Original research paper

Dasatinib enhances curcumin-induced cytotoxicity, apoptosis and protective autophagy in human schwannoma cells HEI-193: The role of Akt/mTOR/p70S6K signalling pathway

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ABSTRACT

The present study was carried out in human schwannoma cells (HEI-193) to determine the combined anti-cancer effect of curcumin and dasatinib. Cells were treated with curcumin only, dasatinib only, or the combination of curcumin and dasatinib for 24 hours. Cellular toxicity, cell proliferation, and cell death were determined by LDH, MTT, and trypan blue dye assays, respectively. ELISA based kit was used to determine apoptotic cell death. Western blotting was used to determine the expression of apoptotic and autophagy--associated protein markers. Similarly, expression levels of Akt/mTOR/p70S6K signalling pathway-related proteins were studied using Western blotting. Cell death and apoptosis were significantly higher in HEI-193 cells treated with curcumin and dasatinib combination compared to individual controls. The combination of curcumin and dasatinib significantly enhances autophagy markers compared to individual controls. Furthermore, the combination of curcumin and dasatinib significantly activates Akt/mTOR/p70S6K signalling pathway compared to individual controls. In conclusion, our results suggest that the combination of curcumin and dasatinib significantly enhances cytotoxicity, apoptosis, and protective autophagy in HEI-193 cells through Akt/mTOR/p70S6K signalling pathway.

INTRODUCTION

Multi-targeted drugs are supposed to replace therapies aiming at a single target (1). Modulating multiple targets is hypothesized to be a golden formula in treating several disorders. The combinatorial therapeutic approach has been successfully applied for the amelioration of several diseases like cancers, malaria *etc*. Combination therapy has been reported to show enhanced antitumour activity in certain cancers (2, 3).

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Curcumin is a well-known antitumor and anti-inflammatory agent used to treat a wide variety of human diseases, including cancer (4, 5). Curcumin (orally administered) has been reported to stabilize tumour growth in pancreatic cancer patients (6). However, it should be noted here that curcumin has both pro-oxidant and antioxidant properties and therefore, its action is dependent on disease type, curcumin concentration, and immune system of the host (7, 8). Curcumin is reported to target a wide variety of biomolecules like growth regulators, signalling molecules, transcription factors, tumour- and apoptosis-related genes, and adhesion molecules (5, 9). Molecular alterations associated with the curcumin include Akt/mTOR/p70S6K pathway inhibition, overexpression of caspases 3 and 8, NF-kB inhibition, Bcl-2 down-regulation, COX-2 downregulation and Bax up-regulation (10–12). Curcumin is known to target some cancer-related pathways and has, therefore, been used to target cell apoptosis and survival pathways in many cancer cells. Earlier, curcumin has been reported to up-regulate hsp70 expression to avoid apoptotic death (13). Incubation of curcumin together with heat shock protein inhibitor (KNK437) has been found to significantly suppress the growth of the human schwannoma cell line (HEI-193) (14).

Dasatinib (BMS-354825), a multi-targeted tyrosine kinase inhibitor, was developed for use against imatinib-resistant leukaemias with much higher potency against cells expressing wild-type BCR-ABL (15). Dasatinib is now being used against different types of tumours (16, 17). However, some data suggest its osteoblastogenesis enhancing effect from mesenchymal progenitors (18). Low dasatinib concentrations have been observed to enhance the differentiation and function of mesenchymal osteoprogenitors. The present work aims to explore the protective role of curcumin in combination with dasatinib in HEI-193 cells.

Autophagy plays an important role in the development and progression of cancer by causing the self-degradation of damaged organelles as well as proteins (19, 20). Autophagy performs dual effects on cancer and can either suppress or enhance a tumour (21). It has been reported that cellular integrity is preserved by autophagy and therefore prevents tumour formation. Cells deficient in autophagy are more likely to form a tumour *in vivo*. On the other hand, by allowing cellular survival and suppressing apoptosis, autophagy maintains oncogenesis. Natural agents with anti-tumour activities are known to enhance autophagy in various types of cancers (22–24). However, the exact molecular mechanism by which natural agents induce autophagy-associated cell death in cancers is still unknown.

