigation [25]. Among them, Olaparib (Ola) (AZD2281) is the first FDA approved drug used for the treatment of relapsed platinum-sensitive BRCA mutated advanced ovarian cancer [26].

Strategy for reducing cancer by inducing DNA damage as well as inhibiting the DNA repair partway is a promising approach to treat cancer. Hence, if a combination of chemotherapeutic agents cause DNA damage and simultaneously inhibit the DNA repair pathway, this will eventually lead to cancer cell death. Taking this into consideration, we chose Cur as a DNA damaging agent in combination with PARP-1 inhibitor Ola for this study. In most of the cases, researchers studied the anti-cancer potentiality of PARP inhibitors in combination with another synthetic chemicals (mainly DNA damaging agent), which is very toxic and offered other secondary complications. Interestingly, there is no report where Ola is used to treat oral cancers in any combination with bioactive compounds.

So, the objective of the current study is to evaluate the role of Cur and Ola in terms of inhibiting the proliferation of oral cancer cells In vitro as well as In vivo and to study the mechanism of their anticancer action. We have shown that Cur and Ola synergistically caused apoptosis in oral cancer cells. Ola enhances the DNA damaging capability of Cur in oral cancer cells by inhibiting the BER cascade, which causes persistent irreparable DNA damage in cells. Increased irreparable DNA damage ultimately leads to apoptosis in cancer cells.

Materials and Methods

Cell Culture and Chemicals

The OSCCs (SCC-25 and H-357), Colon-HCT-116, Breast (MCF-7, MCF-10A-Tr and MDA-MB-231), Lung-A-549 and Cervical (HeLa) cancer cell lines were maintained in DMEM (50:50, *v*/v) medium supplemented with 1% antibiotic (100 units of penicillin and 10 mg streptomycin per ml in 0.9% normal saline), 10% fetal bovine serum (HIMEDIA, India) while H-357 cells need DMEM-F12 (50:50, *v*/v), 0.5 μg/mL of hydrocortisone and 1% (*w*/*v*) of l-glutamine [27] for their growth. Non-transformed breast epithelial cells, MCF-10A were grown in DMEM-F12 (50:50, v/v) medium supplemented with 10% FBS, 1% antibiotic, 0.5% of hydrocortisone, 100 ng/mL of cholera toxin, 10 μg/mL of insulin, 10 ng/mL of epidermal growth factor and 1% (*w*/*v*) of L-glutamine. All the cell lines were maintained in a humidified atmosphere in 5% CO2 at 37 °C [28]. Most of the experiments were carried out in H-357 cells. The drugs Cur and Ola were purchased from Sigma Chemicals Co. (St Louis, MO, USA) and AdooQ BioScience LLC, USA, respectively. Cell culture-based chemicals were purchased from Himedia (Mumbai, India). Anti-PARP-1 (#ab6079), anti-RAD51 (#ab63801), anti-mouse (TRIT-C conjugate) (#ab6786) and anti-DNA-PKcs (#ab70250) were purchased from Abcam, MA, USA. Anti-FEN-1 (#2746), anti-XRCC1 (#2735), anti- Phospho-Histone H2A.X (Ser139) (20E3) (#9718), anti-rabbit (#7074) and anti-WRN (#4666) antibodies were procured from Cell Signaling Technologies Inc., MA, USA. Anti-BAX (#SC-7480), anti-BCL-XL (#SC-8392), anti-DNA ligase-III (#SC-135883), anti-mouse (#SC358914), anti-CASPASE3 (#SC-7272) and anti-POL-β (#SC-376581) were purchased from Santa Cruz Biotechnology Inc., CA, USA. Anti-APE-1(#NB-100-101) and anti-LAMIN-B1 (#NBP2-59783) were purchased from Novus Biologicals, CO, USA. Anti-α-TUBULIN (#T5168) antibody was purchased from Sigma Chemicals Co. (St Louis, MO). Anti-PAR (#4335-MC-100) antibody was purchased from Trevigen, Gaithersburg, MD.

Treatment with the Drugs

At first, we have determined the IC_{50} (50 % inhibition of cell growth in culture) for individual Cur, Ola and their combination. For the individual, cells were grown to 60-70% confluency and treated with increasing concentrations of Cur (0-75 μM) and Ola (0-50 nM) for 30 h and the MTT assay was carried out. But for combination, cells were pre-incubated with varying concentrations of Cur (0-75 μM) for 6 h, then in each dose of Cur, increasing concentrations of Ola (0-50 nM) were added and cells were grown for another 24 h prior to carry out the MTT assay. Data were calculated and IC_{50} value was determined. In very low dose of Cur and Ola combination did not show significant cell death and very high dose of Cur and Ola caused significant cell death that might be due to toxic effect of the drugs (data not shown). So we chose comparatively lower dose of Cur and Ola for combination treatment.

In rest of the experiments, cells were treated with IC_{50} values of Cur and Ola for individual treatment but in combination treatment, cells were pre-exposed with 10 μM Cur for 6 h and then 2 nM Ola was added for the indicated time.

Measurement of Cell Viability Using MTT Assay

To check the short term cytotoxic ability of drugs in different cancer cells, a colorimetric based MTT [3-(4, 5-dimethylthiazol-2yl-)-2, 5-diphenyl tetrazoliumbromide] assay was performed according to the protocol described earlier [27]. In brief, approximately, 8000 to 10,000 cells/well of different cancer cells along with MCF-10A cells were seeded in 96-well plates in triplicate and grown to 60-70% confluency. Then, cells were treated with Cur and Ola individually along with their combination with indicated concentrations as described above. After that, media was removed, 100 μL of 0.05% MTT reagent was added to each well and incubated at 37 °C overnight for the formation of purple formazin crystals. Purple formazan crystals were dissolved in 10% NP-40. The color intensity was measured by spectrophotometer at 570 nM using microplate reader (Mithras LB 940, Berthold, Germany). Data were calculated and represented as percent survival.

Analysis of Combined Drug Effect

Synergistic, additive or antagonist action of Cur and Ola was determined by isobologram analysis as described earlier [29]. Isobologram plots were drawn by plotting the individual IC_{50} values (data obtained from MTT assay) of Ola and Cur in H-357 cells in X- and Y-axis, respectively. The IC_{50} value in the combination treatment of Cur and Ola was pointed on the graph. In principle, if the spotted point (IC_{50} value of drugs) falls on the line then it is considered as an additive, whereas, if it falls below or above the line, then it is considered as a synergistic or antagonist drug effect, respectively.

Measurement of Long Term Cell Survival Using Clonogenic Cell Survival Assay

Clonogenic assay was performed to determine the long term cytotoxic effect of Cur, Ola and their combination in H-357 cells [27]. In brief, approximately, 500-600 viable cells/well were cultured in 12 well plate and treated with 10 μM Cur, 2 nM Ola and their combination respectively for 72 h. Then, media was aspirated, fresh media was added and the cells were allowed to grow for 5-6 doublings. After colony formation, the media was removed, washed, stained with 0.2% crystal violet and incubated for 1 h. After that, crystal violet stained plate was washed with water and air dried. Colonies were counted using a gel documentation system (UVP, Germany). Data were calculated and presented as percent survival against drug treatment conditions.

