Abstract. Verbascoside (Verb) may exhibit potential antitu­mour activities in leukaemia. The present study investigated the effect of Verb, in combination with imatinib (IM), dasatinib (Das), lipopolysaccharide (LPS) and TNF, on cell survival, Abl expression, apoptosis, oxidative stress and the MAPK pathway in chronic myeloid leukaemia (CML) cells. Cell viability was determined using the WST-8 assay in K562 and R-K562 cells treated with Verb and/or IM, Das, LPS and TNF. Apoptosis and DNA damage in CML cells was detected by caspase-3 and comet analysis. The protein levels of Abl (Phospho-Tyr412), and total/phosphorylated p38, JNK and ERK in CML cells were analysed using a Colorimetric Cell-Based Assay. Oxidative stress was examined using total antioxidant and oxidant status assays. Treatment with Verb and/or tyrosine kinase inhibitors (TKIs), LPS and TNF resulted in a significant decrease in the Tyr-412 phosphorylation of Abl in K562 and R-K562 cells. In addition, cotreatment with Verb and IM or Das additively induced apoptosis by activating caspase-3 levels in both cell lines. Activation of p38 and JNK can result in growth arrest and cell death, whereas ERK stimulation results in cell division and differentiation. The present study demonstrated that cotreatment with Verb and TKIs suppressed phosphorylated-ERK1/2, whereas the levels of phosphorylated-p-38 and phosphorylated-JNK were significantly elevated by Verb and/or IM, Das, LPS and TNF, thus suggesting that Abl and Src inhibition could be involved in the effects of Verb on MAPK signalling in R-K562 cells. Furthermore, Verb elevated reactive oxygen species levels additively with TKIs in both cell lines by increasing the oxidant capacity and decreasing the antioxidant capacity. In conclusion, anti-leukemic mechanisms of Verb may be mediated by Abl protein and regulation of its downstream p38-MAPK/JNK pathway, caspase-3 and oxidative stress in CML cells.

Introduction

Verbascoside (Verb) (1) is naturally occurring in many plants such as Paulownia tomentosa, Buddleja brasiliensis, Lantana camara, Olea europea, Striga asiatica, Lippia citriodora, Lippia javanica. Verb is hydrophilic in nature and has various pharmacological activities such as antimicrobial, antioxidant, neuroprotective, anti-inflammatory, and wound healing effects along with pro-apoptotic, anti-proliferative, and pro-differentiative, chemopreventive-chemotherapeutic potential effects in cancer (2-6). Bcr/Abl protein is encoded by the Bcr/Abl fusion gene, which causes chronic myeloid leukaemia (CML) due to uncontrolled tyrosine kinase activity. Hematopoietic cell differentiation and proliferation are destroyed by Bcr/Abl (7). It was reported that differentiation of leukemic cells could induced by Verb towards the macrophage-monocyte lineage (8). Verb has been proposed to be the investigational drug for the treat­ment of leukaemia patients (9,10). Tyrosine kinase inhibitor; Imatinib mesylate (IM) targets the inhibition of Bcr/Abl and involved in the stimulation of apoptosis in leukemic cells (11). Unfortunately, relapse and resistance can be seen in some patients (12). IM resistance is mainly related to Abl mutations (Val256Gly, p.Thr315Ile, p.Gly250Glu, p.Tyr253His, p.Phe317Leu and p.Met351Thr) and amplification of the Bcr/Abl oncogene, but, some patients lacking these mutations could be unresponsive to IM treatment (13). Bcr/Abl independent resistance mechanisms are still being investigated (14,15). To overcome the resistance, 2nd generation Abl tyrosine kinase inhibitors have been developed. Dasatinib (Das) is 325-fold more potent against cells expressing wild-type Bcr/Abl compared to IM (16). Utilization of elevated concentrations of chemotherapeutic drugs to treat cancers might possibly cause cytotoxic effects on the healthy cells. So, possible usage of...
the least drug concentrations is the main aim in the treatment of various cancers. Natural compounds with low toxicity can influence the resistance mechanisms (17). Complete destruction of resistant leukemic cells may require other therapeutic agents in combination with Bcr/Abl inhibitors that modify the molecular pathways to control cell survival. Only a few studies have investigated the role of Verb in leukaemia. It remains unclear whether Verb regulates the biological functions of leukaemia cells and overcomes the resistance. Therapeutic applications targeting the inflammatory environment might perturb CML cells (18). Tumour necrosis factor (TNFα) is a cytokine that plays a role in inflammation, stress response, and apoptosis and produced mainly by activated macrophages (19). In our study Lipopolysaccharide (LPS) and TNFα were utilized to trigger inflammation in sensitive (K562) and resistant K562 (R-K562) cells and the effect of Verb on oxidative stress, apoptosis, and mitogen-activated protein kinase (MAPK) signalling pathway was investigated. The abnormal activation of MAPK signalling pathway by Bcr/Abl tyrosine kinase plays an important role in the progression of leukaemia (20). Extracellular signalling molecules such as cytokines, cellular stress, and growth factors could activate the MAPK pathway. MAPK is involved in the regulation of cell proliferation, differentiation, cell cycle, survival, and induction of leukemogenesis. p38 MAP kinase, c-Jun N-terminal kinase (JNK), extracellular signal regulated kinase (ERK) are three main members of the MAPK pathway (21). As a biological material we used rapidly proliferating human myelogenous leukaemia cell line K562. In the present study we aimed to investigate the effects of Verb alone and its concomitant use with IM, Das, LPS and TNFα on apoptosis, oxidative stress and MAPK pathway in both IM-sensitive and IM-resistant K562 CML cells in order to assess possible interactions between inhibitors of tyrosine kinase and Verb.

