Synthetic antioxidants from a natural source can overtake the oncogenic stress management system and activate the stress-sensitized death of KSHV-infected cancer cells

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Abstract. Synthetic and modified natural derivatives are reported as potential bioactive compounds and are being used therapeutically against various diseases in a widespread manner nowadays. Cancerous cells exhibit high levels of reactive oxygen species (ROS) internally, and thus successfully manage to sustain themselves and proliferate via antioxidative mechanisms that maintain a redox balance. On this note, various antioxidants are applied as anticancer compounds, which strategically affects the ongoing oncogenic stress management system in both a pro- and antioxidative manner, resulting in cancer restriction, as well as sustaining cell proliferation via antioxidative mechanisms that promote cancer progression. Alike non-viral cancers, viral cancers exhibit varying levels of ROS during different stages of cancer progression. Hence, successful stress balance should be addressed, depending on the cancer cell stress response during the therapeutic management. The application of antioxidants is crucial and needs to be carefully designed in such cases; the respective underlying mechanisms are less understood. The role of antioxidants controlling the varied levels of stress response at different stages of Kaposi's sarcoma-associated herpes virus malignancy have not been fully reported. Therefore, the present study aimed to analyze the activity of certain antioxidants in KSHV-infected oncogenic cells. For this purpose, two naturally derived flavonoid-based antioxidants (theaflavin and novel curcumin derivatives) were selected and tested in different KSHV-infected cell lines. The findings presented herein demonstrate that these compounds can successfully induce the death of different KSHV-positive cells and can restrict the growth of KSHV-infected cell lines restricting viral reactivation by counteracting the oncogenic stress management system.

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Introduction

Acquired immunodeficiency syndrome (AIDS)-associated malignancies are a major cause of co-morbidity among patients with AIDS worldwide. Kaposi's sarcoma-associated herpes virus (KSHV)/human herpesvirus 8 (HHV-8) is an important herpes viral agent associated with viral malignancy, related to HIV-AIDS (1,2). In the case of KSHV, both the latency and lytic phases play critical roles in the process of oncogenesis, as with other viral malignancies. In a KSHV-associated malignant environment, the virus maintains a delicate spontaneous balance between these two phases. Depending on the need for the maintenance of the oncogenic process, the virus can switch between the two phases and maintain a persistent infection inside the host in response to varied stress exposures by controlling several cellular events (3,4). Several factors influence this lytic-latency balance in a KSHV-infected tumor microenvironment. Reactive oxygen species (ROS) have been found as the key determining factor for maintaining the balance between the two phases of the KSHV life cycle at different stages of KSHV infection. In response to varying levels of intracellular ROS, KSHV can modulate several cellular ROS regulatory pathways required for malignancy (5). The virus strategically maintains a moderate ROS level during latency, allowing unregulated cell proliferation. While there is a slight increase in the ROS level, the virion senses it and converts it towards lytic reactivation. This avoids the maintenance of a stressed cell; the virus utilizes the stress influence to secure its own persistence. The replicative cells again utilize the high ROS level and infect new healthy cells (6,7). KSHV has been found to overread the cellular mechanisms under hypoxic conditions and perform hypoxia-induced viral reactivation. During this process, viral and cellular replication machineries are strategically protected from KSHV-encoded latency-associated nuclear antigen (LANA) cellular degradation. This interactive understanding of the hypoxic stress response in virus-associated oncogenesis clearly reveals a continuous oncogenic stress management system, which is maintained inside the KSHV-infected cell for its pathogenesis (8,9).

Due to this viral stress responsive strategy, a number of potent anticancer drugs are not applicable against

KSHV-associated malignancies. A number of cytotoxic drugs are capable of inducing significant cell death in KSHV malignant cells; however, in parallel, they can reactivate the virus by moderating the increase in cellular ROS levels (10). Alternatively, cytotoxic drugs can induce the cell death process, triggering oxidative stress, which exerts a toxic effect on the normal cellular environment. Therefore, the long-term chemotherapeutic application of such drugs results in severe side-effects in the case of several malignancies, including KSHV-associated ones (11,12). Thus, potential targeted therapeutic strategies need to be developed against the 'cancer cell oxidative stress balancing system', which can effectively break the ROS homeostasis inside the cancer cell, along with a minimal effect of chemotherapeutic stress on the body.

Antioxidants have been reported as oxidative stress balancers, which can function both as pro- and antioxidant agents and are widely applied in anticancer therapeutics (13). On this note, some naturally derived bioactive compounds are well reported as ROS scavengers. However, in the majority of cases, it has been observed that either these compounds are used as adjuvants or are supplemented along with chemotherapy (14,15). Due to a lack of knowledge about their stress balance mechanisms in an oncogenic environment with different ROS levels during cancer and chemotherapy, these natural antioxidants are very limited as regards their clinical applicability. The application of ROS scavengers is dependent on the status of ROS in cancer cells and the malignant environment, as they can be associated with cancer progression and cancer inhibition (13). KSHV-infected malignant cells have a flexible stress management system of the virus, which maintains a lytic-latency balance for a continuous oncogenic process, and may be an ideal model which may be used to understand the role of ROS scavengers as anticancer compounds. The present study aimed to determine the mechanisms through which two very common flavonoid-based antioxidant compounds from natural sources with an effective ROS scavenging capacity can respond against the critical KSHV oncogenic stress balance strategy, and lead to a potential anticancer therapeutic effect.

Materials and methods

Cell lines and cell culture. Two KSHV-infected malignant cell lines (JSC-1 and BC-3) and a non-KSHV cell line (293T) were used in the present study; all cell lines were kindly provided by Dr Erle Robertson from University of Pennsylvania (Philadelphia, PA, USA) and Dr David Blackbourn, from the University of Surrey (Guildford, UK). The non-KSHV control cell line, BJAB, was collected from Dr Avhik Saha (Presidency University, Kolkata, India) and Dr Avra Roy (Calcutta University, Kolkata, India). The cells were grown and maintained in complete RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS and 1% penicillin/streptomycin in 5% CO_2 at 37˚C in a humidified atmosphere. Contamination-free cell lines at passage number 3-10 after thawing were used in the experiments.

Peripheral blood mononuclear cell (PBMC) isolation and processing. PBMCs were isolated using Histopaque (RNBB8622; MilliporeSigma) from blood collected from healthy individuals. The selected healthy individuals were had an average age of 33 years, had a proper height-to-weight ratio (a standard/ideal weight according to an individual's height) and the following clinicopathological characteristics: Hematological parameters (range): Red blood cells (mill/cmm), ~3.9; white blood cells (Cmm), ~6,500; hemoglobin (g/dl), ~12.9; platelets (Lakh/cmm), ~2.88; neutrophils (%), ~60.29; lymphocytes (%), ~32.83; eosinophils (%), ~4; monocytes (%), ~3.36; basophils (%), 0. Liver parameters (range): Cholesterol, ~128.6; aspartate aminotransferase (U/l), ~25; alanine aminotransferase (U/l), ~17; albumin(g/dl), ~4.53. Renal parameters (range): Creatinine, ~0.66; total protein (g/dl) ~7.2; blood glucose (mg/dl), ~98. The PBMCs were maintained in complete RPMI-1640 medium for 24 h. Both the treatment and non-treatment conditions used for KSHV-positive cell lines were maintained for these PBMCs. During FACS analysis, the lymphocyte population was gated from whole PBMCs by the bivariate FSC vs. SSC dot plot. The gated population was used for flowcytometry-based cell death analysis.

Chemical compounds. For the present study, two flavonoid-based naturally derived compounds were selected. Theaflavin was purchased from MilliporeSigma (tea extract from *Camellia sinensis*, T5550, contains ≥80% theaflavins) and a stock solution of 5 mg/ml was prepared by dissolving it into 50% EtOH in distilled water. The other group of flavonoids, the novel curcumin derivatives (the Cl and F derivatives) were used and were synthesized from mother curcumin (C1386; MilliporeSigma). A stock solution (5 mg/ml each) was prepared in a similar manner by 50% EtOH in a distilled water solution for the following experimental purposes. The detailed synthesis process and structure of a series of curcumin derivatives has been previously described (16); the two compounds, 2-Amino-4-(4-chlorophenyl)-5-((*E*)-3-(4-hydroxy-3-methoxyphenyl)acryloyl)-6-((*E*)-4-hydroxy-3-methoxystyryl)-4*H*-pyran-3-carbonitrile (Cl derivative) and 2-Amino-4-(4-fluorophenyl)-5-((*E*)-3-(4-hydroxy-3-methoxyphenyl)acryloyl)-6-((*E*)-4-hydroxy-3-methoxystyryl)-4*H*-pyran-3-carbonitrile (F derivative) were selected from this series for the present study.

Establishment of stress induced in vitro cell line model. The KSHV-associated malignancy cycle is completely dependent on a fine balance between the maintenance of the viral latency and lytic phases inside the cell (17,18). This fine biphasic balance maintenance is stress responsive. A transient serum stress *in vitro* model was thus utilized in different experiments to analyze this varied stress response in a natural manner resembling the oncogenic stress management system during KSHV malignancy. For the present study, the latently infected KSHV cells (B lymphocytes) were grown in serum-free (without FBS) RPMI-1640 medium for 24 h. The surviving cells were revived by growing in complete RPMI-1640 (+10% FBS) medium for the following 24 h. These stressed and recovered KSHV cells (as an *in vitro* model) were used in further experiments with all three different drug treatments at their respective concentrations. A similar cell line-based stress induced model for viral malignancy was previously used in HIV-associated cancers (19). It has been observed that alterations in serum levels in the growth medium affects various virus-associated factors, such as infectivity, replication etc., in virus-infected cell lines (20,21). Thus, the stress-altered

cell line model may be a useful model which may be used to understand the stress responsive behavior of KSHV-infected oncogenic cells and to examine any alteration(s) with antioxidant treatments.

Cell viability assay. Different KSHV-infected cell lines in treated and non-treated conditions [use of antioxidants at various concentrations (10, 20, 30, 40 and 50 μg/ml) and time durations (6, 12 and 24 h) were subjected to cell viability assay. Cells ($1x10^6$) in each set treated and incubated (37˚C in 5% CO_2) for this analysis. These cells were then washed with PBS and incubated with 50 μg/ml propidium iodide (PI) at room temperature for 15 min. Following treatment, the cells were analyzed using a Bio-Rad ZOE fluorescent cell imager (by air drying the treated suspension cells over slides) red channel (excitation, 556/20 nm; emission, 615/61 nm; Bio-Rad Laboratories, Inc.) and a flow cytometer (BD FACSVerse; BD Biosciences) in a specific PI channel. The percentage of PI-positive cells was measured with the loss of membrane integrity and viability compared to untreated (EtOH-treated) control cells using BD FACSuite™ software (version 1.0.6; BD Biosciences).

Annexin V-FITC apoptosis detection using flow cytometry. KSHV cells from different treatment sets and control cells were incubated (37˚C in 5% CO_2) for 24 h with their effective low concentration and a comparatively high effective concentration (low concentrations: Theaflavin, 30 μg/ml; curcumin derivatives, 10 μg/ml; high concentrations: Theaflavin, 50 μg/ml; curcumin derivatives, 30 μg/ml). Following incubation, they were washed with PBS and subsequently prepared for Annexin V-PI staining following the manufacturer's protocol (FITC Annexin V apoptosis detection kit, 51-6710AK, BD Biosciences). The cells were then analyzed using a BD FACSVerse flow cytometer and data were analyzed using BD FACSuite™ software (version 1.0.6) by the FITC vs. the PI densitometry plot. To examine specific the caspase-mediated cell death process, the caspase inhibitor, Z-VAD-FMK (50377, BD Biosciences), was used along with the different treatments.