Measurement of DNA Damage by Alkaline Single Cell Gel Electrophoresis or Comet Assay

DNA damaging potentiality of the drugs was measured by alkaline single cell gel electrophoresis (SCGE) or comet assay. In brief, 4×10^4 H-357 cells were seeded in 12 well plate and after 24 h cells were treated with indicated concentrations of Cur, Ola and their combination for 30 h. Then cells were harvested by trypsinization and resuspended in ice-cold 1X phosphate buffer saline (PBS). Comet assay was performed according to the protocol described earlier [27]. Sample slides were stained with SYBR® green dye (Sigma-Aldrich), incubated in the dark for 30 min at room temperature and the migration of DNA was observed at 20X using a fluorescence microscope (Nikon, Japan). Comet lengths were analyzed by TriTek CometScore™ software (Tritek Corporation, VA, and USA) and represented graphically as arbitrary comet length vs different treatment conditions.

Immunocytochemical Staining for γH2AX Formation and Cleaved CASPASE-3 Expression

Further to check the DNA damaging and apoptotic potentiality of Cur, Ola and their combination, γH2AX (a DNA damage marker) and cleaved CASPASE-3 (an apoptosis marker) immunofluorescence was performed in H-357 cells according to the immunofluorescence assay protocol [27]. Briefly, cells were grown in 24 well tissue culture plate up to 70-80% confluency and were treated with the indicated concentration of Cur, Ola and their combination. Then cells were fixed with methanol:acetone (1:1) for 15 min at −20 °C, blocked with 2% BSA and 0.02% triton X-100 in 1X PBS followed by washing with 1X PBS. Cells were incubated with primary anti-γH2AX

and anti-CASPASE-3 antibodies (1:500 dilutions in 1X PBS), respectively. Unbound antibodies were removed by washing with 1X PBS. Secondary antibodies conjugated with TRIT-C were added and incubated for 2 h in room temperature. Then, the cells were washed with 1X PBS, nuclei were counter-stained with DAPI and images were captured using an inverted fluorescence microscope (Nikon, Japan) at 20X magnification.

In Vivo BER Assay

To measure the BER activity of cells, a plasmid-based In vivo BER assay protocol was followed as described earlier [27]. In brief, a closed circular DNA containing the p21 (pGL2-p21) promoter downstream of the luciferase reporter gene was de-aminated by 3 M sodium bisulphite in the presence of 50 mM hydroquinone that modifies cytosine into uracil-residues (U-p21p) which is a substrate for SP-BER. The resulting U-p21p was further treated with uracil-DNA glycosylase (UDG), and the formed abasic site was reduced with 0.1 M sodium borohydride to R-p21p which is a substrate for LP-BER. Approximately, 60-70% confluent cells were transfected with 2.0 μg/ml of R-p21p cDNA and U-p21p along with 0.5 μg/ml of β-gal using 10 μl/ml of Lipofectamine reagent. β-gal was used as an internal control to determine the transfection efficiency of the cells. After 8 h, transfection media was replaced with fresh serum-containing medium and cells were treated with individual Cur, Ola and their combination (Cur + Ola) as aforementioned for 30 h. Next, the cells were harvested by trypsinization and the SP-BER and LP-BER activities were measured in cellular lysates using a DLR luciferase assay instrument (Berthold, Germany).

Determination of PARylation of PARP-1 in Vitro

The PARylation of PARP-1 was performed after the cells were exposed to Cur, Ola and their combination according to the protocol [27]. In brief, approximately, 5×10^5 H-357 cells were grown and treated with the aforementioned concentrations of above drugs. Then, cells were harvested and the nuclear lysate was prepared according to the protocol [29]. The nuclear extracts were immunoprecipitated with anti-PARP-1 and incubated in the PARylation reaction buffer [50 mM Tris HCl (pH 7.8), 25 mM $MgCl_2$, 1 mM DTT and 100 μM NAD^+, protease inhibitors] for 30 min at 37 °C. The PARylation reaction was terminated by adding SDS-PAGE sample loading buffer. Proteins were separated by SDS PAGE and transferred onto nitrocellulose membrane for western blotting. Then, the membrane was probed with specific antibodies as the manufacturer's protocol and band intensity were analyzed by densitometry.

Measurement of Apoptosis by Annexin-V-FITC/PI Dual Staining

Annexin-V-FITC/PI dual staining was performed to detect the distribution of cells in early apoptotic, late apoptotic and necrotic phases in the individual Cur, Ola and their combination treated cells according to the protocol [30]. In brief, 1×10^5 H-357 cells/well were seeded in 6 well plate. 70-80% confluent cells were exposed to the aforementioned drugs for 30 h. Then, cells were harvested, washed with 1X PBS, stained with Annexin-V/FITC and PI according to the manufacturer's protocol (Sigma). Stained cells were incubated for 10 min and sorted with an event count of 10,000 cells per sample by using Flow Cytometry (FACS CANTO II, Becton & Dickinson, CA, USA). Data were analyzed using FACS Diva software.

Measurement of Topoisomerase Activity

To assess the ability of Cur, Ola and their combination in term of inhibiting topoisomerase, topoisomerase activity was assayed according to the protocol [29] after treating H-357 cells with the indicated concentration of drugs. In brief, 80-90% confluent cells were treated for 30 h with the aforementioned drugs and 20 μM etoposide (well-known topoisomerase II inhibitor, used as a positive control). After that, nuclear lysate was prepared and processed for the measurement of topoisomerase activity. Topoisomerase reaction tubes, containing 20 μg of nuclear lysate, 1 μg PGL_2-luc-p21 plasmid as a substrate and 1X reaction buffer (200 mM Tris-Cl, pH 7.5, 100 mM $MgCl_2$, 10 mM ATP, 10 mM EDTA, 10 mM dithiothreitol, 1.5 mM KCl and 300 μg/ml bovine serum albumin), were incubated at 37 °C in thermo mixer for 30 min. The reaction was stopped by adding sodium dodecyl sulfate (SDS) and agarose gel (0.9%) electrophoresis was performed by loading a fixed amount of those samples. After 1 h (30 V) of electrophoresis, the image was taken and quantification was done by gel documentation system (UVP, Germany).

Western Blot

Western blot analysis was done according to the protocol mentioned earlier [31]. In brief, 1×10^6 cells/plate were cultured to 80-90% confluency prior to Cur, Ola and Cur + Ola treatment for 30 h. Then, cells were harvested and lysed with modified radioimmunoprecipitation assay (RIPA) lysis buffer and cellular lysates were processed for western blotting. A fixed amount (60 μg) of protein was loaded and separated by 10% SDS- polyacrylamide gel electrophoresis (PAGE) (4.5% SDS-PAGE for DNA-PKcs). Proteins were transferred to nitrocellulose membrane. Then, the membrane was probed with specific antibodies (1:1000 dilutions in 1X PBS) according to the manufacturer's protocol and band intensity was analyzed by densitometry.

Development of Mice Xenograft Model

In vivo animal experiment was carried out according to the protocol [30]. Briefly, female Balb/C mice (6 weeks old), were housed in a proper light/dark cycle of 12 h/12 h. All the animal work and the experimental protocol were approved by the Institutional Animal Ethical Committee (IAEC, KIIT University, Bhubaneswar, India). The mice were grouped in to five groups. The first group the mice were injected with only PBS (designated as control). Then, H-357 cells (1×10^7 in 200 μL PBS) were injected into the left mammary fat pads of the remaining 4 groups. Among them one group of mice is untreated and other three groups were treated with Cur, Ola, and Cur + Ola respectively. Each group contained 6 mice. The mice health condition and tumor formation were monitored every alternate day. Tumor dimension was measured using slide caliper and tumor volume was calculated using the formula: $(W^2 \times L)/2$, where width (W) ≤ length (L). After 25 days when a measurable amount of tumor was detected, the aforementioned drugs (20 mg Cur and 20 nM Ola per kg body weight dissolved in PBS) were administered (Cur, Ola alone and their combination) orally in one group every alternate day. After another 25 days of treatment, the mice were sacrificed, tumor tissues were collected and processed for further experiments (western blotting, immunohistochemistry and PARylation).