Materials and methods

Cell cultures. K562 (CCL-243); human chronic myelogenous leukaemia cell line were purchased from ATCC (American type culture collection). Authentication of the cells was performed by the American Type Culture Collection using short tandem repeat analysis on the cells. Cells were cultured in Iscove's Modified Dulbecco's Medium (Wisent Bioproducts) supplemented with 10% foetal bovine serum (Gibco) and 1% of penicillin-streptomycin antibiotics (Bio Ind). Cells were maintained under 5% CO₂ at 37˚C conditions. Increasing concentrations of IM were applied to K562 cells stepwise for 12 months between 0.1 µM-10 µM (22). Resistant cells were separated using a Ficoll-Hypaque gradient, washed with IMDM, and kept in IMDM with 10% FBS and 10 µM IM. Subpopulation cells which were able to grow in 10 µM IM, 200 µM Das, 2,500 nM Das, 100 ng/ml LPS (26), 10 ng/ml TNFα (19) for a duration of 48 h at 37˚C.

Cell viability. Cell viability of untreated controls and drug-treated cells were assessed by the WST-8 Cell Proliferation Kit (Elabscience). 20x10⁵ cells were seeded into 96-well plates in 100 ml IMDM medium for each well. At the end of the assay, absorbance of each sample was measured using a microplate reader (Biotek).

Abl (Phospho-Tyr412) colorimetric cell-based analysis. Tyr412 is necessary both for activity and for regulation of Bcr/Abl, by stabilizing the inactive or the active conformation of the enzyme in a phosphorylation-dependent manner (27). The Colorimetric Cell-Based ELISA Kit (Cat#EKC2643, Boster Biological Technology, USA) allows for the detection of Abl (Phospho-Tyr412). The effects of Verb alone and in combination with IM, Das, LPS and TNFα on target protein expression determined in K562 and R-K562 cells. The Abl protein is captured by target-specific primary (1°) antibody while the HRP-conjugated secondary (2°) antibody binds to the Fc region of the 1° antibody. Through this binding, the HRP enzyme conjugated to the 2° antibody catalysed a colorimetric reaction upon substrate addition. The phosphorylated target protein's OD values were normalized using the non-phosphorylated target protein's OD values (Anti-ABL2(Phosphorylated)Antibody)/OD450(Anti-ABL2 Antibody). The measured OD450 readings were normalized to the OD595 values obtained for crystal violet staining (OD450/OD595). Crystal violet solution was provided as a ready-to-use solution in the kit. Briefly, 50 µl crystal violet solution was added to each well and was incubated for 30 min at room temperature on a shaker. Crystal violet binds to cell nuclei and gives absorbance readings proportional to cell counts at 595 nm. GAPDH specific monoclonal antibody was used to serve as an internal positive control in normalizing the target absorbance values.