Akt (serine/threonine-specific protein kinase) has emerged as an important player in the survival, proliferation, and migration of cancer cells (25, 26). Increased activation of Akt has not only been reported as a marker for tumour growth, tumorigenesis and metastasis but also for cancer therapy resistance (27). It has been found that Akt/mTOR/p70S6K signalling regulates apoptosis and autophagy in chemo-resistant cells (28). Inhibition of the PI3K/AKT signalling pathway has been found to restore the sensitivity of colon cancer cells towards 5-fluorouracil (5-FU) (29). Furthermore, inhibiting AKT phosphorylation in prostate cancer cells prevents their metastasis potential (30).

EXPERIMENTAL

Chemicals

Cell culture reagents were purchased from Sigma-Aldrich (USA). Dasatinib (Selleck Chemicals, USA, GmbH: BMS-354825) purity ≥ 99 % HPLC) and curcumin (CAS No.: 458-37-7,

purity ≥ 94 %) from Sigma were prepared in DMSO. A final concentration of 0.01 μmol L⁻¹ (dasatinib) and 18 μmol L⁻¹ (curcumin) was used for carrying out all the experiments. MTT assay kit, LDH leakage assay kit and Trypan blue assay kit were all purchased from Abcam (USA). Halt Protease Inhibitor Cocktail was obtained from Thermo Scientific (USA). All other chemicals were purchased from Sigma-Aldrich.

Cell culture and treatments

Human schwannoma cells HEI-193 (31) were grown and maintained in DMEM-F12 media plus 10 % fetal bovine serum at 37 °C and 5 % CO_2. Cells were grown in culture media for 24 h and treated with curcumin alone (18 μmol L⁻¹), dasatinib alone (0.01 μmol L⁻¹) or curcumin and dasatinib together for 24 h.

MTT assay

HEI-193 cells (1×10^3) were cultured in 96-well plates for 24 h and treated with curcumin alone (18 μmol L⁻¹), dasatinib alone (0.01 μmol L⁻¹) or curcumin and dasatinib together for 24 h. The percentage of cell proliferation was taken as an average of the results of triplicates.

LDH leakage assay

HEI-193 cells (1×10^3) were cultured in 96-well plates for 24 h and treated with curcumin alone, dasatinib alone (0.01 μmol L⁻¹) or curcumin and dasatinib together for 24 h. For LDH activity analysis, 300 μL of the medium was taken out and analysed using Cy-QUANT™ LDH Cytotoxicity Assay Kit (Invitrogen, USA).

Trypan blue assay

HEI-193 cells (1×10^3) were cultured in 96-well plates for 24 h and treated with curcumin alone, dasatinib alone (0.01 μmol L⁻¹) or curcumin and dasatinib together for 24 h. For the analysis of trypan blue assay, 0.4 % trypan blue dye was used to determine cell viability. Dead cells take up the dye and can be counted using a haemocytometer, whereas viable cells exclude the dye.

Apoptotic assay

To carry out the apoptotic assay, cells were cultured in 96-well plates for 24 h HEI-193 cells (1×10^3) were cultured in 96-well plates for 24 h and treated with curcumin alone, dasatinib alone (0.01 μmol L⁻¹) or curcumin and dasatinib together for 24 h. ELISA-based In Situ Cell Death Detection Kit (Roche Molecular Biochemicals, USA) was used to determine apoptosis.

Protein extraction

Preparation of HEI-193 cell lysate was achieved using lysis buffer (NP-40). To prevent proteolysis of cell lysate, Halt Protease Inhibitor Cocktail was used. Centrifugation (3000 rpm

for 10 min) of cell lysate was performed to obtain supernatant. Bradford assay was used to determine protein concentration. The preparation of protein samples was carried out as performed by Waza *et al.* (32).

Western blotting

For immunoblotting purposes, proteins were separated on SDS–PAGE and trans-ferred to the PVDF membrane. Detection of target proteins was determined using pri-mary antibodies; anti-Bax, anti-Bcl-2, anti-caspase-3, anti-caspase-9, anti-LC3, anti-Beclin1, anti-Atg7, anti-Atg12-Atg5, anti-p-Akt, anti-Akt, anti-p-mTOR, anti-mTOR, anti-p70S6K, anti-p-p70S6K and anti-GAPDH. Primary antibodies were incubated with the membrane overnight. The membranes were rinsed and further incubated with secondary HRP (horseradish peroxidase)-conjugated antibodies. Detection and quantitative analysis of the antibodies were performed with a gel image processing system.