Immunohistochemical (IHC) Analysis

IHC was performed to check the expression of proteins in tissue extract as the protocol described earlier [31]. Tissue sections were deparaffinized with xylene, rehydrated with graded series of ethanol (100%, 90%, and 70%, respectively), washed in 1X PBS, and then, the antigen was retrieved by citric acid buffer (pH 6). Nonspecific sites in fixed slides were blocked by 5% 100 μL fetal bovine serum (FBS) blocking solution. Endogenous peroxide activity was blocked by hydrogen peroxide. Then, sections were immunostained with anti-POLβ and anti-FEN-1 primary antibodies (1:500 dilutions in 1X PBS) at 4 °C overnight. Then slides were washed with 1X PBS and incubated with Horseradish peroxidase (HRP)-conjugated secondary antibody (1:500 dilutions in 1X PBS) for 30-60 min at room temperature. Sections were washed in 1X PBS and immunoreactivity was visualized using 3,3-Diaminobenzidine (DAB) peroxidase substrate kit (SK-4100, Vector Laboratories, CA, USA) followed by hematoxylin counter stain. Images were captured at 20X magnification using bright-field microscope (Leica DM2000, USA).

Statistical Analysis

Statistical analysis was performed by using Graphpad Prism version 5 software, USA. The data were analyzed by one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test and expressed as mean ± SD of 3 independent experiments. Statistical significance of mean differences were represented as '*' ($p < 0.05$), '**' ($p < 0.005$), '***' ($p < 0.0001$) and '$$' represents statistically non-significant ($p > 0.05$) mean differences with respective control.

Results

Ola Induces Cur Mediated Inhibition of Oral Cancer Cell Proliferation

Eight different cancer cell lines along with one non-transformed breast epithelial cells (MCF-10A, used as a control) were used for this study. To check the short term cytotoxic activity of Cur, Ola and their combination in these cell lines, an MTT cell viability assay was performed. It was noted that Cur and Ola caused 50 % cell death approximately at 15-50 μM and 25-40 nM in different cell lines, respectively. Interestingly, Cur and Ola individual treatment having more cytotoxic effect in cancer cells compared to MCF-10A cells. In all tested cell lines, combination drug treatment showed significantly less IC_{50} value compared to individual exposure of Cur and Ola. Combination of Cur and Ola showed approximately 1.7-3 fold and 6-15 fold lower IC_{50} value compared to their individual treatment respectively. It was noted that combination treatment caused 50 % cell death when 10 μM Cur combined with 2 nM of Ola in H-357 cells; which is the lowest IC_{50} of both drugs compared to the rest of the tested cell lines (Fig. 1a). Therefore, H-357 cells were used for the rest of the experiments. Some of the major experiments were also carried out in other oral cancer (SCC-25) cells (Supplementary figures). Data appeared that Cur and Ola might cause cell death in a synergistic manner. To confirm the synergism of their combined action, an isobologram diagram was plotted. The point 'P' appeared far below the line of additivity, which indicates Cur and Ola action on the cells is synergistic rather than additive to inhibit cell proliferation (Fig. 1b).

Earlier, it was reported that DNA damaging agent (e.g. Cur) causes DNA damage in cancer cells [10, 11] and when cells were provided a favorable environment for their growth, the damaged DNA got repaired by using various cell's internal DNA repair system [27]. But, if somehow cell's internal DNA repair system gets compromised, then irreparable DNA damage will persist. During the progression of time, the DNA damage will increase and ultimately cells will undergo apoptosis. To check whether similar things were also happening or not, several experiments were carried out. Cells were treated in five separate ways with the aforementioned drugs prior to perform an MTT assay: (1) cells were grown for 30 h and

a

Name of cell line	IC_{50} (μM)	IC_{50} (nM)	IC_{50} of combination	
	Cur	Ola	Cur(μM)	Ola(nM)
HCT- 116	30.0 ± 1.2	36.0 ± 0.5	12.3 ± 0.5	2.5 ± 0.2
H-357	26.0 ± 0.5	28.0 ± 0.3	10.0 ± 0.1	2.0 ± 0.4
SCC-25	31.0 ± 0.4	34.0 ± 1.4	11.5 ± 0.3	2.5 ± 0.3
HELA	15.5 ± 0.1	33.0 ± 1.0	12.5 ± 0.4	5.0 ± 0.1
MCF-7	48.0 ± 0.9	30.0 ± 1.7	15.6 ± 0.1	5.5 ± 0.1
MDA-MB-231	17.5 ± 0.5	32.5 ± 1.1	10.5 ± 0.5	2.2 ± 0.6
A549	39.2 ± 0.3	36.0 ± 0.6	15.0 ± 0.7	5.0± 0.2
MCF-10A-Tr	27.2 ± 0.6	33.2 ± 2.1	12.5 ± 0.5	2.5 ± 0.5
MCF-10A	>75	>50	30.0 ± 0.7	10.0 ± 0.3