ROS determination. KSHV-infected and non-KSHV cells (in both the latent condition and exposed to serum stress) were seeded with or without various concentrations doses of the drugs (the three compounds) and the FDA-approved antioxidant, N-acetyl cysteine (NAC; MilliporeSigma) for different periods of time. Subsequently, following incubation (37˚C for 6, 12 and 24 h), the cells were washed in PBS and resuspended in serum-free RPMI-1640 medium with 50 μM 2',7'-dichlorofluoresin diacetate (DCFH-DA; D6883; MilliporeSigma) for 30 min at 37˚C in the dark. Following incubation, the fluorescence intensity of DCFH-DA was determined using a flow cytometer (BD FACSVerse; BD Biosciences) at 488 nm excitation and 530 nm emission (FITC channel).

Cell cycle detection. To analyze the different cell cycle stages, the treated and non-treated KSHV (latent, serum starved and revived) cells were incubated (24 h at 37˚C in 5% CO_2) with or without the respective drugs. They were then washed with PBS and fixed with chilled 70% EtOH on ice for 15-30 min. Following fixation, the cells were washed with PBS again. The cells were then stained with 10 μg/ml PI in Triton X-100 (RM845; HiMedia) buffer and RNase (DS0003; HiMedia) for 30 min at room temperature and examined using a BD FACSVerse flow cytometer. The DNA contents at different phases of the cell cycle were assessed using BD FACSuite™ software (version 1.0.6).

Mitochondrial membrane potential analysis. Latent, serum stress-exposed cells were subjected to the various treatment sets for different periods of time followed by analyses using a flow cytometry-based mitochondrial membrane potential detection kit (BD Mito Screen, JC-1 kit, 55132; BD Biosciences). The cells were washed with PBS and then processed for JC-1 staining following the manufacturer's protocol. The cells were then examined using a BD FACSVerse flow cytometer and analyzed using BD FACSuite™ software (version 1.0).

Autophagy detection. Treated and untreated cells were incubated for different periods of time (12 and 24 h) with theaflavin, Cl and F curcumin at their respective concentrations and stained with monodansylcadaverine (MDC; BCBX0277; MilliporeSigma) at 50 μM for 30 min and the fluorescence intensity was measured using a Bio-Rad ZOE fluorescent cell imager (by air drying the treated suspension cells over slides) in the blue channel (excitation, 355/40 nm; emission, 433/36 nm) and a flow cytometer (BD FACSVerse) at a wavelength of 488 nm. The autophagy inhibitor, bafilomycin-A1 (B1793; MilliporeSigma; 20 nM working concentration) was used to mark the specific autophagic process inside the cell, following different treatment conditions in stress-exposed and revived cells.

Western blot analysis. Cells were lysed using RIPA buffer containing protease inhibitor cocktail (P8340, Millipore Sigma) and following centrifugation (14,000 x g for 15 min at 4˚C), the supernatant containing protein samples was collected. The protein concentration was determined using a Lowry protein assay kit (23240, Thermo Fisher Scientific, Inc.). An equal amount of protein lysate from each sample (~20 μg) for each lane was loaded and subjected for separation by SDS-PAGE electrophoresis. Following transfer to a nitrocellulose membrane, the membrane was blocked (using 5% skim milk at room temperature for 1 h) and probed with specific primary antibodies (overnight incubation at 4˚C) followed by HRP-tagged secondary antibody (1 h of incubation), at room temperature. Following proper incubation, the membrane was visualized using a Chemidoc XRS+ image system (Bio-Rad Laboratories, Inc.) using Bio-Rad ECL chemiluminescent substrate and captured using Image Lab software (version 5.2). The intensity of the protein band was analyzed and normalized to the loading control (mouse mAb GAPDH; 1:1,000, cat. no. sc-166545, Santa Cruz Biotechnology, Inc.) and finally with the untreated control using ImageJ software (version 1.8.0, National Institutes of Health). Primary antibodies against rabbit mAb BAX (cat. no. 2772T), rabbit mAb Bcl-2 (cat. no. 4223T), mouse mAB p53 (cat. no. 2524S) (all from Cell Signaling Technology, Inc.) were used at a dilution of 1:1,000. Goat anti-S_6 K (S_6 kinase) antibody was obtained from Boster Biological Technology (cat. no. A0147, 1:500 dilution).

Rabbit anti-Beclin1 (6176, IMGENEX India Pvt Ltd.), mouse anti-p21 (80191; IMGENEX India Pvt Ltd.) were used at a dilution of 1:1,000. Mouse mAb anti-checkpoint kinase (Chk)-1 (sc-8408) and mouse mAb anti-Chk-2 (sc-5278) were obtained from Santa Cruz Biotechnology, Inc. (1:1,000 dilution). 12% SDS PAGE was used for identifying these proteins. For the detection of the autophagy representative marker protein, LCIIIB, rabbit pAb anti LCIIIB was used (cat. no. NB100 2220; Novus Biologicals, Ltd.) at a dilution of 1:500 with 10% SDS PAGE. Anti-mouse (cat. no. 11-301; Abgenex); anti-rabbit (cat. no. AS014; ABclonal Biotech Co., Ltd.); anti-goat (cat. no. 20401; Imgenex) HRP-tagged secondary antibodies were used at a 1:10,000 dilution.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA from different KSHV-associated cell lines with various treatment and experimental conditions were isolated using the TRIzol-based method (TRI reagent; T9424; MilliporeSigma). cDNA was synthesized from isolated cellular RNA using the reverse transcription method according to the manufacturer's protocol (Bio-Rad iScript cDNA Synthesis kit; cat. no. 170-8891; Bio-Rad Laboratories, Inc.). The cDNA was then subjected to amplification using specific primers (LANA forward, 5'-CATACGAACTCCAGGTCTGTG-3' and reverse, 5'-GGTGGAAGAGCCCATAATCT-3'; vFLIP K13 forward, 5'-GGATGCCCTAATGTCAT GC-3' and reverse, 5'-GGCGATAGTGTTGGAGTGT-3'; replication and transcription activator (RTA) forward, 5'-CAGACGGTGTCA GTCAAGGC-3' and reverse, 5'-ACATGACGTCAGGAA AGAGC-3'; and GAPDH forward, 5'-CCACATCGCTGA GACACCAT-3' and reverse, 5'-TTCCCGTTCTCAGCCTTG AC-3') used in a previous study, purchased from Integrated DNA Technologies (22,23). qPCR was performed done using SYBR-Green Supermix (Bio-Rad iTaq Universal SYBR-Green Supermix, cat. no. 172-5124, Bio-Rad Laboratories, Inc.) on a Bio-Rad CFX connect Real-Time PCR System. Thermal cycling has been performed with an initial denaturation at 95˚C for 3 min, following 40 cycles of 95˚C for 10 sec; 55˚C for 30 sec and 72˚C for 30 sec. mRNA expression levels were analyzed using Bio-Rad CFX Manager with the $2^{-\Delta\Delta Cq}$ method from the fold change triplicate values, considering the fold change of untreated cells as the fold induction (24). The expression for each was normalized using GAPDH as the reference gene.

Statistical analysis. All statistical analyses were performed using Graph Pad Prism software (version 6; GraphPad Software, Inc.). The data presented are the average of obtained from triplicate values for each independent experiment repeated three times. The differences between the means of two groups were analyzed using an unpaired t-test and ordinary one-way ANOVA used to analyze differences between multiple groups. Two-way ANOVA was performed followed by Bonferroni's post hoc test to assess statistical significance, in the case of analyzing comparisons or interrelationships between more than two set of groups/multiple set of groups. A probability (P)-value <0.05 was considered to indicate a statistically significant difference. All the statistical values in the different experiments represent the mean ± SD, where n=3.

Chemical structure of the compounds. The 3D structure of theaflavin was obtained from PubChem (ID 135403798). The structure of the novel curcumin derivatives (Cl and F) was drawn using ChemDraw (16).

Results

The compounds exert cytotoxic effects on and the efficient caspase-mediated apoptosis against different KSHV cell lines. In order to examine whether the selected flavonoid compounds exert any cytotoxicity against KSHV-positive cells, a cell viability assay was performed to determine cell cytotoxicity in a concentration- and time-dependent manner, with all three compounds in the preliminary screening. Treatment with all three compounds efficiently led to a decrease in cell viability in a concentration- and time-dependent manner. The *in vitro* PI cytotoxic assay clearly revealed that all three compounds exerted significant cytotoxic effects against two different KSHV-infected cell lines compared to the control (Fig. 1A and B).

The FITC Annexin-V assay clearly demonstrated that the cell death process was mediated through caspase-dependent mechanisms in the presence of all three compounds. The densitometry plots of the concentration-dependent treatment indicated the gradual shifting of the pro-apoptotic population towards late apoptosis. These plots also demonstrated that the caspase inhibitor with three of the test compounds successfully restricted the late-stage apoptotic event (Fig. 1C and D). The bar charts representing the comparative assessment of apoptosis with the inhibitor, indicate a significant increase in the apoptotic cell population in the treated sets only, which was decreased with the inhibitor (Fig. 1C and D). These results strongly suggest that both theaflavin and curcumin derivatives are capable of inducing the specific caspase-mediated death of KSHV-infected cells. All the compounds led to significant cell death from their starting concentrations to the higher concentrations (apart from the 10 μg/ml concentration of theaflavin against JSC-1 cells). Thus, among these concentrations, considering the more significant results, the minimum effective concentrations (low dose) and higher effective concentration (almost 50% cell death; high concentration) for these drugs can be predicted from the aforementioned results. For theaflavin, the lower concentration of 30 μg/ml can be the starting level for the cell death process, which reaches >50%, and the effective concentration is 50 μg/ml. The concentrations were comparatively much lower for the curcumin derivatives, starting from 10 μg/ml with the significant effectiveness being observed and reaches high at 30 μg/ml. Thus, for theaflavin, the effective low concentration was selected at 30 μg/ml and the effective high concentration at 50 μg/ml, whereas in the case of curcumin derivatives, the effective low concentration was selected at 10 μg/ml. and the effective high concentration at 30 μg/ml. The threshold timeline for the drugs was found to be 12 h, reaching optimum levels at 24 h, and finally decreasing to a constitutive level after 48 h. Thus, in further experiments, incubation for 24 h was selected for the assessment of the drug effectiveness.