Statistical analysis

SPSS software was used to carry out statistical analysis. Experimental values were given as mean and standard error of the mean. Statistical significance was measured with ANOVA, and for multiple comparisons, post-hoc test was used ($p < 0.05$ as statistically significant).

RESULTS AND DISCUSSION

Effect of curcumin and dasatinib on cellular toxicity

The combination therapies own the main advantage of reduced development of drug resistance and hence enhanced efficacy (2). Moreover, combination therapies also possess the advantages of a lower rate of treatment failure and less fatality (33). Curcumin has been reported to possess anticancer properties with minimal toxicities against normal cells (34, 35). On the other hand, dasatinib was primarily used against imatinib-resistant leukae-mias and is now being used against various types of tumours (36, 37). The present study aims to study the combined role of curcumin and dasatinib against HEI-193 cells. Herein, we report that the combination of curcumin and dasatinib increases lactate dehydroge-nase (LDH) leakage and cell death in HEI-193 cells.

LDH is found in cells and is responsible for cellular respiration. Breakage of plasma membrane causes LDH release from the cells to outside medium. In cell culture experi-ments, the presence of LDH in the culture medium represents cell death.

Treatment with curcumin resulted in membrane damage in HEI-193 cells as deter-mined by LDH release. However, curcumin together with dasatinib treatment increased LDH leakage more than the individual treatments as shown in Table I.

HEI-193 cell proliferation and cell death were measured using MTT assay and trypan blue assay, respectively. Curcumin treatment decreases cell viability. However, curcumin, together with dasatinib, significantly decreased cell viability. Similarly, curcumin treat-ment increased cell death. However, curcumin together with dasatinib treatment signifi-cantly increased cell death, as shown in Table I.

Table I. Cell viability and death of HEI-193 cells with different treatments

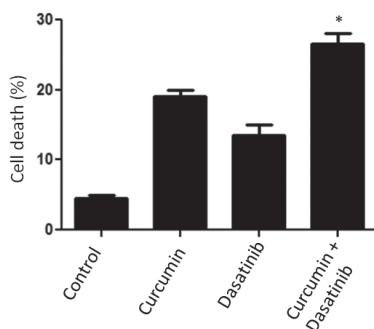| Samples	LDH assay (% cell death compared to control)	Trypan blue dye uptake (% cell death)	MTT assay (% cell proliferation)
Control (cells only)	100	2.7 ± 1.3	100
Curcumin	115.2 ± 3.1	13.1 ± 2.7	72 ± 3.3
Dasatinib	110.4 ± 2.7	11.7 ± 1.9	84 ± 2.7
Curcumin + dasatinib	130.5 ± 4.7	15.7 ± 3.1	66 ± 4.7

Fig. 1. Individual treatments of cells with curcumin, dasatinib and curcumin/dasatinib combination (control – untreated cells).

Effect of curcumin and dasatinib on apoptotic cell death

The apoptotic assay was carried out by using Cell Death Detection ELISA method. During apoptosis, cells exclude fragmented DNA and histone from the nucleus to the cytoplasm. ELISA based method can be used to detect the excluded DNA and histone in the cytoplasm. We found that individual treatments with curcumin and dasatinib significantly ($p < 0.05$) increased cell apoptosis compared to the control (Fig. 1). However, curcumin in combination with dasatinib showed a significant ($p < 0.05$) increase in apoptosis compared with each of them separately.