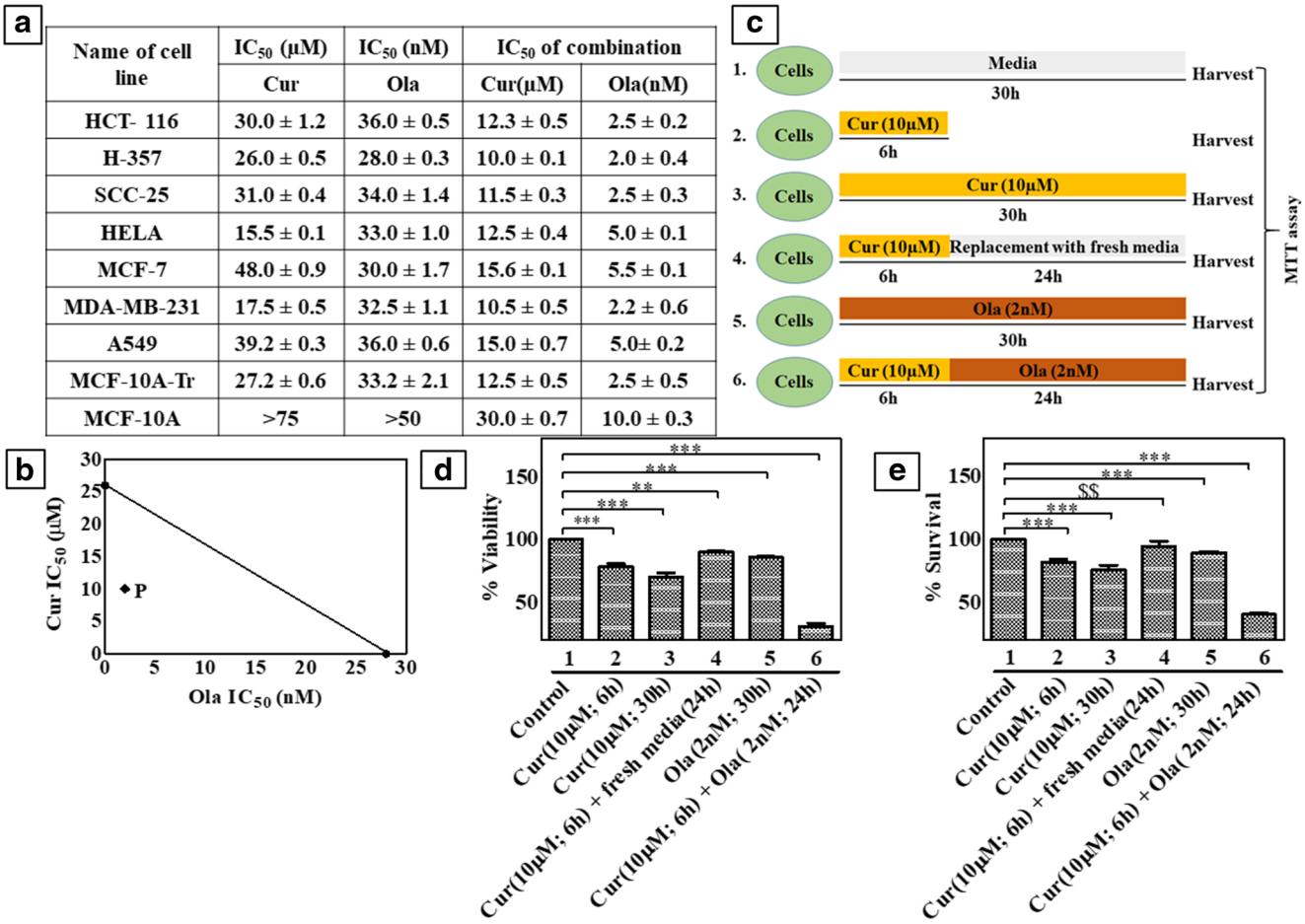

Fig. 1 Cur and Ola synergistically increased cancer cell death. **a** The IC_{50} value of Cur, Ola and their combination treatment (Cur + Ola) in different cell lines for 30 h. Data represent mean ± SD of three independent experiments. **b** Isobologram diagram to study the synergism between Cur and Ola. The "P" represents the IC_{50} value of the combined drugs (Cur + Ola) in H-357 cells. **c** Represents the treatment procedure of experiments. **d** Cell viability after the treatment with Cur, Ola and their combination (Cur + Ola) in H-357 cells according to the treatment procedure mentioned in **c**. **e** Clonogenic cell survival after challenging Cur, Ola and their combination (Cur + Ola) accordingly in H-357 cells. Statistical significances were determined using one-way ANOVA, where '***' represents statistical significant ($p < 0.0001$) and $$ represents non-significant ($p > 0.05$) data with respect to control. Data represent the mean ± SD of three independent experiments

then proliferation was noted, (2) cells were exposed to 10 μM Cur for 6 h and immediately proliferation was measured, (3) cells were treated with 10 μM Cur continuously for 30 h prior to harvest and then proliferation was observed, (4) cells were challenged with 10 μM Cur for 6 h, media was aspirated and grown for another 24 h with fresh media before performing the MTT assay, (5) cells were exposed to 2 nM Ola continuously for 30 h prior to perform cell viability assay, (6) cells were treated with 10 μM Cur for 6 h, media was aspirated and exposed to 2 nM Ola for another 24 h before measuring cell viability. The treatment protocol and percent cell viability were provided in Fig. 1c and d, respectively. Cell viability was decreased in all the treatment conditions, but highest cell death (70%) was noted ($p < 0.0001$) in combination treatment (Set#6) compared to untreated control, while lowest cell death (15%) was observed in presence of Ola only ($p < 0.0001$) (Set #5). Interestingly, it was noted that Cur 30 h continuous treatment caused more cytotoxicity (more than 30% cell death) than Cur 6 h treatment followed by grown another 24 h with fresh media (10% cell death) (Set#3 vs. Set #4). It was observed that there was 20% reduction (30% death in set#3 and 10% death in Set# 4) of cell death, where the cells were treated with Cur for 6 h and kept for another 24 h in fresh media compared to Cur 30 h continuous treatment. This 20% reduction of cell death might be due to the fact that replacement with fresh media and incubate for another 24 h, allows the cells to get sufficient time to repair the damaged DNA in comparison to 30 h continuous treatment of Cur (Set#3). As a result, cell survival was found to be increased. On the other hand, it was noted that cells were unable to repair the damaged DNA when Ola has incubated in Cur pre-treated cells (Set #6). It might be due to the reason that Ola inhibits the DNA repair capacity of the cells, hence enhancing cell death. Thus, in agreement with our hypothesis, the above data suggested that Cur + Ola treatment increased cell death in oral cancer cells.

To support the above data, long term cell survival potentiality was measured after individual and combined exposure of indicated concentrations of the aforementioned drugs to H-357 cells by clonogenic cell survival assay. The percentage of cell survival was decreased by approximately 2.5 fold in Cur + Ola treated cells compared to untreated control further strengthened our above result (Fig. 1e).

Ola Increased the DNA Damaging Ability of Cur in Oral Cancer Cells

The relative comet formation in H-357 cells after Cur, Ola and Cur + Ola treatment were demonstrated in Fig. 2a. A measurable amount of comet formation was noted in Cur treated cells. Approximately, 4 and 2 fold enhanced ($p < 0.0001$) comet formation in H-357 cells was noted in 10 μM Cur exposure for 30 h and 6 h, respectively. Interestingly, when 6 h of Cur treated media was replaced with fresh media, a very little amount of comet tail was noticed. However, in the combination treatment of Cur and Ola, approximately 7 fold enhanced ($p < 0.0001$) comet formation was measured (Fig. 2a and b). Further to support the cell line independent phenomenon of Cur and Ola, comet assay was performed in SCC-25 cells and the highest comet was found in the combination treatment of Cur and Ola than their individual treatments (Supplementary Fig. 1).

Again to validate the DNA damaging potentiality of the above-mentioned drugs, γH2AX immunofluorescence was performed. Here also, maximum enhanced (60 fold) expression ($p < 0.0001$) of γH2AX was found in the combination treatment of Cur and Ola in comparison to untreated control. Interestingly, it was noted that there was no significant γH2AX formation