The compounds are not cytotoxic against healthy lymphocytes and non-KSHV lymphoma cells. KSHV malignancy has often been found to be associated with HIV. Highly

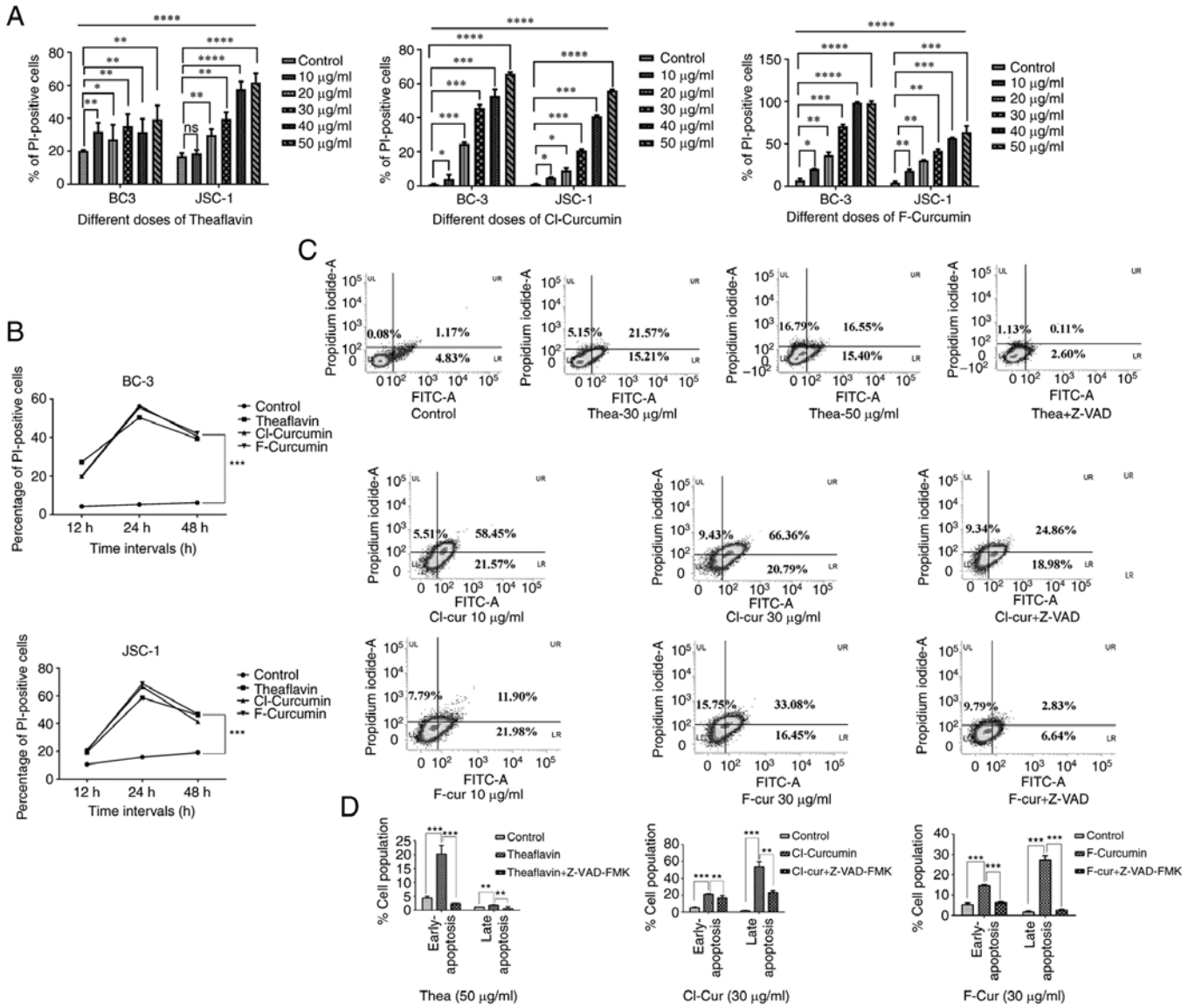

Figure 1. Cell viability and apoptosis assessment in different KSHV-positive cell lines with treatment. (A) Concentration-dependent increased loss of cell viability with an increase in PI positivity with all the three compounds in both BC-3 and JSC-1 cells treated for 24 h. ^{ns}P>0.05, *P≤0.05 **P≤0.01 ***P≤0.001, ****P≤0.0001 significant differences for all data sets vs. the control. (B) Time kinetic increase in cell viability with an increasing PI florescence intensity with all the three compounds at their respective concentrations (theaflavin, 50 μg/ml; both curcumin derivatives, 30 μg/ml) against BC-3 and JSC-1 cells. Significant change in compound treated cells compared to control is indicated by ***P≤0.001, significant differences vs. the control. (C) FITC-Annexin V apoptosis analysis in JSC-1 cells treated with the three compounds represented by densitometry plots (one low dose and one high dose). Apoptosis assay following treatment with the compounds along with Z-VAD-FMK was performed to examine caspase dependency. All the FITC-Annexin V plots with the percentages of the cell population in each section are mentioned, representing the apoptotic event. (D) Bar charts demonstrating the comparative pro- and late apoptotic cell percentage following treatment with the three compounds alone (at their particular effective concentration as indicated) and treatment along with Z-VAD-FMK application compared to the control **P≤0.01 and ***P≤0.001, significant differences vs. control and differences with respect to treatment in the case of Z-VAD). KSHV, Kaposi's sarcoma-associated herpes virus; Thea, theaflavin; Cl-cur, Cl-curcumin; and F-cur, F-curcumin.

active antiretroviral therapy (HART) therapy is the only effective therapeutic option for both cases. However, prolonged HART treatment leads to a stressful effect and creates an extra burden of drug-induced cytotoxic after-effects which hamper the normal cellular environment with a dysfunctional anti-oxidative system along with a distorted immune response. Immune reconstitution inflammatory syndrome (IRIS) is the most common example in such cases (25). This rare incident is associated with the deterioration of the body's response against previous subclinical or present clinical conditions and as a result, the risks of developing further pathogenic co-infections, such as KSHV are augmented (11,26). Thus, the present study then assessed whether the compounds used for treatments exert any cytotoxic effects against normal healthy lymphocytic cells. The isolated PBMCs treated with each of the three test compounds at their effective concentrations for 24 h were subjected to cell death analysis. The lymphocyte population from the control set was isolated (Fig. 2A) and this population was selected for determining the effects of the compounds. The results clearly indicated that the compounds eliminated the chance of any toxic stress burst and helped towards maintaining a normal healthy cellular environment along with treatment (Fig. 2A).

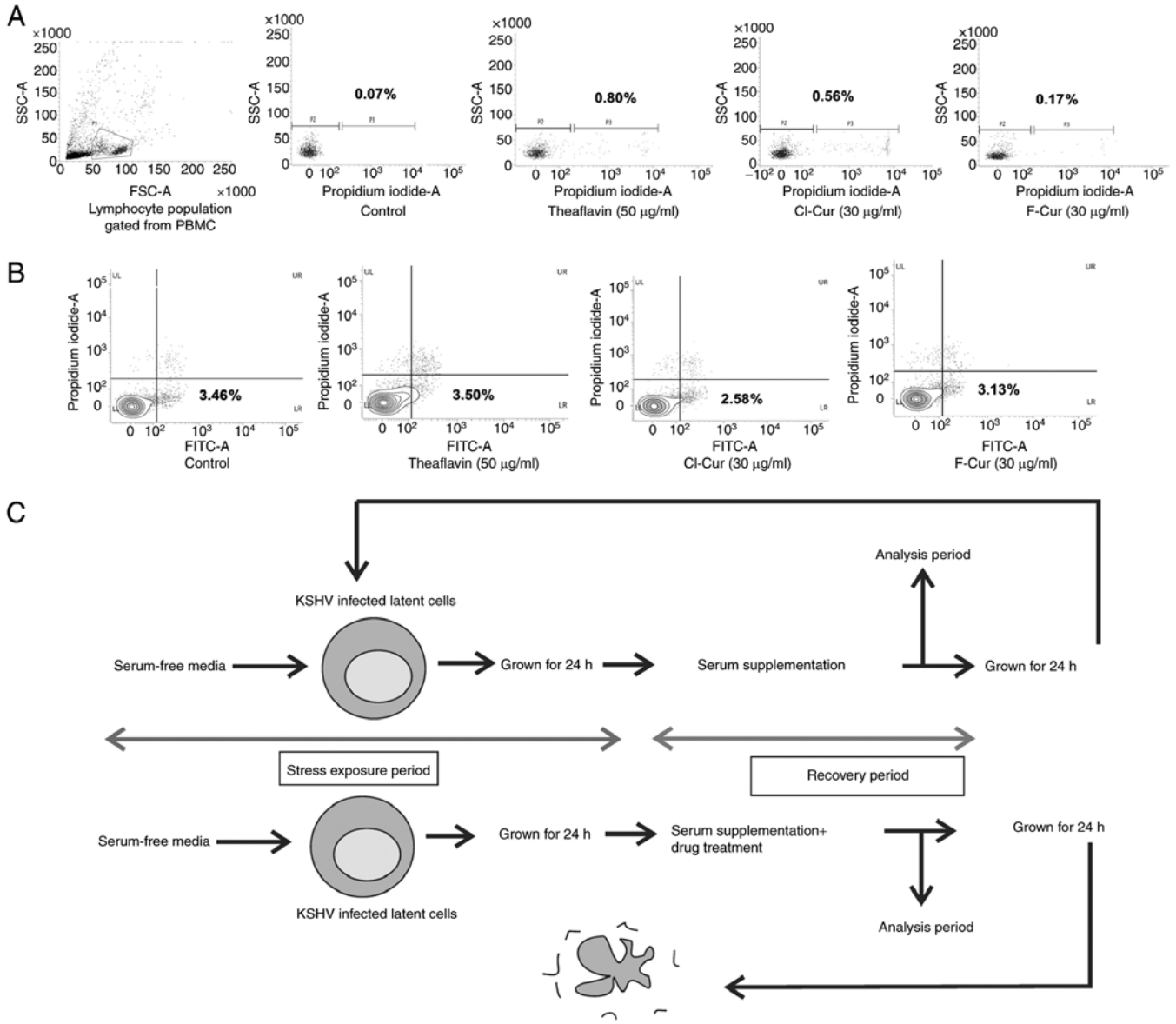

Figure 2. Effect of the three antioxidants on healthy control cells and non-KSHV B-cell lymphoma. (A) Cytotoxic effect of the three compounds on the lymphocyte population compared with the non-treated control. Cytotoxicity was measured using cell viability assay by measuring the propidium iodide fluorescence intensity. (B) Apoptotic cell death induction in the non-KSHV B cell lymphoma cell line BJAB was measured by Annexin V-FITC assay following treatment with the three compounds at their effective concentrations. (C) Schematic diagram of the stress -induced in vitro model for KSHV-positive cell lines. KSHV, Kaposi's sarcoma-associated herpes virus; Cl-cur, Cl-curcumin; and F-cur, F-curcumin.

Similarly, the non-KSHV B cell lymphoma cell line, BJAB, was also investigated with the same test compounds, which did not exert any notable apoptosis-inducing effects compared with the aforementioned KSHV-positive cell lines (Fig. 2B). This indicates that, apoptotic induction is specific against virus induced altered cell death mechanisms.

Status of oncogenic stress response in KSHV-infected cells following treatment with antioxidants. KSHV-infected cells maintain a redox homeostasis via a stress responsive antioxidant mechanism during different phases of pathogenesis; this begins from infection, is then maintained and is reactivated until neoplasty, resulting in the regulation of the pathogenesis process. The resulting oncogenic stress management system eventually helps throughout the malignancy process. As a result, the total cellular oxidative stress is in an unbalanced condition throughout the process of oncogenesis. The role of the anti-oxidative therapeutic approach against this malignant environment with a distorted stress balance needs to be fully understood as a stress responsive anti-oncogenic strategy.