Effect of curcumin and dasatinib on the expression of apoptotic markers

Cell survival and death are mainly decided by pro- and anti-apoptotic proteins (38). Protein levels of caspases (pro-apoptotic proteins) are known to be up-regulated during cell death (38). On the other hand, protein levels of anti-apoptotic proteins are down-regulated during cell death (39). We observed a significant increase in the expression levels of pro--apoptotic proteins like caspase-3 and caspase-9 expression during combined treatment of curcumin and dasatinib compared to individual treatments (Fig. 2). Similarly, the combination of curcumin and dasatinib significantly decreased expression levels of anti-apoptotic proteins like Bcl-2, while the expression levels of Bax were increased compared to individual treatments.
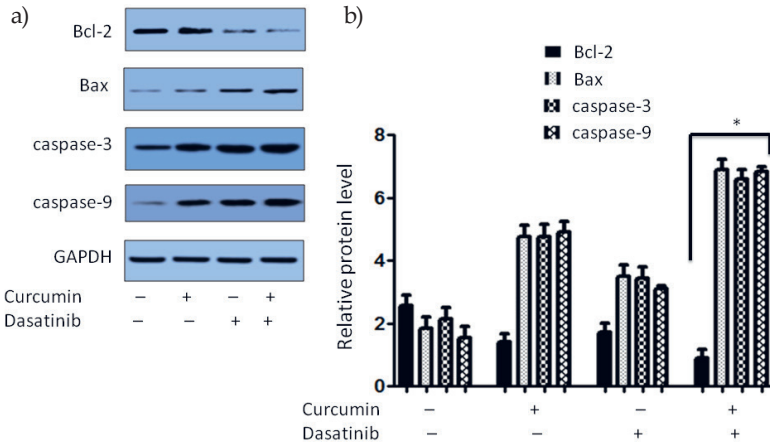
Fig. 2. a) Expression analysis of pro-apoptotic and anti-apoptotic proteins using immunoblotting
(untreated cells – control; GAPDH – internal control), b) densitometry analysis.

To detect the underlying mechanism of cell apoptosis in HEI-193 cells, Western blot-
ting was carried out. Expression levels of Bcl-2 got decreased by individual treatments
with curcumin and dasatinib compared to control (untreated cells). However, the combi-
nation of curcumin and dasatinib was more effective in decreasing the Bcl-2 expression
compared to the individual treatments. Bax expression levels were increased by individual
treatments of curcumin and dasatinib compared to the control. However, the combination
of curcumin and dasatinib significantly increased Bax expression compared to individual
treatments. Similarly, caspase-3 and caspase-9 expression was increased by individual
treatments with curcumin and dasatinib compared to control. However, the combination
of curcumin and dasatinib significantly increased caspase-3 and caspase-9 expression
compared to individual treatments.

Effect of curcumin and dasatinib on the expression of proteins related to autophagy

Autophagy has been reported to allow cell survival and therefore, promote tumour
growth. Different natural agents are known to enhance autophagy in various types of
cancers (22). Increased autophagy causes cell death *via* self-digestion and degrades cellular
organelles and proteins (40). Microtubule-associated protein light chain 3 (LC3) II is an
autophagy-related factor that has been widely studied as an autophagic protein (41). LC3II
serves an important role in the development of autophagosomes and their maturation and
is therefore used to monitor the autophagic activity in cells (42). Previous studies have
demonstrated that curcumin-induced activation of autophagy via promotion of mTOR and
LC3 II expression (43).

We set out an experiment to investigate the effect of the combined treatment of cur-
cumin and dasatinib on autophagy markers in human schwannoma cell HEI-193 cells. We
found that the combination of curcumin and dasatinib more significantly increased
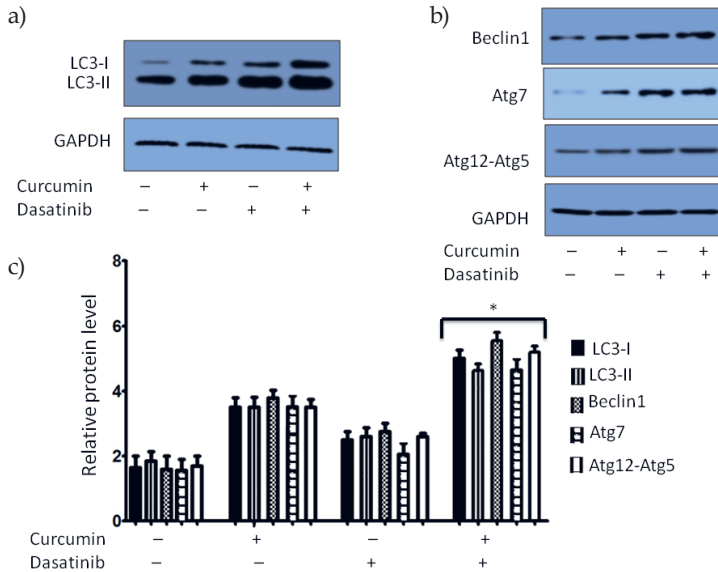expression levels of autophagy markers like LC3 I, LC3 II, Beclin1, Atg7 and Atg12-Atg5