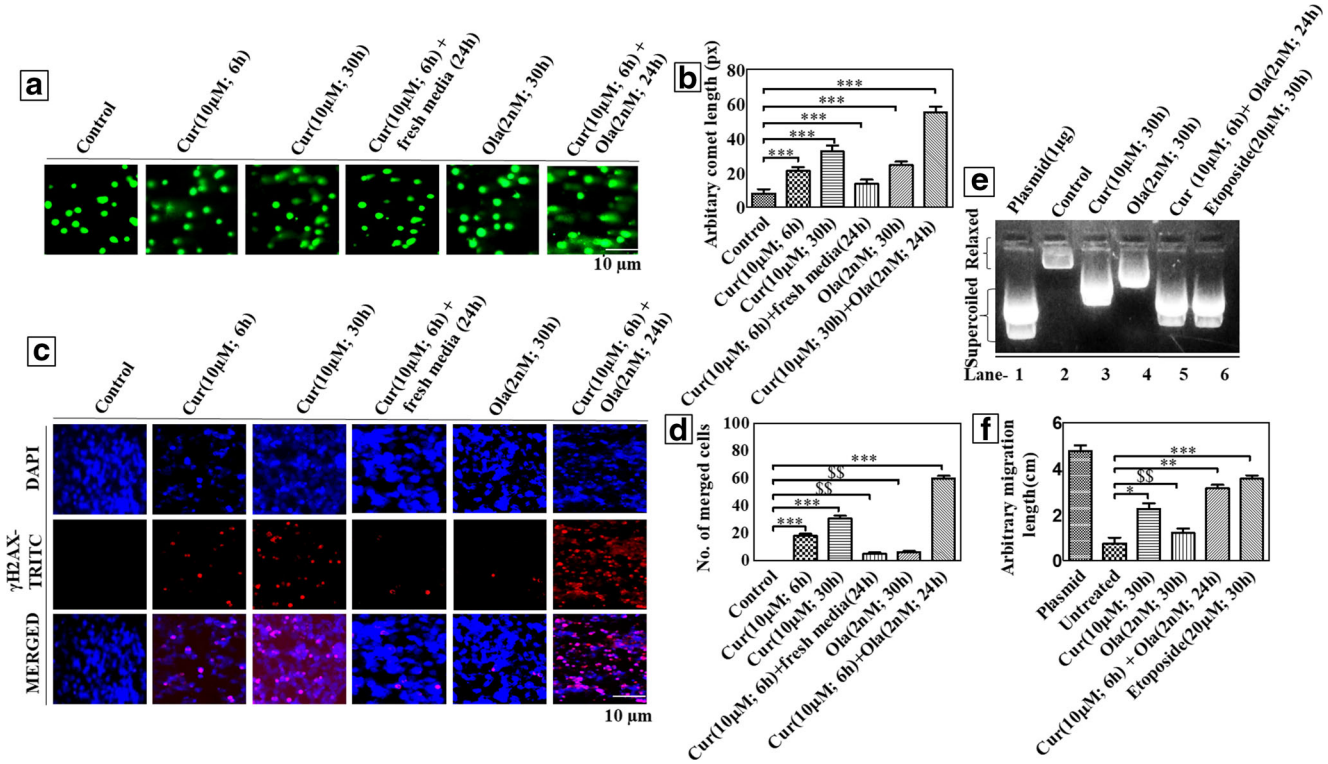

Fig. 2 Assessment of DNA damage/repair after Cur, Ola and combination treatment in H-357 cells for 30 h. **a** Comet formation in H-357 cells after treated with Cur, Ola and their combination. Scale bar is 10 μm. **b** Bar graph representing the quantitation of the average comet length in drug-treated H-357 cells analyzed with TriTek comet score software and plotted against each treatment. **c** Images represent immunocytochemical staining of γH2AX in H-357 cells after exposed to Cur, Ola and their combination accordingly. Scale bar is 10 μm. **d** Bar graph representing the quantitation of γH2AX formation in merged cells (DAPI and γH2AX-TRITC) of **c**. **e** Represents inhibition of topoisomerase activity by Cur, Ola and Cur + Ola treatment in H-357 cells. The experiment was performed according to the protocol described in the methods and material section. Lane 1, 2 represents plasmid (only) and plasmid+reaction buffer+untreated nuclear lysates respectively. Lane 3-5 represents plasmid+reaction buffer+Cur treated nuclear lysate, plasmid+reaction buffer+Ola treated nuclear lysate, and plasmid+reaction buffer+ (Cur + Ola) treated nuclear lysate respectively. Where lane 6 plasmid+reaction buffer+Etoposide (the known topo II inhibitor used as a positive control) treated nuclear lysate. **f** Quantitative representation of DNA migration of **e**. The bar graph shows arbitrary migration length plotted against different treatment conditions as mentioned above. All the data presented here were the representation of the mean ± SD of three independent experiments. Statistical significance was determined by one way ANOVA; where '***', '**' and '*' represents statistical significant ($p < 0.0001$, $p < 0.005$ and $p < 0.05$ respectively) and $$-represents non-significant ($p > 0.05$) data with respect to untreated control

($p > 0.05$) when cells were exposed to Cur (6 h) and grown for another 24 h in fresh media and in Ola treated cells, respectively (Fig. 2c and d). This data further indicated that Cur mediated DNA damage might have been repaired when internal DNA repair system was not inhibited, but treatment with Ola in Cur pre-treated cells could inhibit the DNA repair and finally causing cell death. The similar observation of γH2AX formation was also noted in SCC-25 cells after drug treatments (Supplementary Fig. 2) suggesting the fact that Ola enhanced the Cur mediated DNA damage in both oral cancer cells.

Induction of comet and γH2AX formation indicated that the combination of Cur and Ola caused extensive DNA damage and that might de-regulate the replication process. Topoisomerase is an essential enzyme which plays a vital role in replication [32] and many studies showed the involvement of DNA damaging agents in term of inhibiting topoisomerase II [33–35]. Research also suggested the anti-topoisomerase II activity of Cur [34]. Thus, it was hypothesized that Ola may enhance the anti-topoisomerase II activity of Cur. To test the hypothesis, topoisomerase II activity was measured in H-357 cells. Lane 1 showed the migration of plasmid only. In the untreated sample (lane-2), there was no migration of DNA in the gel due to fully active topoisomerase II-induced complete relaxation of DNA. But in Cur and Ola individual treatment (lane-3 and lane-4), the migration of supercoiled DNA was observed. The maximum migration of DNA was noticed in combination treatment (lane-5) as the supercoiled structure was more. This might be due to the inhibition of topoisomerase II by the combination treatment and their combination is more effective to inhibit topoisomerase II than individual treatment. Lane 6 showed the effect of etoposide, a known inhibitor of topoisomerase II, and served as a positive control. Comparatively higher migration of DNA in the combination treatment suggested the fact that Ola enhanced the Cur mediated topoisomerase II inhibition (Fig. 2e and f).

Cur and Ola Combination Caused Apoptosis in Oral Cancer Cells

From immunocytochemical staining, it was found that the combination of Cur and Ola caused higher expression of cleaved CASPASE-3 than their individual treatment conditions in H-357 cells. Approximately, 30 fold elevated expression ($p < 0.0001$) of CASPASE-3 (cleaved) was found in the 10 μM Cur (30 h) treated cells where the expression was found to be 80 fold higher ($p < 0.0001$) in the combination treatment of Cur and Ola as compared to control (Fig. 3a and b). An induction of BAX/BCL-XL (40 fold) ratio indicated that cells were undergone apoptosis after Cur + Ola exposure (Fig. 3c). To further confirm the apoptosis causing ability of Cur, Ola and their combination treatment, Annexin-V-FITC/PI dual staining were performed. From the data, it was observed that the combination treatment of Cur and Ola increased the percentage of apoptotic cells as compared to their individual treatments. Approximately 11.1% (1.2% necrotic +9.9% late apoptotic cells) and 7.4% (1.7% necrosis +5.7% late apoptotic cells) cell death was noted in Cur and Ola treated cells respectively. However, 80.8% cell death (2.8% necrotic +78% late apoptotic) was observed in Cur + Ola treated cells (Fig. 3d). Taken together, these data revealed that the combination of Cur and Ola having more apoptotic potentiality than their individual treatments.