The present study thus aimed to understand the status of oxidative stress with the application of different antioxidants in KSHV-infected cells. The estimation of intercellular ROS generation in KSHV-infected cells clearly revealed that the KSHV-infected latent cells expressed a significant high level of ROS, which was higher than normal non-cancerous human PBMCs (Fig. 3A). PBMCs are a primary cell model with a heterogeneous cell population, used for various research purposes (27). Thus, in order to supplement the finding with a control cell line, 293T cells were used as a non-viral non-cancer control cell line in this experiment, exhibiting low intracellular ROS levels compared to KSHV-infected cells (Fig. 3A).

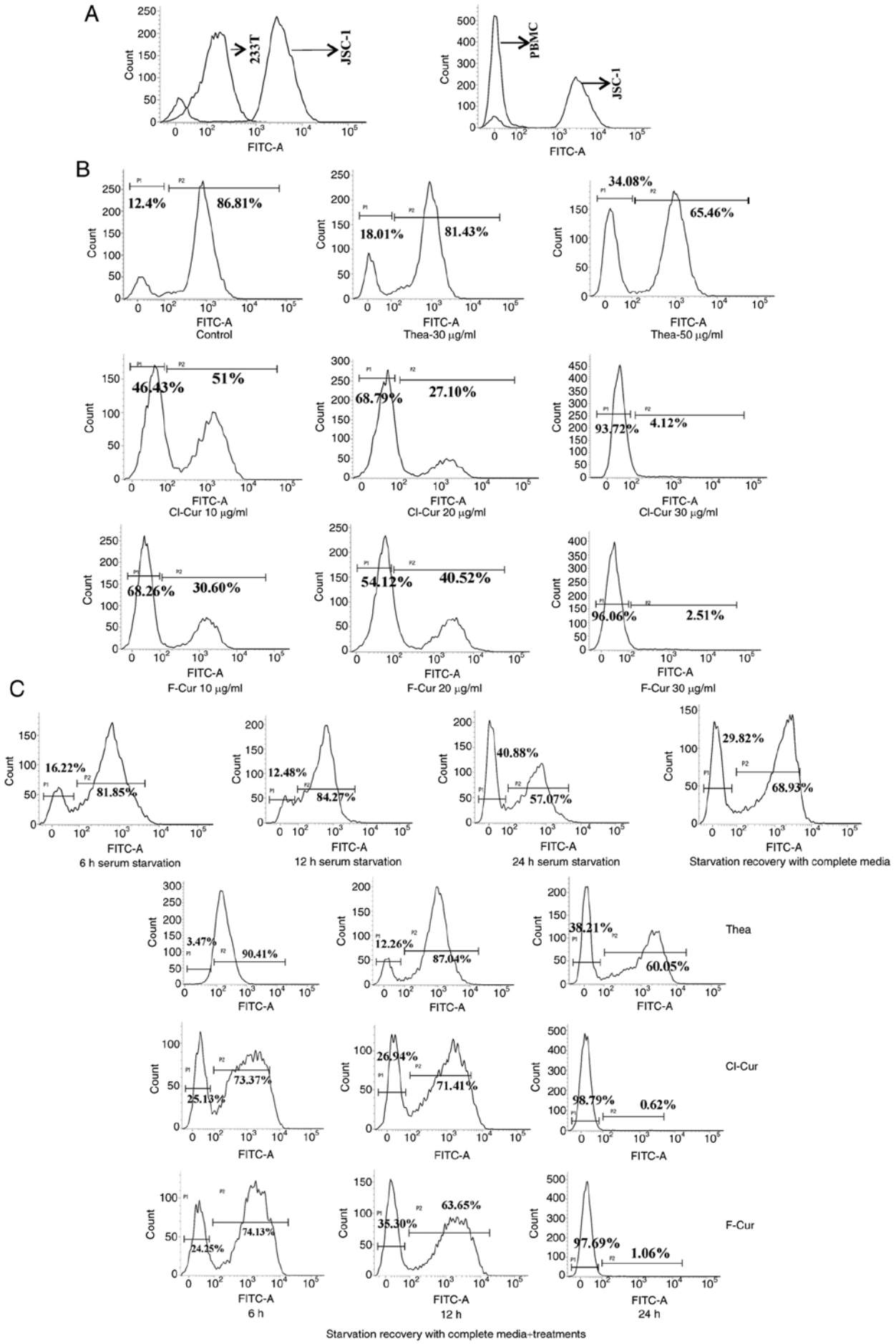

Figure 3. Continued.

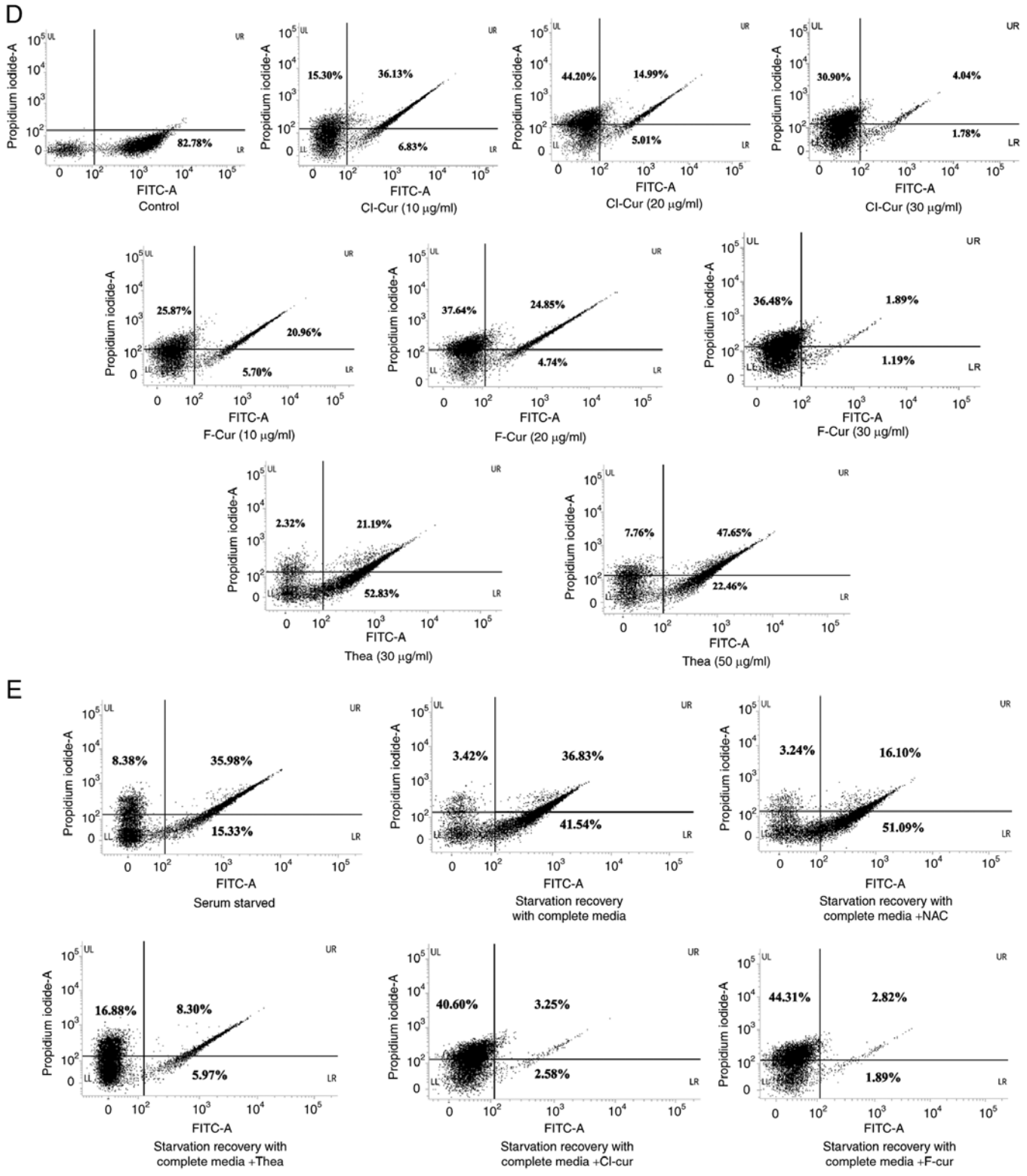

Figure 3. Alteration of intracellular ROS levels along with the death of KSHV cell lines with exposure to different stress and treatment conditions at the indicated concentrations. FITC channel in the x axis in all the plots represents the fluorescence signal for ROS. (A) Histogram overlay illustrating intracellular ROS levels in KSHV cells in comparison with non-KSHV epithelial cancer cells (293T) and PBMCs. (B) Effect of the three compounds on the alteration of intercellular ROS in KSHV cells compared with untreated latent cells. (C) Time kinetic ROS alteration in stress induced *in vitro* model established in JSC-1 cell line at the representative concentrations (theaflavin, 50 μg/ml; curcumin derivatives, 30 μg/ml) of the antioxidants represented by histograms. (D) Representative dot plots illustrating the comparative analysis of concentration-dependent cell viability along with ROS alteration in KSHV-infected JSC-1 cells. (E) Comparative ROS vs. cell death analysis in the stress-induced JSC-1 *in vitro* model with untreated, treated and NAC treatment sets. KSHV, Kaposi's sarcoma-associated herpes virus; Thea, theaflavin; Cl-cur, Cl-curcumin; and F-cur, F-curcumin; ROS, reactive oxygen species; PBMCs, peripheral blood mononuclear cells.

293T cells are a highly transfectable daughter cell line variant of 293, which also supports high viral gene expression

This high level of cellular ROS was completely decreased following treatment with the curcumin derivatives at their

respective concentrations, whereas in the case of theaflavin, it was observed that a moderate level of ROS was maintained in the treated cells. Thus, in order to examine the ROS alteration pattern, a concentration-dependent assay was performed against the KSHV-infected cells with the three antioxidants. The gradual increment of the ROS-negative population from the histogram plots clearly revealed the concentration-dependent efficacy of the compounds to suppress the intracellular ROS generation (Fig. 3B).

Following the confirmation of this alteration in ROS levels in latent KSHV cells, the present study aimed to confirm whether the therapeutic treatment indeed targets the stress balancing system of the virus-infected cells. The latently infected cells were subjected to serum stress for the generation of a time kinetic stress-induced *in vitro* model and to mimic the situation of varied oxidative stress levels during reactivation, cytotoxicity induction, or infection to a new cell (Fig. 2C). It was observed that following the starvation period, an extra stressful cellular environment was created, which tended to markedly decline after 24 h. After 24 h of stress exposure when these cells were supplemented with complete growth medium, the intracellular ROS levels increased, gradually balanced and maintained at a moderately high level, as found in latent cells (Fig. 3C). When these stress-exposed cells were revived with normal growth medium along with treatment, the stress balance mechanism was initiated without any further elevation in the intracellular ROS levels. Collectively, these results indicate that the compounds can interact with the oncogenic stress response of the virus-infected cells along with the cell death process, by utilizing the existing oxidative stress response.