Fig. 3. a) and b) Expression analysis of autophagy-related marker proteins using immunoblotting (untreated cells – control; GAPDH – internal control), c) densitometry analysis.

expression compared to individual treatments (Fig. 3). Curcumin has been reported to increase expression levels of LC3 I and LC3 II in breast cancer (44). Similarly, dasatinib was reported to increase expression levels of LC3 I and LC3 II in ovarian cancer (45). We observed increased levels of LC3 I and LC3 II after individual treatment with curcumin and dasatinib in HEI-193 cells compared to control. However, the combination of curcumin and dasatinib significantly increases LC3 I and LC3 II expression compared to individual treatments. Similarly, increased protein expression levels of Beclin1, Atg7 and Atg12-Atg5 were observed after individual treatments with curcumin and dasatinib compared to control (untreated cells). However, the combination of curcumin and dasatinib significantly increases Beclin1, Atg7 and Atg12-Atg5 expression compared to individual treatments.

Effect of curcumin and dasatinib on Akt/mTOR/p70S6K signalling pathway

Akt/mTOR/p70S6K signalling pathway has been linked to both apoptosis and auto-phagy (46, 47). It has been reported that D6 (a curcumin analogue), possesses antiprolife-rative properties in melanoma partially due to the downregulation of the PI3K/AKT path-way (48). PI3K/AKT/mTOR/P70S6K pathway is considered a critical intracellular signalling pathway for cell survival and death (49). It has been reported that dasatinib induced Src inhibition, which further suppressed the activation of mTOR via the PI3K-Akt signalling pathway in NSCLC Cells (50). Furthermore, the combination of dasatinib and rapamycin was found to induce cell migration and invasion inhibition by suppressing Src/PI3K/AKT signalling (50).
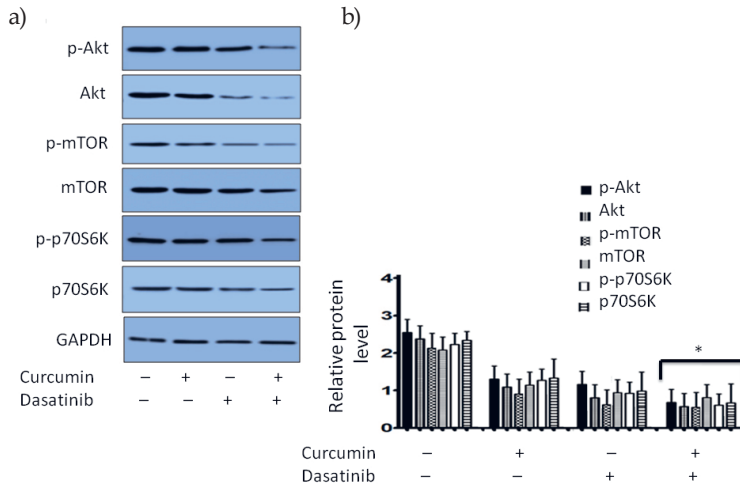
Fig. 4. a) Expression analysis of Akt/mTOR/p70S6K signalling pathway-related proteins using immunoblotting (untreated cells – control; GAPDH – internal control), b) densitometry analysis.

We, therefore, started investigating the effect of the combined treatment of curcumin and dasatinib on the Akt/mTOR/p70S6K signalling pathway in HEI-193 cells. The combination of curcumin and dasatinib significantly decreased protein levels of Akt, p-Akt, mTOR, p-mTOR, p70S6K and p-p70S6K compared to individual treatments (Fig. 4).

CONCLUSIONS

Screening drugs that are previously well-known for curing specific diseases and then coming up with new ways to combine their constituent molecules into a single therapeutic agent could represent a new frontier for pharmacology. In the present study, the combination of curcumin and dasatinib was used against human schwannoma cells HEI-193 to look for their possible synergistic anticancer effects. Our results suggest that curcumin combined with dasatinib significantly enhances cytotoxicity, apoptosis and protective autophagy in HEI-193 cells through Akt/mTOR/p70S6K signalling pathway as compared with the individual treatments. We concluded that the combination of curcumin and dasatinib can be a promising combination when considering the future development of a new therapeutic approach for the treatment of human schwannoma, a devastating tumour of the peripheral nervous system, consisting of different cell types, for which the unmet medical need still exists.

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