Combination of Cur and Ola Inhibits in Vivo BER Activity and PARylation in Oral Cancer Cells

The above result appeared that Cur and Ola combination caused apoptosis by inducing excessive DNA damage in the cells. Now, the question arises whether DNA damage is solely responsible for Cur + Ola mediated cell death and/or inhibition of DNA repair also contribute to Cur + Ola driven cell death. To address this question, at first, some of the major DNA repair involved proteins expression was checked. There was no significant alteration in the expression of RAD51 and DNA-PKcs (representative HR and NHEJ pathway intermediates) were found in all the treatments. Interestingly, 10 fold down-regulation of POL-β (BER component) was noted in the Cur + Ola treated cells as compared to control. Further to confirm the involvement of Cur and Ola in BER, other pivot BER-involved proteins (WRN, FEN-1, APE-1, DNA ligase III and XRCC-1) were checked. The expressions of WRN, FEN-1, APE-1, DNA ligase III and XRCC-1 were found to be decreased approximately by 3, 5, 10, 5 and 2 fold, respectively in combination treatment compared to control. Moreover, it was also noted that the expression of PARP-1 induced by 4 fold compared to control in Cur treated cells which might be due to the extensive DNA damage caused by Cur. But almost 40 fold decreased expression of PARP-1 was observed in the combination treatment of Cur and Ola in comparison to Cur treatment (Fig. 4a). Secondly, plasmid-based In vivo BER assay was performed to measure BER activity. Here in the combination treatment, approximately 3 fold ($p < 0.005$) and 2 fold decreased luciferase activity in U-p21p (SP-BER) and R-p21p (LP-BER) transfected cells was observed respectively. Decreased luciferase activity in both U-p21p and R-p21p after Cur + Ola treatment further supported that the combination of Cur and Ola inhibits both SP and LP mediated BER (Fig. 4b). A similar observation was noted in SCC-25 cells after treatment with drugs (Supplementary Fig. 3).

It is known that PARP-1 is an essential enzyme for DNA repair in cancer cells and it works through PARylation to form PAR and recruit DNA damage repair enzymes to DNA damage site for the repair [36]. So we wanted to check whether Cur mediated damage caused PARylation and addition of Ola inhibit the PARylation process by inhibiting PARP-1 activity at the site of DNA damage. It was noted that there was 1.5 fold increased PARylation (PAR product) after 5 min exposure of Cur to H-357 cells, where the expression of PAR was found to

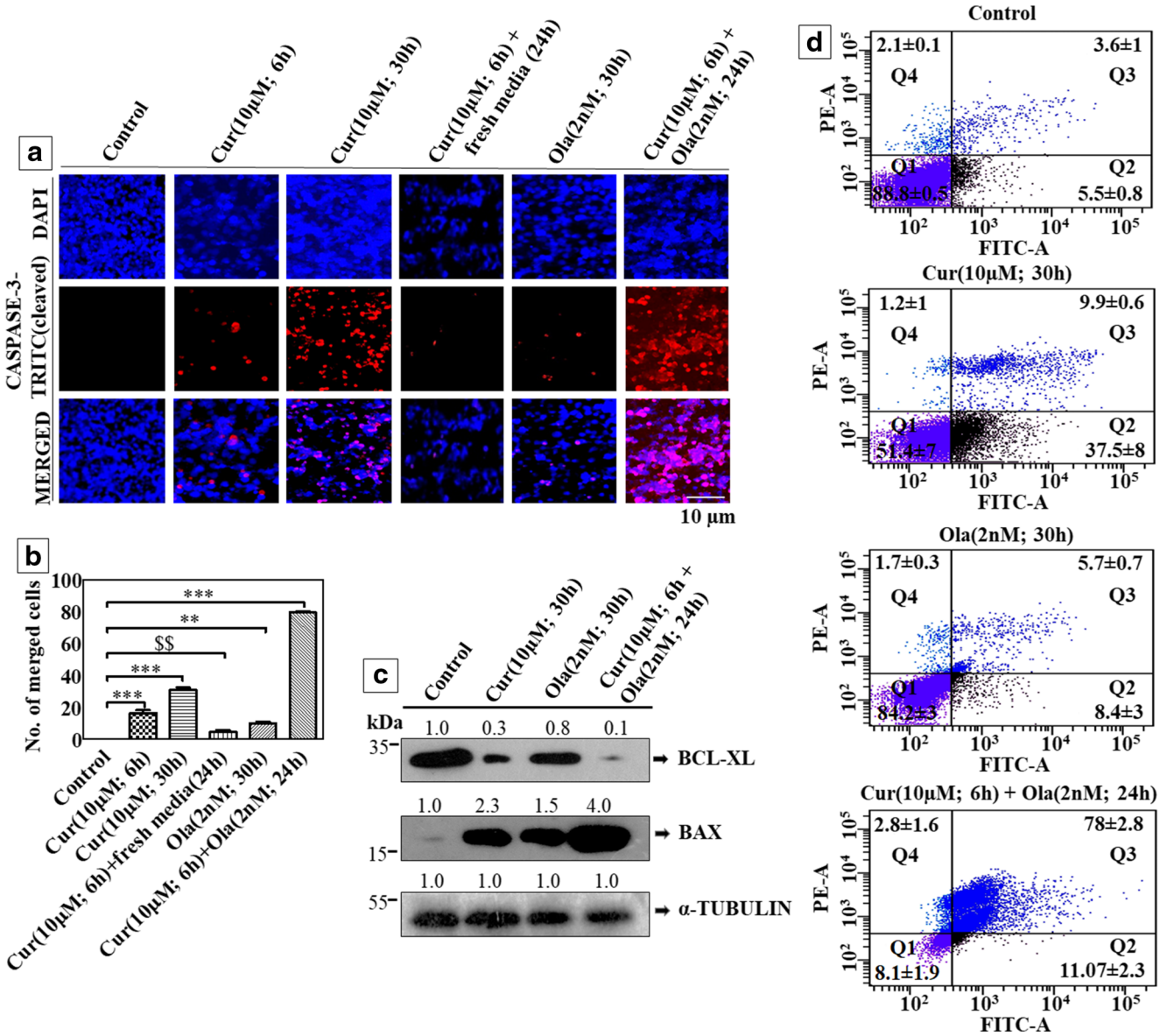

Fig. 3 Cur, Ola and their combination treatment caused apoptosis in Oral cancer cells. **a** Images represent immunocytochemical staining of cleaved CASPASE-3 expression in 30 h treated H-357 cells**.** Scale bar is 10 μm. **b** Quantitative representation of CASPASE-3 expression in merged cells (DAPI and cleaved CASPASE-3-TRITC). Statistical significance were determined using one-way ANOVA; where ***, ** represents statistical significant ($p < 0.0001$ and $p < 0.005$ respectively) and $$ represents non-significant ($p > 0.05$) data compared to control. Here, data represent the mean ± SD of three independent experiments. **c** Protein expression of apoptotic markers in Cur, Ola and Cur + Ola treated H-357 cells. α-TUBULIN served as a loading control. The numerical values above each blot represent the relative fold change with respect to control. Blots were the representative of three independent experiments. **d** Analysis of apoptosis by Annexin-V-FITC/PI dual staining in H-357 cells after treating with Cur, Ola and their combination using FACS. The data was one of the representatives of three independent experiments

be 6 fold higher after the 10 min exposure of Cur as compared to untreated control. Interestingly, 2 and 3 fold reduction of PAR was seen after 5 and 10 mins (respectively) exposure of Ola in Cur pre-treated (10 mins) cells (Fig. 4c). A similar observation was noted in SCC-25 cells after treatment with drugs (Supplementary Fig. 4). The interaction between PARP-1 and FEN-1 was not found to be altered significantly. It is reported that PARP-1, XRCC-1, POL-β and APC physically interact with each other in BER Pathway [20]. Our data also indicated that PARP-1 might interact with FEN-1 in the BER pathway (Fig. 4c). All together, our data suggested that Cur exposure caused DNA damage and Ola further increased the damage through inhibition of BER pathway.