Of note, up to this point, these results demonstrated that the antioxidants induced apoptosis with a reduction in intracellular ROS levels. Thus, there may be a contradictory fact between these two events of ROS alteration and apoptosis induction. In order to further clarify this fact, a ROS vs. cell death percentage assay was performed on the same cells using a flow cytometer. This analysis clearly revealed that the JSC-1 cells without treatment exhibited only a positive ROS signal. When they were treated with both theaflavin and curcumin derivatives, the total cell population gradually tended to switch towards the both PI- and ROS-positive zone and ultimately, towards only the PI-positive zone (Fig. 3D). This finding was then verified and supplemented, utilizing the stress-induced *in vitro* model. It was clearly revealed that the stress-induced cells were distributed in the ROS-PI dual-positive zone, which indicated stress-induced cell death. This event was recovered by complete medium supplementation, after which the cells were observed to be redistributed more in only the ROS-positive area. Following the treatment of these cells at the significant concentration the whole population shifted towards only the PI-positive area. The activity of these antioxidants was also analyzed in comparison with the FDA-approved antioxidant, NAC. NAC has been reported to exert effective chemopreventive and inhibitory effects on angiogenesis in an *in vivo* oxidative stress-induced KSHV model (28,29). It was observed that, in the *in vitro* stress-induced cell line model, NAC supplementation recovered stress-induced cell death and led to the distribution of the cells in only the ROS-positive zone (Fig. 3E).

These experiments supplement the fact that antioxidants drive all the ROS-positive latent KSHV-infected cells towards death and at their higher concentration, a total cellular destruction occurs, represented by only the PI-positive cell population. From these aforementioned results, it was confirmed that the theaflavin and curcumin derivatives can effectively control KSHV-infected cell growth by specifically targeting the oncogenic stress response of these cells. It was clearly observed that the initial pattern of stress management by activating the antioxidative strategy in stress-exposed KSHV-infected cells was similar for both serum-recovered, and serum-recovered cells with treatment. However, recovery from stress exposure with normal growth supplement gradually balanced this by switching it to an oncogenic stress balance, which maintained the latency. However, when these cells were treated with antioxidants, a successful stress response towards apoptosis was observed. This result strongly revealed that the virus was evolved in such a manner, which utilized the cellular antioxidant mechanism for its survival and the antioxidants utilized the same mechanism, but sensitized these virus-infected cells against stress, leading to cell death. The difference was in the case of theaflavin, of which the significant effective concentration was higher compared to that of curcumin.

The compounds exert a significant alteration in mitochondrial membrane potential. The mitochondria are the major cellular energy hub for different cellular functioning and naturally release a certain amount of ROS as by-product. However, hypoxic pressure, or stress, influence the mitochondria to generate high levels of ROS, which are stabilized with time. The cells continue to grow with these elevated ROS levels by escaping senescence and death. These persistent dysfunctional cells with high mitochondrial ROS levels induce mutations in DNA, eventually resulting in malignant transformation. Thus, dysfunctional mitochondrial functions elicit malignant transformation (30,31). Pathogenic cancer cells, such as Epstein Barr virus-associated cancers, have been reported to persist with such dysfunctional mitochondria and to exhibit an unregulated cell proliferation (32). Thus, the status of the mitochondria following cancer treatment against needs to be understood in the case of the antioxidative therapeutic approach. Therefore, the present study further proceeded to analyze the status of mitochondrial membrane potential following the treatment of the normal and stress-exposed KSHV-positive cell lines with the antioxidants. It was observed that all the three test compounds initiated the deterioration process of the damaged mitochondria at their respective effective concentrations. The compounds effectively increased the mitochondrial membrane potential in a concentration-dependent manner in the latent KSHV-infected cells (Fig. 4A). When the stress-exposed cells were revived with complete growth medium, initially they exhibited a destroyed mitochondrial load, which was maintained in the same manner for few more hours of supplementation, which later stabilized. Ultimately, the latently infected cells exhibited a normal balanced mitochondrial potential and continued to grow. While these stress-exposed cells were grown with the stress balancers (both theaflavin and curcumin derivatives), they were successfully able to switch the rescue process of the damaged mitochondria inside the oncogenic cell, with a notable loss of mitochondrial membrane

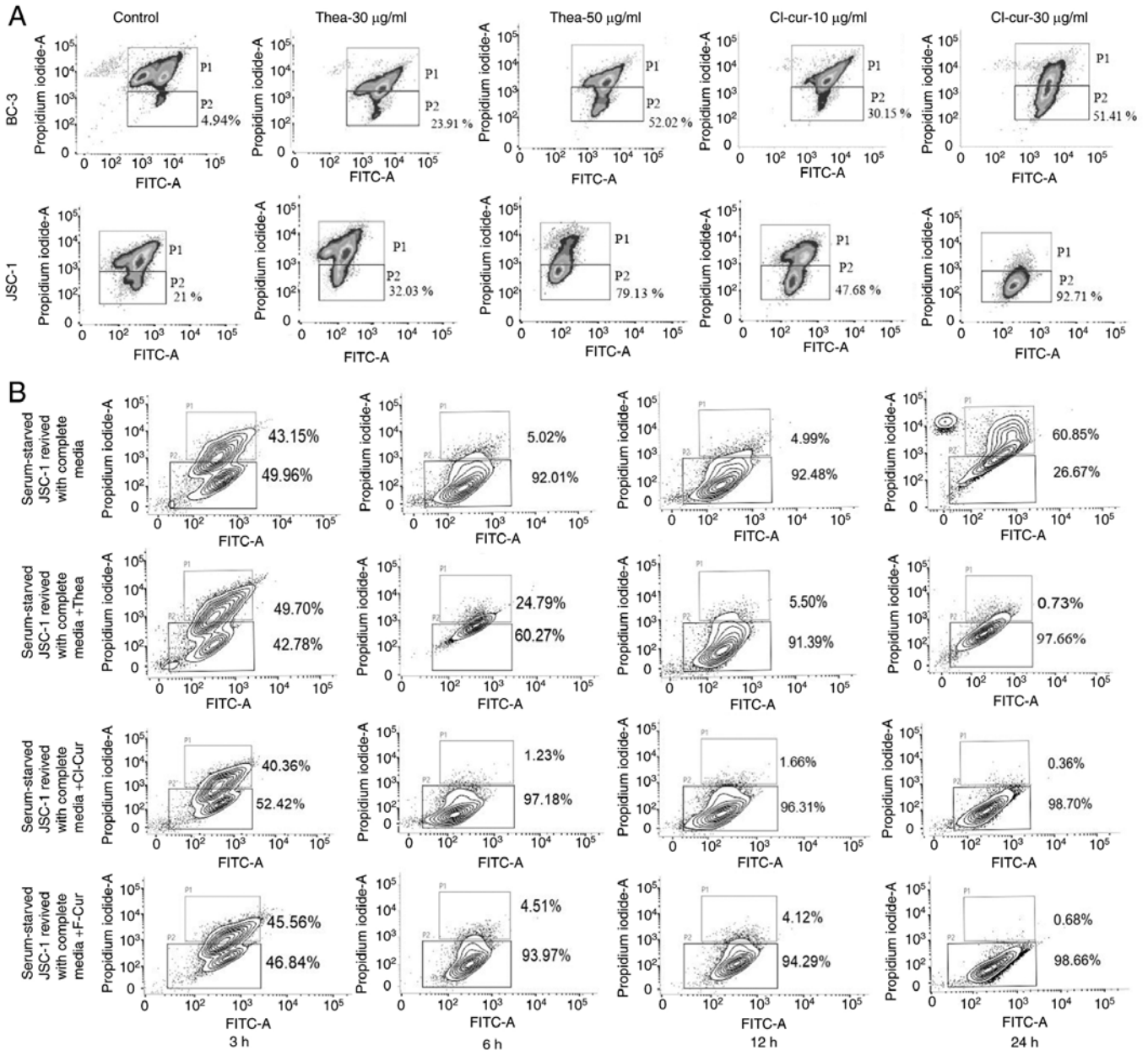

Figure 4. Effect of ROS scavengers on mitochondria. The FITC channel in the x axis represents the JC-1 signal. (A) Mitochondrial membrane potential alterations in BC-3 and JSC-1 latent cells, treated with theaflavin and one of the curcumin derivatives (Cl-Cur) at the indicated concentration. (B) Serum-starved (24 h) JSC-1 cells were supplemented with complete growth media alone, theaflavin (50 μg/ml), Cl-curcumin and F-curcumin (30 μg/ml). The alteration of mitochondrial membrane potential was assessed for 3 to 24 h. The serum-starved cells revived normal growth media recovered the damaged mitochondria with time, whereas the treated cells represented a gradual degradation of the dysfunctional mitochondria. The indicted percentages in the densitometry graphs represent the percentage of the population on each fluorochrome range.

potential in a gradual time-dependent manner, which drove the cell towards apoptosis (Fig. 4B).

The antioxidants can break the evolved tactics of the virus to utilize stress-induced cell cycle check points. The oncogenic herpes virus has been evolved to utilize the stress-induced cell cycle regulatory check points for efficient viral replication, leading to cell cycle arrest during malignancy in a p53-mediated manner (33). Whenever there is certain type of stress induction, such as drug or metabolic stress and nutrient deprivation, the affected cell has an indigenous management system which halts the cells and does not allow the cells to undergo further growth with the stress-affected defects. However, the virus is evolved in such manner that it utilizes this stress for its replication. The results of the present study clearly indicated that with the stress induction to the latent cells, there was a halt in the cell cycle process, which was recovered after 24 h of growth supplementation; this is how the virus maintains the latency. The serum-stressed cells underwent normal cell cycle phases after they were replaced with complete growth media. The graphical representation of different phases of the cell cycle clearly revealed with significant difference that the cells treated with theaflavin and curcumin that were stress-exposed exhibited a restricted cell cycle arrest at the sub G_0-G_1 phase compared to stress recovered nontreated control cells (Fig. 5A). No such proper synthesis phase was also