Caspase-3 analysis. Caspase-3 enzyme activity was determined using a colorimetric assay (Elabscience). The cells that had been treated with IM, Das, LPS and TNFα and/or Verb for 48 h were collected by centrifugation at 1,000 g for 10 min. Pelleted cells were treated with 100 µl of cold lysis buffer to obtain the cell lysate. Then, the cell lysates were incubated on ice for 10 min and were centrifuged at 14,000 g for 1 min. Following the centrifugation, supernatants were transferred to new microcentrifuge tubes. For measuring caspase-3 enzyme activity, 100 µl of the samples were added to the wells, after the incubation period 100 µl Biotinylated Detection Ab
working solution, 100 μl HRP conjugate working solution, 90 μl Substrate Reagent and 50 μl Stop Solution applied to each well respectively. Absorbances of the samples were read under 450 nm wavelength of light via a microplate reader.

**Spheroid formation.** It was reported that tumour spheroid formation was observed only in the resistant K562 cells, independently of the absence or the presence of IM in the culture medium (28). 1x10⁴ cells were plated per well in a 6-well plate pre-coated with 1ml of agar-agar (Sigma) in serum-free IMDM and incubated at 37°C in 5% CO₂ for 10 days. The colonies were photographed every 5 days (Olympus CKX41).

**Cell-based ELISA measuring MAPK protein expression levels.** Total/phosphorylated protein levels of ERK, p38 and JNK were determined using cell-based ELISA (RayBio® Cell Based Assay cat. no. CBEL-ERK-SK; RayBiotech Life, Inc.). These assays can be used for determination of the relative amount of p38 MAPK (Thr182/Tyr185), JNK (Thr183/Tyr185) and ERK1/2 (Thr202/Tyr204) phosphorylation. 50,000 cells were seeded into a 96 well tissue culture plate coated by adding 100 μl poly-L-Lysine. The cells were fixed after the incubation period. After blocking, anti-phospho‑protein specific antibody or anti-pan-protein specific antibody was added into the wells and incubated. The wells were washed, and HRP-conjugated anti-mouse IgG was added. TMB substrate solution was added and incubated. The wells were washed, and HRP-conjugated anti-mouse IgG was added. TMB substrate solution was added and incubated. The colour intensity was assessed by the spectrophotometry method is associated with the amount of total oxidant status (TOS).

**Measurements of total oxidant status (TOS).** Incubation of K562 and R-K562 cells with the IM, Das, LPS and TNFα and/or Verb for 48 h, the cell culture media was discarded. Colorimetric method was applied to the supernatants to detect the TOS (29). In this method, the ferrous ion O-dianisidine complex was oxidized to ferric ion. The glycerol molecules which are abundant in the reaction medium, enhance the oxidation reaction. A coloured complex with xylenol orange appeared due to the ferric ion present in the acid medium. The colour intensity was assessed by the spectrophotometry method is associated with the amount of total oxidant molecules present in the sample. The calibrations were carried out using hydrogen peroxide, and the obtained results were explained as mmol H₂O₂ equiv./l.

**Measurements of total antioxidant status (TAS).** The Fenton reaction produced a hydroxy radical, which is the most powerful biological radical. The dianisyl radical, which has a bright yellowish-brown colour, was also obtained due to the hydroxyl radical's reaction with the colourless substrate O-dianisidine (29). Upon the addition of a cell culture medium sample, the oxidative reactions initiated by the hydroxyl radicals present in the reaction mix are suppressed by the antioxidant components of the sample, preventing the colour change and thereby producing an effective way to measure the TAS level. The assay results are expressed as mmol Trolox equiv./l. The protein concentration of the samples obtained for the TAS-TOS assays were determined by the Bradford method using BSA as a standard.

**Determination of oxidative stress index (OSI).** The OSI was calculated in accordance with the following formula: OSI (arbitrary unit)=TOS (μmol H₂O₂ equiv./l)/TAS (mmol Trolox equiv./l) x10.