Cur and Ola Combination Reduced the Tumor Volume in Xenograft Mice

To confirm the anti-cancer activity of Cur, Ola and their combination In vivo, experiments were carried out in xenograft mice model. After 10 days of implantation of H-357 cells,

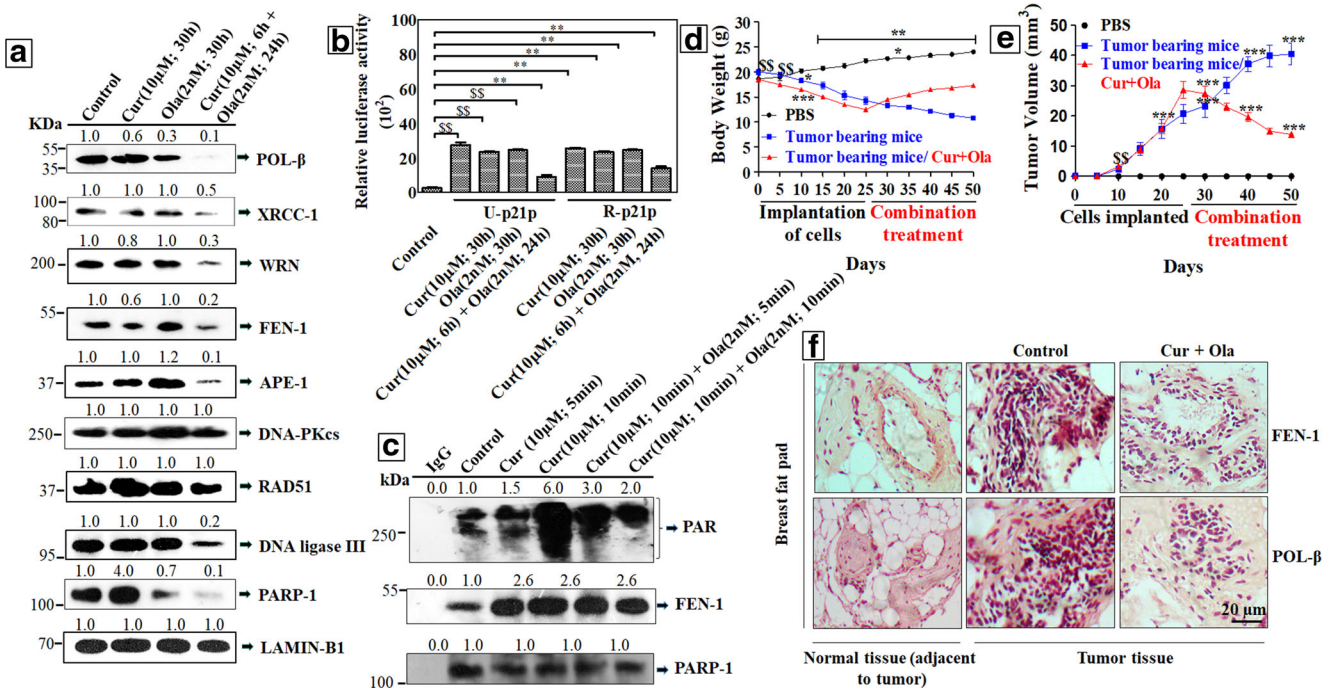

Fig. 4 Combination treatment of Cur and Ola de-regulate the BER pathway in oral cancer cells and reduced tumor volume in xenograft mice. **a** Shows expression of major DNA repair-related proteins in nuclear lysates of Cur, Ola and Cur + Ola treated H-357 cells. LAMIN- B1 served as a loading control. The numerical values above each blot (relative fold change with respect to control) were measured by densitometer. Blots were the representative of three independent experiments. **b** Bar graph representing the relative luciferase activity of U-p21p and R-p21p DNA transiently transfected in H-357 cells after exposure to Cur, Ola and their combination. Control represents the non-transfected cells. Statistical significance was determined using one-way ANOVA; where '**' represents statistical significant at ($p < 0.005$) and $$ represents non-significant ($p > 0.05$) data. Here, data represent the mean ± SD of three independent experiments. **c** Protein expression of PAR and FEN-1 in Cur, Ola and Cur + Ola treated PARP-1 immunoprecipitated H-357 cells. PARP-1 served as the loading control. IgG represents as a negative control. The numerical value above each blot was measured by densitometer and showed the relative fold change with respect to control. **d** Graphical representation of changes in average body weight of mice after implantation of H-357 cells and then, treating with the drugs. **e** Data represents changes in the tumor volume after administration of the drugs. Statistical significances were determined using one way ANOVA; where '***' represents statistical significant ($p < 0.0001$) and $$ represents as non-significant ($p > 0.05$) data. Data represent the mean ± SD of three independent experiments. **f** Immunohistochemical expression of FEN-1 and POL-β in tissues of untreated and combination of Cur and Ola treated mice. Images used here were representative of three independent experiments. Scale bar is 20 μm

tumor formation was noticed. The body weight of the animals was significantly reduced and the tumor volume was increased day-by-day. After 25 days of administration of cells, tumor volume was significantly increased ($p < 0.0001$) and the body weight of animals was reduced ($p < 0.0001$). The treatment was done for 25 days for every alternative day. It was noticed that there was little recovery from lost body weight and little reduction of tumor volume was noted at the end of Cur and Ola individual treatment (Supplementary Fig. 5). Interestingly, animals significantly gained their lost body weight ($p < 0.0001$) and a significant reduction of tumor volume ($p < 0.0001$) was also observed after Cur + Ola treatment (Fig. 4d and e). Then, animals were sacrificed and the tumor tissue was removed for further experiments. The expression of some representative BER proteins (FEN-1 and POL-β) was monitored by immunohistochemistry using tumor sample. The expression of POL-β and FEN-1 was increased in cancer tissue but significantly reduced after Cur + Ola treatment (Fig. 4f).

Further, tumor tissue was lysed and processed for western blot and PARylation experiments. Cur and Ola combination mediated apoptosis was checked by measuring the protein expression level of BAX, BCL-XL and cleaved CASPASE-3 in mice tissue lysates. 25 fold increased BAX/BCL-XL ratio and 3 fold enhanced expression of cleaved CASPASE-3 were noted in Cur + Ola treated tumor tissue lysate in comparison with untreated tumor (Fig. 5a). Finally, the PARylation activity was measured after Cur + Ola treated tumor tissue lysate. Here also, approximately 4 fold rise in PAR expression was found in Cur treated tumor tissue, where the complete reduction of PAR product was noted after Cur + Ola treatment in tumor tissue lysates (Fig. 5b).

Discussion

Although the combination of PARP inhibitors with synthetic DNA damaging agents inhibit the growth of cancer cells and