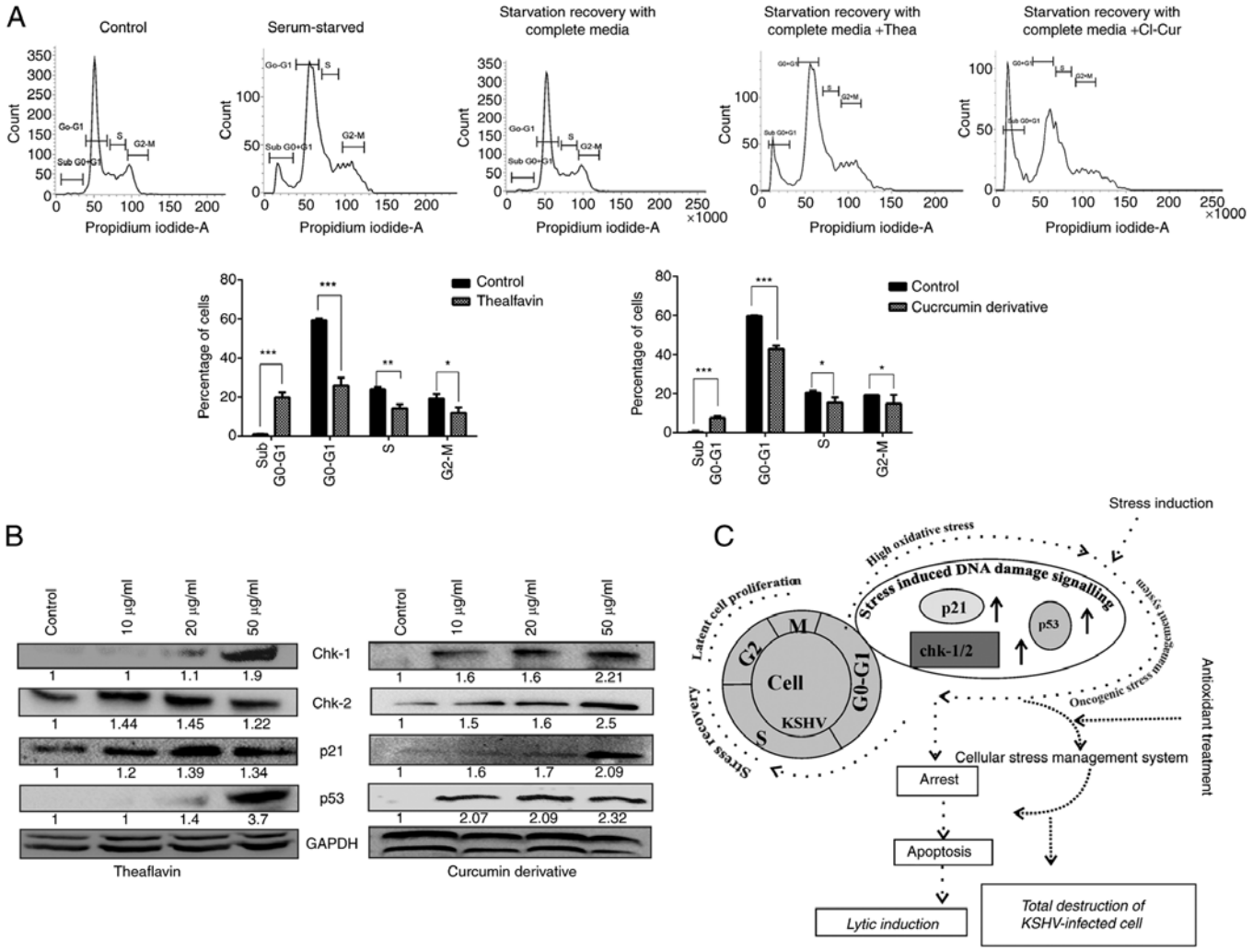

Figure 5. Alteration in cell cycle regulation with treatment. (A) Changes in the different phase of cell cycle in JSC-1 cells following treatment in the stress-induced *in vitro* cell line model. $^{*}P\leq0.05$, $^{**}P\leq0.01$ and $^{***}P\leq0.001$, significant differences vs. control. (B) Western blot analysis of the expression of Chk-1 and Chk-2, and p53 and p21 in JSC-1 cells treated with the compounds at the respective concentrations. GAPDH was used as the loading control. The relative quantified intensity values were normalized to the untreated control and the final value is presented in the western blots under each lane. (C) Schematic diagram depicting the mechanisms through which antioxidants can utilize stress-induced cell cycle arrest regulation and promote KSHV cell death. KSHV, Kaposi's sarcoma-associated herpes virus.

observed in these cases. On the other hand, more specifically, the expression of the DNA damage-induced cell cycle regulatory proteins, Chk-1 and Chk2-, was found to be enhanced in a concentration-dependent manner (Fig. 5B). We have also found a significant increase of p53 and p21 protein which indicates the upregulation of stress induced DNA damage signaling in a p53 dependent manner (Fig. 5B). These results suggest that the virus can manipulate the stress-induced cell cycle regulation, so following a stress exposure, with supplementation, they are able to manage the cell growth with KSHV infection. However, when the cells were treated with the antioxidants, cell growth was arrested at a particular stage; the compounds rendered the cells sensitive to the existing high ROS levels and the stress-induced cell cycle restricting kinases were then activated, leading to apoptosis (Fig. 5C).

Status of lytic-latency gene in KSHV-positive cells. A marked imbalance in the oxidative stress response is a major consequence of viral pathogenesis. The aforementioned findings demonstrated that KSHV latency was evolved in such a manner that it maintained a redox homeostasis against the infection-induced oxidative stress response by interacting with inbuilt defense and death-associated cellular mechanisms, such as apoptosis. It managed to maintain a moderate intercellular ROS concentration and allowed the cells to proliferate. On the other hand, when the latency-associated cells are exposed to various stress inducers, they are unable to proliferate by managing the stress; they better decide to degrade the cell instead of maintaining it and are directed to be reactivated (34). This high oxidative stress favors the process until the establishment of infection. Thus, there is a continuous fluctuation of ROS in a KSHV-infected cancer cell, which is associated with a cycle of latency and lytic stages and thus, both latency and lytic genes correlate with each other to help in sustaining the virus. Likewise, the KSHV latency-associated gene, LANA, regulates the expression of the lytic-associated gene, RTA, and protects the infected cell from hypoxia-induced damage. RTA in turn, following infection, regulates the expression of LANA for the maintenance of latency (9). Thus, when an antioxidative approach is designed against an infected cancer cell to disrupt

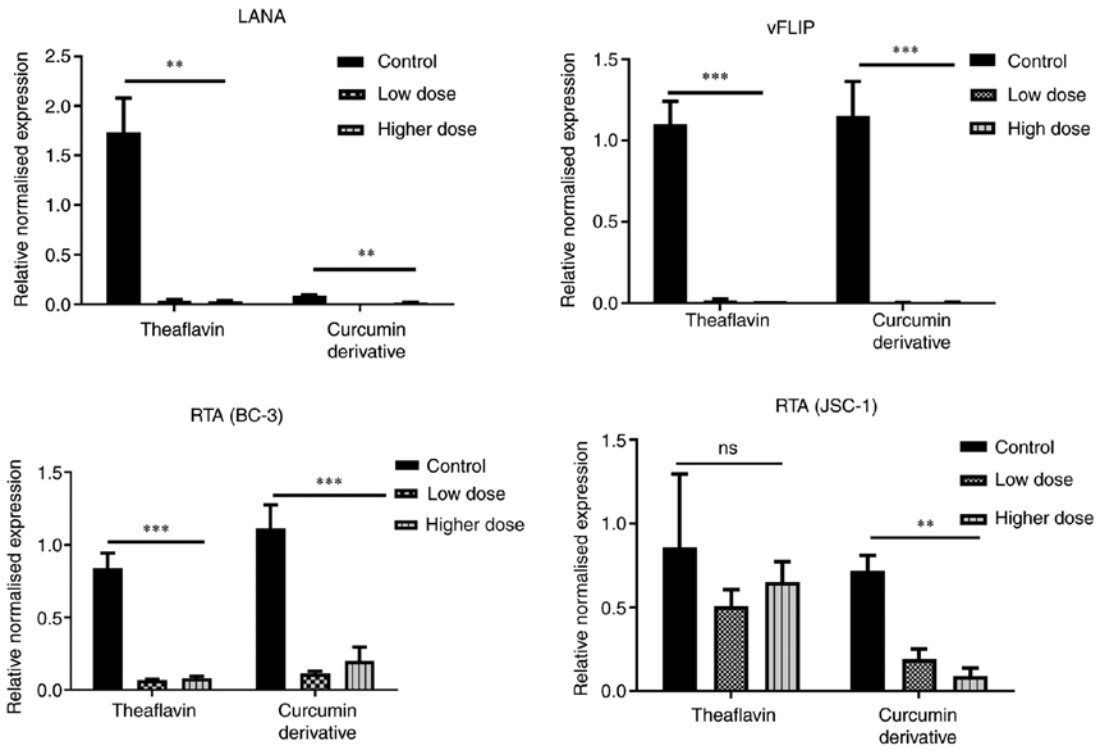

Figure 6. Reverse transcription-quantitative analysis of Kaposi's sarcoma-associated herpes virus latency and lytic proteins following treatment with antioxidants. Theaflavin and curcumin derivative treated JSC-1 cell for 24 h at the indicative doses was used for analyzing the expression of LANA and vFLIP. JSC-1 and BC-3 were used for analyzing RTA expression. Alterations in mRNA expression are represented by alterations in the relative fold change (calculated by $2^{-\Delta\Delta Cq}$ method) of the target genes, considering the expression of the untreated control as one-fold. $^{ns}P>0.05$, $^{**}P\leq0.01$ and $^{***}P\leq0.001$, significant differences vs. control. LANA, latency-associated nuclear antigen; RTA, replication and transcription activator.

the oxidative balance, both lytic and latency gene profiles need to be analyzed well. In the case of KSHV malignancy, the role of two major latency-associated genes of KSHV, LANA and vFLIP is critical (5). The present study found that the expression of both LANA and vFLIP was decreased following treatment with theaflavin and curcumin derivatives, and exhibited significant differences in the latent KSHV-infected cells with respect to the untreated control cells (Fig. 6, upper panel). On the other hand, to understand whether lytic upregulation is associated with cell death, the present study also examined whether the antioxidants can specifically lead to an imbalance in the oncogenic stress response of the infected cells and induce any early lytic gene expression. Increased RTA and KSHV reactivation is known to be associated with oxidative stress induced by several anticancer drugs (3,6). The results clearly demonstrated that there was no such expression of the early lytic associated protein, RTA, with any of the drug treatments with respect to stress-recovered untreated control cells; the downregulation of RTA was instead observed, which may be due the probable antiviral mechanism of the compounds effective against stress recovered cells showing slight high RTA level (Fig. 6, lower panel). Collectively, these results prove the fact that both theaflavin and curcumin derivatives successfully destroy the latency-associated viral proteins and eliminate the chance of latency-mediated stress-induced viral reactivation and maintenance.

Autophagy-apoptosis switching functions as the intermediate regulatory mechanism behind the cell death process. Viruses need a functional host cell for survival and multiplication; this is the reason they evolve in such a manner, where they are not intended to exert any harmful effect to their host. Viruses utilize several host cell mechanisms (3). Autophagy is one such mechanism of the host cell, which is a ROS responsive process and known as the stress balancer of the cell. It has been reported that in KSHV-infected cells, a basal level of autophagy is always present that helps in the maintenance of latency by balancing the harmful consequences of infection and maintaining the host cell to a certain level of healthy state so that the virus can reside inside the cell and receive an adequate nutrient supply (35). On the other hand, increased autophagy is observed during viral reactivation, eventually helping in KSHV replication (36-39). Thus, the present study then aimed to examine the autophagic status during the therapeutic applications of the test antioxidants to determine the role of autophagy in this stress balance mechanism.