**Comet assay.** DNA damage and any genotoxic effects of IM, Das, Verb, IM + Verb, Das + Verb were determined using Comet Assay IV Version 4.3.2 for Basler FireWire in K562 cells. K562 cells were incubated with the agents for 48 h. Then the cell mediums were removed and cells were washed in 0.1 M PBS for three times, separated, and then suspended in 0.1 M PBS (2x10³ cells in 75 μl PBS) with low melting agarose (LMA). The cells of all groups were divided into three layers. The first layer contained 1% normal melting agarose. The cells were suspended in a second layer and 1% LMA was added to the solidified first layer. A third layer containing 1% LMA was subsequently added. All these stages were performed in the cold. After the solidification process, all slides were kept in the lysing solution for 1 h at 4°C. Next, the slides were placed in the electrophoresis buffer for 20 min. Then, the slides were electrophoresed at 25 V (300 mA, approx. 0.74 V/cm) for 30 min at 4°C. The samples were washed 3 times with the neutralization buffer for 5 min, and then the slides were immersed in methanol for 5 min at -20°C. To prevent exogenous DNA damage, these processes were carried out in a dark medium. Then, the slides were placed on a smooth area to dry. The slides were saturated with ethidium bromide (60 μl) and then monitored the 590 nm emission and 510-560 nm excitation filters using a Nikon fluorescent microscope. Images of 50 randomly chosen comets on each triplicate slide were captured per sample using x20 magnification (30).

**Statistical analysis.** Data are expressed as mean ± standard deviation of three independent determinations. An error probability with P<0.05 was selected as significant. All experiments were performed in triplicate and the mean value was used for analysis. A one-way analysis of variance (ANOVA) was used to test significant (P<0.05) differences between the groups (Control, IM, Das, LPS, TNF and/or Verb) datasets, separately. When significant, the differences were further tested with a post hoc Tukey-HSD test. Shapiro-Wilk test was applied, prior to ANOVA routine, to test for normality and homogeneity of variances, respectively. When those conditions could not be met, a Kruskal-Wallis analysis and Dunn's test were used.

**Results**

**Cell viability assessment via WST8 cell proliferation assay.** To analyse the effectiveness of Verb on cell viability we used K562 and R-K562 cells. The cell viability of K562 control cells incubated with no agent was set as 100% and the cell viability of the other groups was compared to the viability of the control cells. In order to determine the % cell viability of K562 and R-K562 cells we have treated the cells with different concentrations of IM (1-10-100 μM) for 24 and 48 h. We have observed a significant change in cell viability in 100 μM IM dosage after 24 and 48 h of exposure compared to untreated control cells in K562 cells. IM had a significantly higher action on 48 h incubation in K562 cells (Fig. 1A). Cell viability was...
observed as 67.2 and 65% after 2,500 nM Das, incubation respectively in 24 and 48 h (Fig. 1B). 48 h incubation was selected for further analysis. And the 48-h treatment was fixed for the treatment of R-K562 cells. We incubated K562 cells with Verb alone and in combination with IM, Das, LPS and TNFα for 48 and 72 h. Cell viability was quantified using the WST8 assay. Relative amount of cell viability of the Verb and/or IM, Das, LPS and TNFα in K562 and R-K562 cells were shown in Fig. 2. Incubation with 100 µM IM, 2,500 nM Das, 100 ng/ml LPS, 10 ng/ml TNFα and 50 µg/ml Verb led to a significant decrease in cell viability after 48 and 72 h. Concomitant incubation of Verb with selected TKI drugs and inflammation inducers, LPS and TNFα, caused a dramatic diminution in K562 and R-K562 cell viability. Cell viability was observed as 51, 65, 52, 45% after IM, Das, LPS and TNFα incubation respectively in 48 h treatment resulted in 43, 52, 35% cell viability after IM, Das, LPS and TNFα incubation, respectively. Verb treatment was more effective when combined with the IM, Das, LPS and TNFα on cell viability than its single use in 48 or 72 h timeline. 48 h treatment of Verb was seen as sufficient for obtaining the inhibitory effect on cell viability combined with the selected drugs. Hence, 48 h treatment was applied to the R-K562 cells for further experiments. Treatment with Verb alone or in combination with IM, Das, LPS and TNFα resulted in reduced cell viability after 48 h in R-K562 cells (Fig. 2B). Cell viability was observed as 51.5, 42.7, 52.8, 52.2% after IM, Das, LPS and TNFα incubation respectively in 48 h treatment in R-K562 cells. Cell viability was observed as 51.5% after IM incubation and 70.23% after Verb incubation in R-K562 cells (Fig. 2C). Concomitant use of Verb with IM, led to a significant decrease in cell viability (52.29%) compared to single treatment of Verb. But the viability of R-K562 cells were not significantly increased in the IM + Verb treatment compared to single IM treatment. This observation could result from the increased proliferation rate of R-K562 cells compared to their non-resistant counterparts. But Verb shows its anti-leukemic effect by downregulating of