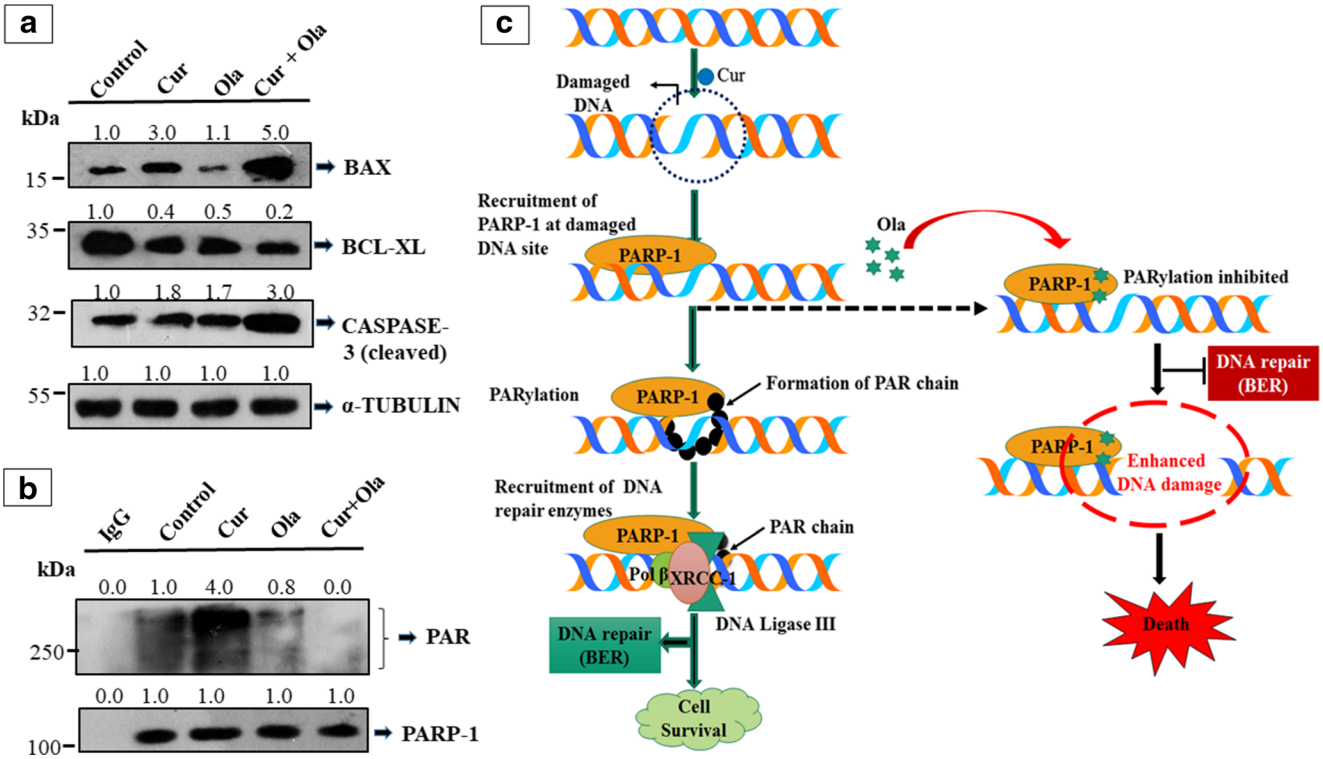

Fig. 5 Cur and Ola treatment caused apoptosis in xenograft mice. **a** Expression of pro-apoptotic and anti-apoptotic proteins in tissue lysate after drug treatment to xenograft mice. α-TUBULIN served as a loading control. The numerical value above each blot represents the relative fold change with respect to control measured by densitometer. Blot was the representative of three independent experiments. **b** PAR formation in tissue lysate after Cur, Ola and their combination treatment to tumor mice. The numerical value above each blot represents the relative fold change with respect to control measured by densitometer. **c** The schematic representation of the probable mechanism of Cur + Ola mediated apoptosis

some of them are in a clinical trial and/or clinically approved against specific cancers [26] but their toxicity, off-target impact, resistance, etc. are the real clinical issues [27, 37]. In cancer treatment, non-toxic, bioactive, DNA damaging drug in combination with PARP inhibitor would avoid the up-regulation of BER, NHEJ, HR and multiple drug-resistant (MDR) proteins to overcome drug resistance [3, 38]. Here, we investigated the anti-cancer effect of Cur in combination with Ola in oral cancer cells (In vitro) and mice xenograft model system (In vivo). Cur + Ola caused significant cell death in not only oral cancer cells but also other cancer cells too. This combination is relatively non-toxic to non-cancerous cells (non-transformed breast epithelial cells) but synergistically caused cell death in oral cancer cells (Fig. 1). Cur caused a decrease in cell viability and post-treatment of Ola in Cur-treated cells induced the process which ultimately resulted in more cell death (Fig. 1). Comet assay, immunocytochemical staining of representative DNA damage and apoptotic markers, Annexin-V-FITC/PI dual staining and western blot analysis confirmed that Cur caused DNA damage and post-treatment of Ola in Cur-treated cells caused persistence of unrepaired damaged DNA, which ultimately leads the oral cancer cells towards apoptosis (Figs. 2 and 3). Combination of Cur and Ola also resulted in inhibition of topoisomerase activity which further disturbed the replication process of cells and ultimately led to apoptosis. Reduction of tumor volume and recovering from lost body weight, increased BAX/BCL-XL ratio and decreased BER protein expression (immunohistochemistry and western) after administration of Cur + Ola in tumor-bearing mice also suggested that this combination caused apoptosis in In vivo too (Figs. 4 and 5). Thus, data revealed that Cur caused DNA damage and Ola inhibited endogenous DNA repair system which ultimately increased the Cur mediated DNA damage, as a result cells undergo apoptosis.

Unchanged expression of RAD51 and DNA-PKcs suggested that there was no involvement of HR and NHEJ pathway in Cur and Cur + Ola mediated cell death (Fig. 4a). Down regulation of POL-β, APE-1, FEN-1, DNA ligase III and WRN indicated the involvement of BER in Cur and Cur + Ola mediated apoptosis in H-357 cells. Alteration in POL-β, XRCC-1, DNA ligase III and FEN-1 expression in western blot analysis as well as decreased in luciferase activity of Cur + Ola treated U-p21p and R-p21p transfected H-357 cells indicated the involvement and inhibition of both SP and LP-BER (Fig. 4a and b).

Activation of PARP-1 during extensive DNA damage showed the formation of PAR polymers from NAD^+ which

vary in length from 16 to 60 ADP-ribose polymers, depending upon the amount of genomic stress [39, 40]. Auto modification of PARP-1 increased the accessibility of DNA repair protein complexes (e.g. DNA POL-β, XRCC-1, DNA ligase III and FEN-1) and recruited them to the damaged site; hence, the damaged DNA gets repaired. Cur treated H-357 cells showed the formation of smaller PAR polymer after 5 min of treatment which increased approximately by 6 fold with increasing duration of treatment (10 min) and it allowed us to demonstrate the action of Ola. It was observed that Ola was able to inhibit Cur induced PARylation which further validated the role of Ola as PARP-1 inhibitor (Fig. 4c). It was reported that an overwhelming amount of DNA damage and inhibition of PARP-1 activity have some significant impact on the existence of AP sites in which PARP-1 may form DNA protein crosslink on this site [36]. In line with this report, our data suggests Cur caused extensive DNA damage, Ola inhibits PARylation which might cause PARP-1 trap in the chromatin and hence, needs further study to check the formation of PARP-1- DNA cross-links due to the above reasons.

Finally, we have provided a plausible explanation of the anti-cancer action of Cur + Ola treatment in oral cancer cells (Fig. 5c). Short exposure to Cur caused DNA damage in cells. Generally, this type of DNA damage is repaired by the cell internal DNA repair system. In the DNA damage site, PARP-1 is activated and make a PAR polymer and recruit DNA repair proteins to repair the damaged DNA. But in the presence of PARP inhibitors, PARP-1 is unable to form PAR chain as a result DNA damage increased. The enhanced irreparable DNA damage ultimately leads to apoptosis.

In summary, our result suggested that Ola induced DNA damaging ability of Cur via inhibition of PARP-1 activity and ultimately caused apoptosis through compromising the BER pathway.

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Authors' Contribution Sefinew Molla carried out most of the experiments. Subhajit Chatterjee, Krushna Chandra Hembram, Deepika Nayak, Chinmayee Sethy and Rajalaxmi Pradhan help to analyze the data and writing the draft of the MS. Chanakya Nath Kundu conceived the idea design experiments and wrote final MS.

Compliance with Ethical Standards

Conflict of Interest Authors declare that there are no conflicts of interest.

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