For this purpose, an autophagy vs. cytotoxicity assay was performed using a microscope, It was found that with treatment, the cell viability increased, and all thePI-positive cells were also positive for the autophagy indicative MDC stain, indicating an autophagy-associated increase in cytotoxicity in the KSHV-infected cells. It was also observed that the control cells also stained positive for MDC, representing the basal level of autophagy in latent cells (Fig. 7A). The merged images at 12 h of these cells indicated a few autophagy apoptotic co-expressed cells (indicated within the white circles in Fig. 7A). At 24 h, a decreased number of cells was observed due to highly active apoptosis. The magnified view of these cells (MDC-stained) with treatment represents an increased size compared to the control with clear punctures inside, marked by

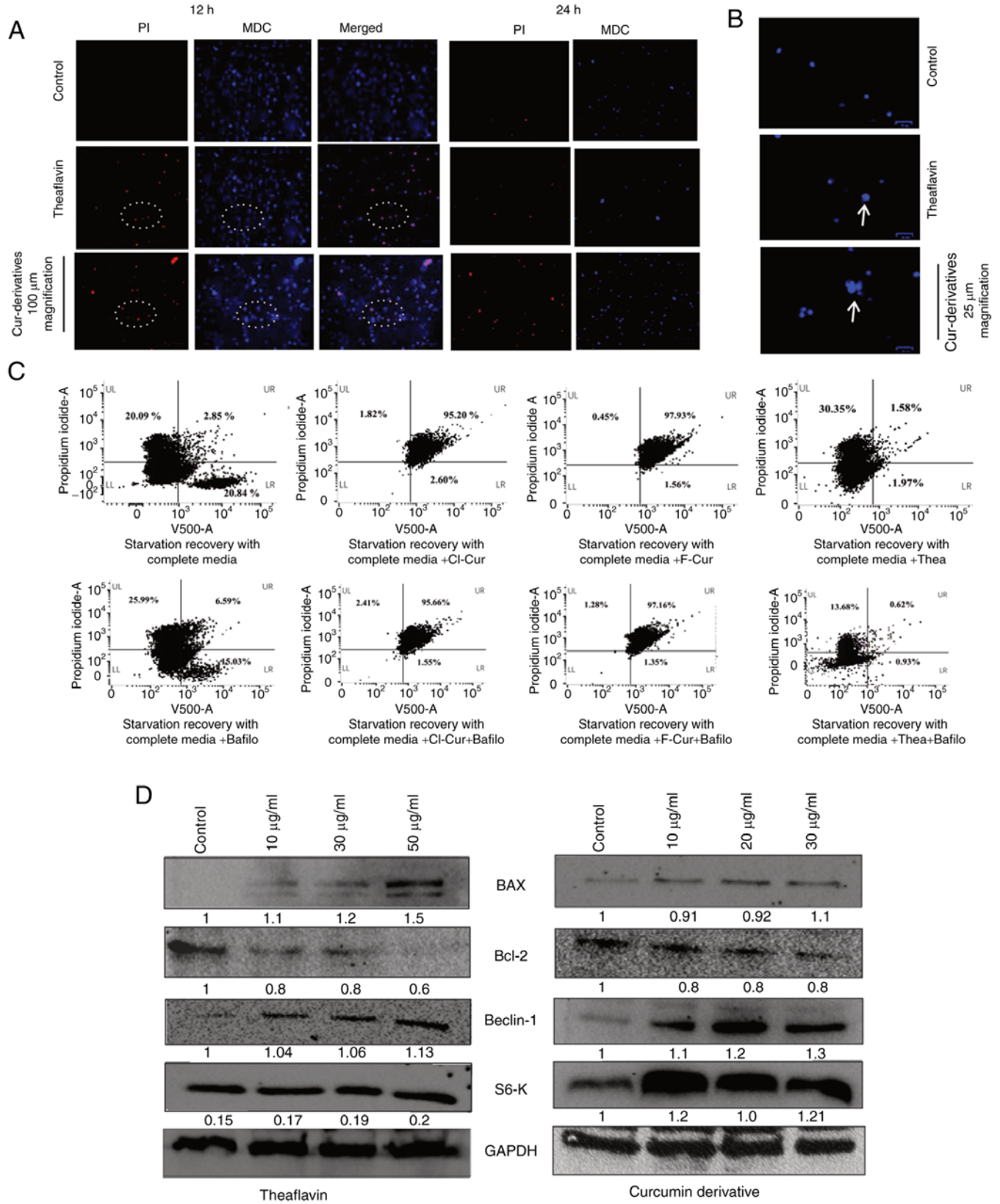

Figure 7. Regulatory mechanisms of cell death in KSHV-positive cells with treatment. The V500 fluorochrome in x axis represents lan staining for flow cytometric analysis. (A) Microscopic observation of the concomitant lack of cell viability along with upregulated autophagy level in JSC-1 cell exposed to different conditions. Increased cell viability was observed compared to the control in a time-dependent manner in both theaflavin and curcumin at their effective dose. Including control all the cells found positive for a basal autophagy indicating MDC stain. The 12 h merged images represents co-expressed cells (MDC and PI), indicated with white circles. (B) MDC-positive image represented with a magnified view showing an increased size of treated cells with more prominent autophagic flux indicated with white arrow marks. (C) Flow cytometric analysis of MDC vs. PI fluorescence in KSHV JSC-1 cells post-treatment at their effective concentration. (D) Western blot analysis of regulatory protein expression following treatment. Representative western blot image of BAX, Bcl-2, Beclin-1 and S6-K protein expression in JSC-1 cell was shown at the indicated doses of theaflavin and one curcumin derivative (Cl-Curcumin). The expression intensity was normalized to the expression of GAPDH as the control gene and then to the untreated control. All the relative quantification values/ratios are presented in the western blots under each lane. KSHV, Kaposi's sarcoma-associated herpes virus; Thea, theaflavin; Cl-cur, Cl-curcumin; and F-cur, F-curcumin.

white arrows (Fig. 7B). A notable change in fluorescence intensity and specifically, for the autophagy event with identifying vesicles was not clearly detected in the microscopy images, as all KSHV-positive cells express a moderate level of autophagy for the maintenance of malignancy. Thus, to determine the role of endogenous or overexpressed autophagy in the cell death process, the present study then performed an MDC vs. PI assay using a flow cytometer in the stress-induced *in vitro* cell line model. The result clearly demonstrated that there was a proper separate MDC-positive population (upregulated autophagy) in the stress-recovered cells with complete medium supplementation. However, a decreased MDC positivity was observed in the cells treated with the autophagy inhibitor (bafilomycin A1) with an increased cell death. This indicated that stress recovery in KSHV-infected cells was achieved by autophagy and the inhibition of autophagy accelerated the cell death process. However, this autophagy cannot protect the cells from drug-induced stress followed by cell death by apoptosis. All the latent KSHV-infected cells with a moderate level of autophagy underwent cell death following treatment, exhibiting a dual MDC-PI positivity. In the case of curcumin derivatives, the apoptosis was not markedly altered with bafilomycin A1 treatment, indicating an autophagy-associated apoptosis, which became independent of autophagy at the respective concentration. However, in the case of theaflavin, the cell death process was completely switched to cell death and was autophagy-dependent (Fig. 7C). Thus, collectively, it was confirmed that ROS responsive autophagy was active in KSHV-infected cells and the cell death process was mediated by autophagic involvement in latent cells. However, there was no such significant autophagic induction in favor of the virus for protection, as this switched towards apoptosis post treatment.

Subsequently, in order to intersect and characterize this autophagy apoptotic switching, a molecular investigation of autophagy-dependent cell death in the theaflavin- and curcumin-treated KSHV-positive cells was performed. Therefore, the expression of specific mitochondria-mediated intrinsic apoptotic protein markers (BAX and Bcl-2) was investigated, in latently infected KSHV cells treated with theaflavin and one of the curcumin derivatives. It was observed that with the loss of mitochondrial membrane potential, BAX expression was increased with a concomitant decrease in Bcl-2 protein expression in the KSHV infected cells compared to the control cells. The anti-apoptotic Bcl-2 oncogene has been reported to negatively regulate the autophagic gene, Beclin-1, and thus contribute to the inhibition of apoptosis (40,41). Moreover, Bcl-2 protein expression in the endoplasmic reticulum and mitochondria can be a stress sensor and can regulate Beclin-1 and caspase-mediated autophagy-apoptosis (42). Thus, after observing Bcl-2 downregulation in the KSHV-positive cells, the present study wished to examine autophagy-mediated apoptosis by investigating the expression level of the Beclin-1 gene. From the results, it was confirmed that there was a concentration-dependent increase in the expression of Beclin-1 autophagy protein, indicating an autophagy to apoptotic switch in the KSHV-positive cell lines treated with both theaflavin and curcumin derivatives (Fig. 7D). The autophagic marker protein, LCIII-B, was also investigated. It was found only found to be expressed in stressed and stress-recovered cells treated with complete medium, but not in cells treated with theaflavin and curcumin derivatives; this supports and clarifies the fact that autophagy is upregulated and plays a vital role in support of the KSHV-infected cancer cells recovering after serum stress, and completely switching towards apoptosis with treatment, thus failing to recover from drug-induced stress and apoptosis (Fig. S1). This type of event can be compared with recent findings on incomplete autophagy supporting severe acute respiratory syndrome coronavirus-2 replication machinery (43). Likewise, in the present study, there was an induction of incomplete cellular autophagy-mediated apoptosis, required during stress recovery where autophagy plays a cytoprotective role, and the complete switch of that autophagy to apoptosis leads to cell demise in treatment sets (44,45). Kinase cascades are ROS-responsive, and this has been reported to modulate and regulate autophagy accordingly during cancers. mTOR is a major connecting pathway between ROS and autophagy in cancer cells, the downregulation of which has been found to restrict cancer progression (46). Activated mTOR is responsible to activate S6K by phosphorylation. Rapamycin, which has been reported to restrict KSHV malignancy by mTOR inhibition, has been found to minimize the phosphorylation of S6K, hence restricting the activated S6K (47,48). Both theaflavin and curcumin have been previously reported as potent mTOR inhibitors (49-51). In the present study, the results of western blot results analysis revealed the visible overexpression of mTOR-regulated intact S_6 post-treatment with the test compounds. The expression of this key kinase is relevant to the present study, by suggesting mTOR inhibition by the test antioxidant compounds (Fig. 7D).

Discussion

Flavonoid compounds, with their structural specificities, are biologically active and exhibit various anticancer, as well as cancer manifesting activities. These compounds are potential ROS scavengers, which can act both as anti- and pro-oxidant compounds by decreasing ROS generation and sensitizing cancer cell unbalanced ROS levels, leading to apoptotic cell death (52). On this note, during viral pathogenesis, the oncogenic stress management system plays a crucial role, where the oncogenic virus takes control over the cellular stress management mechanisms and a continuous oncogenic stress management system persists (Fig. 8A) (53). Thus, a clear understanding of these stress balance tactics at different stages of the cancer progression with a varied ROS response is required to target this mechanism and combat cancer progression. Keeping this in mind, in the present study, two flavonoid based compounds, theaflavin and synthetic curcumin derivatives, were selected, which have been reported as natural antioxidants against various diseases, including cancer. Both theaflavin and curcumin have been reported as natural antioxidants and are therapeutically applied in cancers (49,54). Theaflavin has not been previously reported against any KSHV-associated malignancy, at least to the best of our knowledge; however, curcumin has been found have potential anti-KSHV malignant activity by inducing STAT3 activation and caspase-mediated apoptosis. It is also known that curcumin is a potent inhibitor of APE1-mediated redox signaling in the process of replication, invasiveness and angiogenesis during KSHV malignancy (55-57). Thus, in