Figure 1. Cell viability of K562 cells treated with different concentrations of (A) IM (1-10-100 µM) and (B) Das (1-5-10-100-1,000-2,500 nM) for 24 and 48 h. *P<0.05 K562 cells incubated with 100 µM IM vs. K562 control cells; #P<0.05 K562 cells incubated with 2,500 nM Das vs. K562 control cells (n=5). IM, imatinib; Das, dasatinib.

Figure 2. Viability of cells treated with 100 µM IM, 2,500 nM Das, 100 ng/ml LPS, 10 ng/ml TNF and/or 50 µg/ml Verb K562. Cells were treated for (A) 48 h and (B) 72 h. *P<0.05 untreated control cells vs. all other groups, P<0.05 IM + Verb vs. IM and Verb, P<0.05 Das + Verb vs. Das and Verb, P<0.05 LPS + Verb vs. LPS and Verb, P<0.05 TNF + Verb vs. TNF and Verb, P<0.05 TNF + Verb vs. Verb. (C) R-K562 cells were treated for 48 h. *P<0.05 R-K562 untreated control cells vs. all other groups, P<0.05 Verb vs. IM + Verb, LPS + Verb and TNF + Verb, P<0.05 Das vs. Das + Verb (n=5). IM, imatinib; Das, dasatinib; LPS, lipopolysaccharide; TNF, tumor necrosis factor; Verb, verbascoside.
Abl, inhibiting of MAPK/ERK signalling and ROS mediated DNA damage.

**Abl colorimetric cell-based assay.** Abl (Phospho-Tyr412) levels were determined in K562 and R-K562 cells grown in complete media supplemented with 100 µM IM, 2,500 nM Das, 100 ng/ml LPS, 10 ng/ml TNF and/or 50 µg/ml Verb for 48 h. *P<0.05 untreated control cells vs. cells treated with IM, Das, LPS, TNF and/or Verb; †P<0.05 TNF vs. TNF + Verb; ‡P<0.05 Verb vs. IM + Verb, Das + Verb and TNF + Verb (n=5). IM, imatinib; Das, dasatinib; LPS, lipopolysaccharide; TNF, tumor necrosis factor α; Verb, verbascoside.

**Caspase-3 analysis.** Caspase-3 levels were evaluated in K562 and R-K562 cells grown in complete media supplemented with 100 µM IM, 2,500 nM Das, 100 ng/ml LPS, 10 ng/ml TNF and/or 50 µg/ml Verb for 48 h. *P<0.05 untreated K562 control cells vs. cells treated with Das, TNF, Verb, IM + Verb, Das + Verb, LPS + Verb and TNF + Verb, †P<0.05 IM + Verb vs. IM, ‡P<0.05 Das + Verb vs. Das, ‡P<0.05 LPS + Verb vs. LPS, †P<0.05 untreated R-K562 control cells vs. cells treated with IM, Das, TNF, IM + Verb, Das + Verb and TNF + Verb, †P<0.05 Verb vs. IM + Verb, Das + Verb and TNF + Verb (n=5). IM, imatinib; Das, dasatinib; LPS, lipopolysaccharide; TNF, tumor necrosis factor α; Verb, verbascoside.

**Total/phosphorylated ERK, JNK, p38 protein expression.** Cell-based sandwich ELISA was used to determine total and phosphorylated p38, JNK, ERK protein expression levels in R-K562 cells. Bcr/Abl is activated through the activation of the ERK/MAPK pathway (31). There is no study in the literature...
investigating ERK, p38MAPK, JNK pathway regulated by Verb in resistant CML cells. Regarding MAPK signalling pathway family members, Verb and its combinational therapy with IM, Das, LPS and TNF cause a significant downregulation in ERK1/2 regard to the untreated R-K562 control cells but the Verb and/or TKIs caused an upregulation in the phospho-JNK and phospho-p38 levels in 48 h treatment. Verb increased ERK/MAPK signalling (Fig. 5). There was no significant difference in the total protein levels of ERK1/2 and p-38 following the incubations, compared to the untreated control. Total protein level of JNK increased by the incubations of Verb and/or IM, Das, LPS, TNF. Verb reinforced the TKIs, LPS and TNF’s activity in upregulating p-JNK and p-p38. Concomitant incubation of Verb with IM, Das, LPS and TNF decreased ERK1/2 levels.

**Spheroid formation assay.** The in vitro spheroid formation assay is a common assay used to measure the self-renewal and multipotent nature of the cancer cell subpopulations within cancer cell line. In our study we incubated K562 and R-K562 cells in soft agar culture medium for 10 days. Multicellular tumour spheroid formation was observed on 5 and 10th days. K562 cells do not form tumour spheroids whereas R-K562 cells show spheroid formation. We have observed the spheroid formation in R-K562 cells in time dependent manner (Fig. 6). Spheroid formation assay confirms the resistant characteristics of the R-K562 cells.

**TAS, TOS and OSI.** The effects of Verb and/or IM, Das, LPS, TNF for 48 h on oxidative stress were determined in K562 and R-K562 cells by using TAS-TOS assay. TAS levels significantly decreased in K562 cells treated with IM, Das, LPS and TNF compared to untreated K562 control cells. Verb and its combinational treatment with IM, Das, LPS and TNF also led to a significant decrease in TAS levels in K562 cells. The antioxidant capacity of R-K562 cells was significantly reduced by IM, Das, LPS and TNF. Verb decreased the TAS levels when used together with the Das, LPS and TNF. The TOS levels significantly increased in K562 and R-K562 cells treated with IM, Das, LPS, TNF, Das + Verb, LPS + Verb and TNF + Verb groups. Verb potentiated the TOS inducing activity of Das, LPS and TNF in K562 cells. OSI levels were elevated by all the tested substances (Table I).

**Comet assay.** A Comet assay was measured DNA damage in K562 cells treated with IM, Das, Verb, IM + Verb, Das + Verb for 48 h. Findings were evaluated in accordance with DNA tail length and DNA tail intensity. In K562 cells treated with IM, Das, IM + Verb, Das + Verb tail length, tail intensity of DNA were found to be elevated with respect to the control cells. Verb enhanced the DNA tail length when combined with IM or Das compared to IM or Das alone groups (Fig. 7).
Discussion

Bcr/Abl tyrosine kinase inhibitors have been demonstrated to have significant therapeutic effects in leukaemia. But CML is a life-threatening disease for patients with blast phases, mainly resulting from TKI resistance (32). Resistance and toxicity of tyrosine kinase inhibitors have been frequently reported. Resistant CML cells could have improved DNA repair, impaired apoptosis, modification in membrane transport systems, proteins and enzymes (33). Thus, a TKI in combination with additional drugs, potentiating the efficacy of Bcr/Abl inhibitors for the treatment of leukaemia seems to be an encouraging strategy to tackle the challenges related to CML treatment. There is a demand for continued research into alternative pharmacological approaches with not only more effective but also with less toxic effects. Because of the anticancer effect of natural extracts, they have drawn attention and been investigated in many studies. Verb is hydrophilic in nature and possesses pharmaco-logically beneficial activities for human health, including anti-oxidant, anti-inflammatory and anti-neoplastic properties (1). It has been reported that Verb prevented the proliferation of HL-60 leukaemia cell line in a time and concentration dependent manner. Additionally, cell cycle progression is blocked by Verb at the G1 phase (9). A new delivery system is being investigated by the researchers for boosting Verb’s chemotherapeutic effect against drug-resistant leukaemia cells. Verb containing nanoproduct showed visible tumour suppressor effects by elevating caspase 3 expression (34). The accumulated evidence shows that Verb could have potential antitumour activities in leukaemia, but its activity against sensitive and resistant K562 cells in the presence of TKIs is yet to be investigated. K562 cells have been largely used as a model system for investigating drug development technologies against CML. Different compounds are under investigation to show cell viability changes in K562 cells to induce apoptosis. We explored the cytotoxic activity of Verb and/or TKIs, LPS and TNF against IM sensitive and resistant leukaemia cell lines and found that Verb possesses a remarkable antiproliferative
effect on the both K562 and R-K562 cells additively with the TKIs; IM and Das or inducers of inflammation and apoptosis LPS and TNF (Fig. 2).