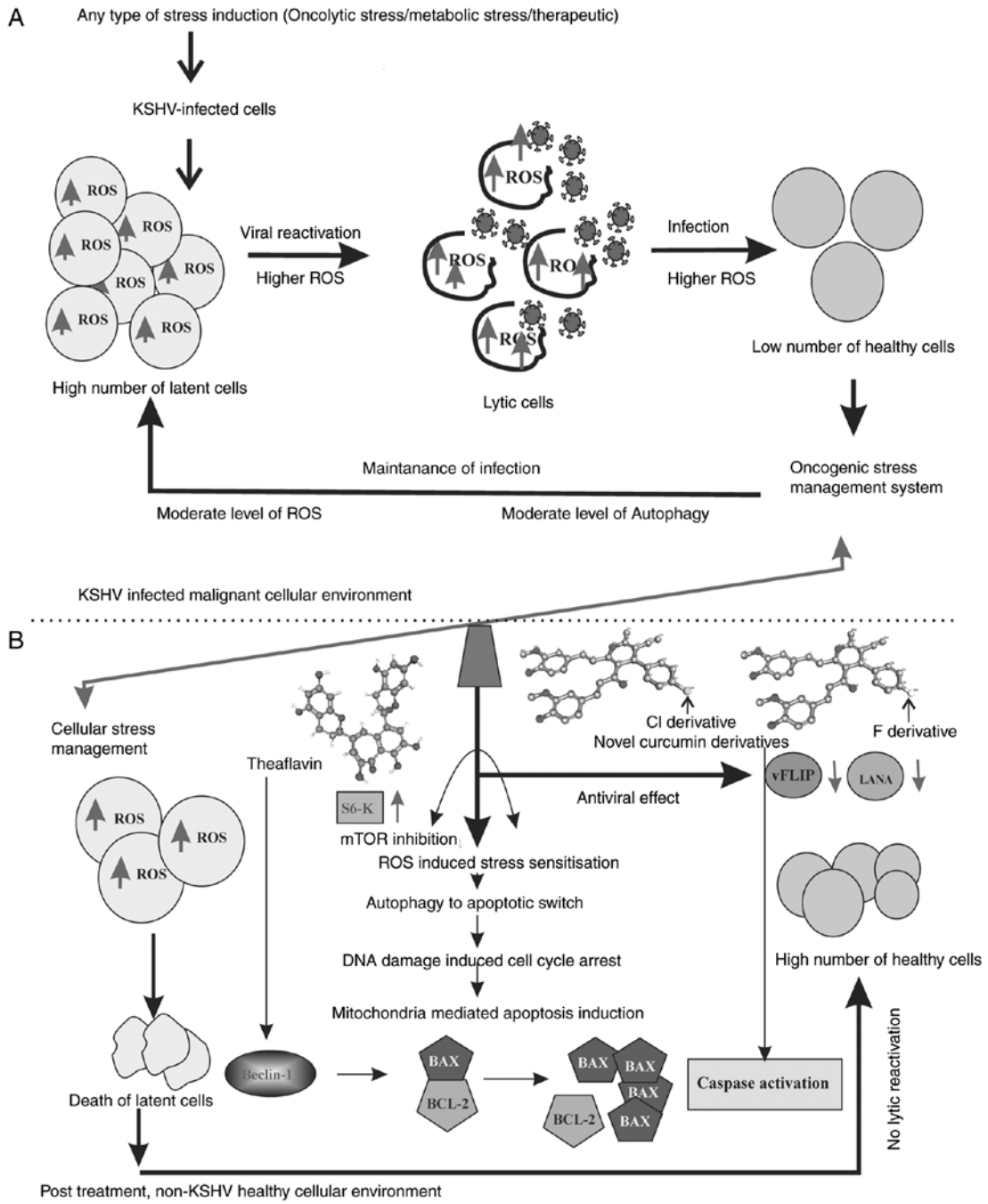

Figure 8. Hypothetical therapeutic regulatory model of the antioxidant activity against KSHV-positive cells. (A) Literature-based model (3-8,35,36) of the oncogenic stress management system inside the KSHV infected malignant cell environment. The latent cell maintain a moderate level of ROS, whereas these levels re higher during lytic reactivation. Further during the infection of new cells, the ROS level is high and during the establishment of infection it is balanced to a moderately high level. This is how the virus makes the cells insensitive to cell death in infected cells by controlling over various stress-related cellular functions and thus respond with different stress responses. (B) Summarized experimental outcomes, representing the antioxidant induced sensitization of cellular stress balance mechanism. The results suggest that theaflavin and curcumin derivatives are potent ROS responders. The therapeutic approach renders the infected cells sensitive against high ROS. As a result, intermediate mTOR signaling and autophagy occur in infected cells; cells undergo senescence and apoptosis in a Beclin-Bcl-2-dependent manner. The ROS responsive mitochondrial damage further initiates caspase-mediated cell death. The downregulation of two major latency associated proteins, LANA and vFLIP, with treatment strengthens the process of cancer cell death without any reactivation or further infection. KSHV, Kaposi's sarcoma-associated herpes virus; ROS, reactive oxygen species; LANA, latency-associated nuclear antigen.

the present study, two different flavonoid compounds were selected, namely theaflavin and modified curcumin derivatives (Cl-curcumin and F-curcumin) for analyzing their bioactivity over the varied level of oncogenic stress balance mechanism of KSHV-infected cells. The novel modified bioactive derivatives were used in the present study, with an intention to magnify the biological efficacy of mother compound curcumin, with increased bioavailability, which is a major drawback of the clinical applicability of this compound.

From the results presented herein, it was observed that both the compounds exhibited potential anticancer activity against the KSHV-associated cell lines via caspase-dependent apoptosis induction. They potentially handle the stress balancing capability of the oncogenic cells as their cancer management

mechanism. The present study clearly demonstrated that the constitutive expression of moderate levels of ROS during KSHV latency, which helps in the maintenance of the virus, was sensitized by these bioactive antioxidant compounds. Moreover, it was revealed that when there was a serum stress induction in the virus-infected cells, this induction was successfully utilized to destroy the energy-deprived cell. When, these stress-exposed KSHV-associated latent cells were supplemented with favorable growth conditions, cellular growth was reverted by managing the high level of ROS, utilizing the same stress balance strategy as a normal cell. When they were grown with theaflavin or the curcumin derivatives, the existing stress balance process inside the cells was destroyed, and cell death occurred. The regulatory mechanism, which controls several downstream signaling pathways for the continuous maintenance of the balance of the latency and lytic phases for the successful survival of the virus, can reside inside the host cell, and is destroyed by these compounds.

Compounds with antioxidative properties are generally considered to minimize the oxidative stress of the cell; however, in some cases, specifically in cancer cells, they can play prooxidative role, particularly when there is a hyper ROS response which drives the cells towards cell death (58,59). How a viral cancer cell responds with ROS fluctuation has been reported during different phases of cancer; however, how this fluctuation is managed by the pathogen-altered cancer cells has not been fully elucidated. Of note, the present study critically analyzed this altered version of cellular stress management in a tumor microenvironment. The present study reveals the mechanism through which the oncogenic stress balance system combats this varied ROS response and how the same standpoint can be an effective therapeutic strategy. These experiments were performed using a stress-induced *in vitro* model with the comparative analysis of different antioxidants. Curcumin and theaflavin have been reported to enhance the ROS level in cancer cells for the induction of apoptosis (60). In the present study, these compounds followed a similar pattern of cell death mechanisms against the KSHV-infected cells. More critically, it was found that against the KSHV-infected cells, theaflavin and curcumin derivatives enhanced the stress-induced cell death process, sensitizing the moderate level of ROS in the latent cells.

The present study clearly demonstrates the regulatory mechanisms behind the stress management and cell death process, where autophagy was found to be the vital player. Autophagy is the major cellular mechanism which is modulated KSHV during malignancy. A moderate level of autophagy helps in latency maintenance and is increased during the lytic phase, assisting in virus reactivation and infection. This autophagy is ROS-responsive and it can also regulate the cellular ROS level. The present study demonstrated that the KSHV stress balance system utilized the existing autophagy, increasing the levels to manage the extra stress induced by serum starvation during latency (Fig. 7C). However, following treatment with theaflavin and curcumin derivatives, the moderate level of autophagy was switched towards apoptosis. Of note, these two antioxidants successfully managed to overcome this autophagy-mediated stress recovery. Moreover, the curcumin derivatives induced autophagy-independent apoptosis at the respective concentrations. The molecular analysis of autophagy in the treated cells revealed that autophagy was actually sensitizing the cells towards death. In both cases of drug treatment, different stress regulatory signaling pathways were altered and drove the cells towards death. The stress-induced DNA damage signaling was found to be activated, where Chk-1 and Chk-2 levels were found to be notably altered. The damaged mitochondria in cancer cells continue to persist and produce excess amounts of reactive oxygen during cancer progression (30). Both compounds used herein were found to destroy these mitochondria and induce mitochondria-mediated caspase-dependent apoptosis. Lastly, mTOR signaling, which is the sensory signaling between the varied ROS response and autophagy, has been found to be (Fig. 7D) altered during treatment. The present study demonstrated that an enhanced cellular autophagy represented with its identifying markers (Figs. 7C and S1) was required for the cancer cell stress management in a cellular environment lacking apoptotic modulators. This autophagy is converted to apoptosis by the altered interplay of several decisive switching molecules, such as p53, Beclin-1, BAX, Bcl-2 and caspase following treatment with the compounds (Figs. 1, and 7). Overall, it was clearly demonstrated that different cellular stress responsive targets, which are utilized by the virus and act in favor of cancer progression, are specifically targeted by the antioxidants for the destruction of the cancer cells. Moreover, in relation to this, different KSHV viral genes have been recently reported to protect the viral replication process during hypoxia-induced stress (9,61). In association to these findings, the present study clearly emphasized the fact that these stress-tolerant oncogenic cells can be targeted by the antioxidants, which specifically affects the stress responsive adaptive regulatory pathways, utilizes the same mechanisms and makes the cells sensitive to the cellular stress response leading to apoptosis (Fig. 8B).

KSHV-associated malignancies are the major secondary type of cancer in patients with HIV. On the other hand, tuberculosis is another common co-infection among patients with HIV. Such an association between these three entities is very common in India (62). It has been found that patients with HIV are in a state of high oxidative stress expression, due to the dual role of infection and the extra burden of prolonged antiretroviral therapy for both tuberculosis and HIV treatment. Thus, this high ROS response in the system enhances the chance of further infections, such as KSHV, which lead to a very poor prognosis without any further treatment (11,25,63). Strategically, treatment modalities need to be identified which can nullify this stressful multi-infectious malignant environment. The findings presented herein demonstrate that the antioxidative approach can withstand such stress recovery mechanisms by the virus. These compounds can efficiently overcome the oncogenic stress management system. In the field of antioxidative anticancer therapy, NAC is an effective FDA-approved agent. NAC has been reported to minimize the KSHV invasiveness *in vivo*, but it is limited in its clinical application with chemotherapy against this malignancy (29). The present study with the comparison of NAC also supports the pharmacological efficacy of these compounds as antioxidants. Collectively, the present study proves the affectivity of the antioxidants against KSHV malignancy which eliminate any chance of deleterious side-effects by chemotherapeutics and their further use as a natural anti KSHV malignant strategy. A

limitation of the present study is that only an *in vitro* cell line model was used. Understanding the therapeutic efficacy of these antioxidants against KSHV-infected *in vivo* models with varied stress responses is mandatory. Thus, further studies are required to fully determine the clinical applicability of these compounds.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

PD contributed to the conception and design of the study, and was involved in conducting the experiment and in data acquisition. PD also analyzed the data and drafted the manuscript. TC performed data interpretation, correction, and the editing and reviewing of the manuscript. GB assisted with the design, synthesis and characterization, as well as with the supply of the novel curcumin derivatives. KC assisted in evaluating the results and reviewing the article. All the authors in the study made substantial contributions to the study and have read and approved the final version of the manuscript. PD and TC confirm the authenticity of all the raw data.

Ethics approval and consent to participate

The present study was performed following the guidelines of the and following the approval of the Visva-Bharati University Human Ethical Committee (Ref. no. IECHR/VB/ 8017/18). Healthy individuals participated in the study. Written informed consent was obtained from each individual participant for sample collection and for utilization of the data for research purposes.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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