Cell viability was observed as 51.5% after IM incubation and 70.23% after Verb incubation in R-K562 cells (Fig. 2C). Concomitant use of Verb with IM, led to a significant decrease in cell viability (52.29%) compared to single treatment of Verb. But the viability of R-K562 cells was not significantly increased in the IM + Verb treatment compared to single the IM treatment. This observation could result from the increased proliferation rate of R-K562 cells compared to their non-resistant counterparts. The expression of Ki67 is strongly associated with tumour cell proliferation and growth and is widely used in the routine pathological investigation as a proliferation marker. Limitations of this study are that the lack of cell viability testing on the range of Verb concentrations on K562 and R-K562 cells and the viability of K562 cells with that of R-K562 cells in response to different concentrations of IM was not compared. We could not evaluate the Ki67 expression levels in our study groups and the establishment of R-K562 cells was confirmed only by Spheroid formation assay and Bcr/Abl expression levels. Another limitation of our study was Verb failed to reduce Ab1 levels when combined with the Das in R-K562 cells compared to Das alone treatment and did not potentiate the Ab1 expression-lowering effect of Das.

Bcr/Abl contributes to the development of CML by enhancing the proliferation potential of hematopoietic progenitors and preventing progenitor cells from apoptosis. To get more insights into Verb's molecular mechanism, we assessed its effect on Ab11 activity, which is amplified in R-K562 cells. As anticipated, Verb inhibited Ab1 phosphorylation at 50 µg/ml concentration. Our results demonstrate that Bcr/Ab1 protein levels are downregulated by Verb (Fig. 3), indicating that Verb's anti-leukemic activity is related to Bcr/Ab1 expression. In our study Verb treatment significantly inhibited Ab1 expressions, showing that Ab1 inhibition by Verb contributed to the growth inhibitions in both K562 and K-562 cells. Although Ab1 is considered the trigger of leukemogenesis, targeting Ab1 kinase activity alone may be insufficient because of Ab1 independent downstream pathways. TKIs, LPS, TNF and/or Verb showed a significant decrease in the Tyr-412 phosphorylation of Ab1 in K562 and R-K562 cells. Verb insignificantly potentiates the inhibitory effect of TKIs, LPS and TNF on Ab1 expression in R-K562 cells. Src family kinases (SFK) lead to the activation of Bcr/Ab1 in the progression of CML. SFK inhibitor Das, may suppress Bcr/Ab1 phosphorylation and decrease the tyrosine kinase activity of Bcr/Ab1. Accordingly, Das treatment significantly alleviated the Ab1 phosphorylation in K562 and R-K562 cells compared to untreated control cells. SFKs are also involved in IM resistance. To overcome resistance problems in CML, combinations of drugs with IM provided an emerging therapeutic concept. Verb induced programmed cell death in CML cells more efficiently than IM, Das, LPS or TNF compared to their single use at 48 h of treatment. It has been reported that Das induces the apoptosis of leukemic cells (17,35). De Martino et al revealed that citrals containing Verb were also apoptotic against leukaemia cells by activation of caspase-3 (36). Our data showed that the combination of Verb with IM or Das induces apoptosis additively in both cells. Verb combined TKIs significantly
cell survival, while high levels of ROS can overcome tumour growth by triggering inhibitors of cell cycle (43). In our study, Verb elevated ROS levels additively with TKIs in both sensitive and resistant cells by increasing the oxidant capacity and decreasing the antioxidant capacity. It is also shown by the other researchers reporting that the Verb has the potential to elevate ROS levels inside the tumour cells (44). Taken together, these data suggest that TKIs and Verb, exert cytotoxic effects on K562 and induce apoptosis by triggering ROS. Inflammation was triggered by LPS in K562 and R-K562 cells and the role of Bcr/Abl, caspase-3 and ROS axis was investigated in our study. The results are compatible with previous studies obtained by Wang et al., whose data confirmed that LPS treatment increased ROS production in K562 cells (45). Verb increased the TOS levels of K562 cells compared to LPS’s single treatment. TAS levels diminished by the LPS and/or Verb in both cells. Speranza et al. reported that Verb has anti-inflammatory activity on LPS treated THP-1, human myelomonocytic leukaemia cells, the anti-inflammatory effects of Verb are related to inhibition of intracellular O$_2^−$-production and the suppression of anti-oxidant enzymes at the post-translational level (46). ROS regulates TNF induced signalling pathways. In our findings it can be observed that TNF significantly increased TOS levels alone or in combination with Verb. González-Flores et al. reported that treatment of K562 cells with TNFα stimulated intracellular ROS levels followed by raised caspase-3 (19). Similarly caspase-3 levels were elevated by TNF and TNF + Verb in both cells in our study. TNFα triggered intracellular ROS generation and led to caspase activation and apoptosis in K562 and R-K562 cells.

The production of ‘comet tails’ during agarose gel electrophoresis can be used to identify DNA damage. Tails with a greater DNA content indicate a more severe DNA damage profile. We have evaluated DNA damage induced by IM, Das and/or Verb in K562 cells. The comet experiment revealed that Verb was potentiated the effect of IM and Das on DNA damage by increasing the length and intensity of DNA tails (Fig. 7). IM and Das, which are commonly used in the treatment of haematological malignancies, and their Verb combination led to increased ROS levels shown by the elevated TOS levels (Table I). Besides the damaging effects on DNA IM, Das and their Verb combination treatment also resulted in apoptosis by activating the caspase-3. Our results indicate that the K562 cells are vulnerable to the genotoxic stress of IM, Das and/or Verb, when compared with K562 control cells. The increased DNA damage with the tested compounds can probably be explained by the fact that the mechanism of DNA damage is oxidative. Mutations in the Abl kinase domain occur by conformational changes that reduce the affinity of the ATP-binding pocket of the TKI, thus leading to drug resistance. The resistance of IM occurs due to point mutations. T212R mutation in Abl kinase domain is determined in the SH2-SH3 domain that generates conformation changes to prevent the binding of TKIs. Abl kinase gatekeeper T315I mutation has proven challenging to inhibit with ATP mimetics. Therefore, severe mutations have urged the second-line therapy (Das, nilotinib, bosutinib) against these particular Bcr-Abl mutants present in the patients (47). Tyr177 and Tyr412 are the two constitutive active fusions of Bcr-Abl. Tyr177 serves as a binding site for the Grb2 adaptor and Tyr412 is required for the activity and the control of c-Abl, which stabilizes the enzyme's inactive or active conformation in a phosphorylation-dependent manner (48). The catalytic domain includes a substrate-binding site. Inhibitors targeting this site would be less affected by mutations. IM works by binding to the ATP cleft of the inactive form of Bcr-Abl and preventing the conformational change required for kinase activation. The literature reported that the amide substituent on the phenyl ring of IM and Das provided the molecule with inhibitory activity against Abl tyrosine kinase. The core phenyl or heterocyclic rings of these drugs occupy the adenine pocket of Abl (49). Verb has many reactive sites with high selectivity for combinatorial chemistry. Verb is a phenylethanoid consisting of a cinnamic acid and hydroxy-phenylethyl moieties attached to a β-glucopyranose through a glycosidic bond. In a recent study the strong inhibition potency of Verb had been shown against Carbonic Anhydrase Enzyme. Docking results confirmed the strong interactions between Verb and the active site of the enzyme (50). Similar binding properties could be found between Abl and the Verb, although further research is needed. Although the biochemical roles and stereochemistry of the Bcr-Abl enzyme active sites have been well identified, and numerous natural and synthetic compounds have been tested for inhibitory action, there is no one-size-fits-all structural type that is the most effective.

Verb is suggested to be possible for the future use of chemotherapy and co-therapy in the clinic (51). We revealed that Verb could repress cell growth and induce apoptosis in K562 and R-K562 cells. The mechanisms involve the inhibition of the Abl oncoprotein and regulation of its downstream p38-MAPK/JNK pathway. Verb effectively suppressed the crosstalk between MAPK and Bcr/Abl signalling, thereby increasing the sensitivity of CML cells towards TKIs without suppressing the inflammation. Thus, Verb may be used additively with Das or IM in the treatment of CML. More research is needed to elucidate the precise mechanisms by that these molecules exert their effects.

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Availability of data and materials

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

Authors’ contributions

ACD designed and conducted all the experimental procedures of the study. GAC interpreted the results. EKT conducted the measurement of TAS/TOS. FA performed the comet assay. ACD and EKT confirm the authenticity of all the raw data. All authors read and approved the final manuscript.
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