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Imatinib mesylate resistance in Chronic Myelogenous Leukemia cells may be overcome by Indian Spice Curcumin

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Abstract

Imatinib mesylate (IM), the drug used in chronic myeloid leukemia (CML) therapy, acts by targeting the Philadelphia chromosome (Bcr-Abl). A disadvantage of IM is development of drug resistance. Expression of various tumor markers like Bcr–Abl, TNF-α, NF-κB, Interleukins(IL-6, IL-8, IL-10), JAK2, STAT3, ERK1/2, PI3K, AKT are anomalously expressed in CML. In IM-resistant CML, having high expression of p-glycoprotein and MRP, levels of these markers are even more aberrant. This study aims to develop a means to overcome drug-resistance using nontoxic phytochemical curcumin, which is a common Indian spice. K-562^R, a drug resistant population was developed from parental K-562 by exposing cells to gradually increasing doses of IM. IM regulates the tumor markers and induces apoptosis in K-562 cells; presence of curcumin enhances the effect. IM, showed no/little effect on aberrant expression of tumor markers in K-562^R cells; however, inclusion of curcumin along with the drug, efficiently regulates these biomarkers, leading to induction of apoptosis even in drug-resistant CML cells. Results obtained indicate that curcumin administration may help to overcome IM-resistance in CML cells. The study highlights the potential of curcumin in combating the problem of drug resistance in leukemia therapy.

Keywords: Chronic Myeloid Leukemia, drug resistance, Imatinib mesylate, Bcr-Abl, Curcumin.

1. Introduction

Myelogenous leukemia is characterized by aberrant proliferation of cells in the bone marrow and spleen and may be acute myelogenous leukemia(AML) or chronic myelogenous leukemia(CML), later being the commonest adult leukemia encountered in the Asian countries [1]. CML is characterised by p210Bcr-Abl, formed due to translocation between 9 and 22 chromosomes [2]. Bcr-Abl shows constitutive tyrosine kinase activity and regulates a number of signalling pathways including Janus kinase-Signal Transducer and Activator of Transcription (JAK-STAT), Mitogen-activated protein kinase (MAPK), Phosphoinositide 3-kinase (PI3K/Akt), Nuclear factor-κB (NFκB) and others [3, 4], aberrant expression of which promotes proliferation, hence survival of leukemia cells. Various cytokines like Tumor necrosis factor alpha (TNF-α), IL-6, IL-8, IL-10 are also aberrantly expressed in leukemia, of which TNF-α contributes to leukemogenesis via the NFκB pathway [5, 6]. Upon nuclear translocation, NF-κB gets activated, regulating the expression of various other genes that lead to haematological malignancies, including pro-inflammatory cytokines like IL-6 and IL-8 [7, 8]. NFκB is linked to the anti-inflammatory cytokine IL-10, promoting development of malignancy [9].

Binding of IL-6 to its receptor activates the JAK/STAT pathway, which is implicated in leukemia. [10]. MAP kinases are serine-threonine kinases mediating malignant transformation and cell-cycle progression[11]. Abnormal regulation of PI3K/Akt network plays pivotal role in the etiology of leukemia. These pathways are activated by cytokines (IL-6, IL-8, IL-10, TNF-α) [12], NF-κB, p210 Bcr-Abl. Studies have shown that cross-talk between these signaling pathways also contribute to leukemogenesis. The standard therapeutic modality in CML is the use of tyrosine kinase inhibitors(TKIs) [13]. Use of tyrosine kinase inhibitors like imatinib mesylate(IM), targeting Bcr-Abl has drastically improved treatment response[14]. Despite its efficacy, administration of IM is associated with various adverse effects and development of resistance to IM poses a serious challenge to therapy [15]. These problems are often encountered with next generation TKIs, like Dasatinib, Poantinib etc [16]. Thus, reversal of drug resistance using nontoxic means needs urgent attention to improve prognosis. Curcumin, extracted from the rhizomes of the plant *Curcuma longa*, is bestowed with strong anti cancer properties [17] and is non-toxic upto a dose of 12 g/day[18]. Due to its efficacy in reversal of drug resistance curcumin may be a good option to cope IM resistance in CML.

Present study aims to address the problem of drug resistance by curcumin.

2. Materials and methods

2.1 Materials

Cell culture media RPMI-1640 was obtained from GIBCO-BRL India Pvt. Ltd, New Delhi, India. Acrylamide, N, N'-methylenebisacrylamide, fetal bovine serum (FBS) and kits for detecting levels of TNF-α, IL-8 and IL-10 were procured from Invitrogen BioServices India Pvt. Ltd., Bangalore, India. Dithiothreitol (DTT), bovine serum albumin (BSA), Ponceau S, RNase A, proteinase K, propidium iodide (PI), ethylene glycol-O,-O'-bis,(2-aminoethyl) N,N,N',N'-tetra acetic acid (EGTA), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), CHAPS, curcumin and IM were purchased from Sigma Chemical Co, St. Louis, MO, USA. Nitrocellulose membrane was obtained from Hybond ECL, Amersham Biosciences, UK. Tris, glycine and sodium dodecyl sulfate (SDS) were purchased from Amresco, Ohio, USA. NFκB p65 Transcription Factor Assay Kit used to detect NFκB activity was procured from Abcam, Cambridge, United Kingdom. Antibodies against PgP, MRP1, JAK2, STAT3, PI3K, AKT, ERK1/2 were purchased from GENETEX, USA. Antibodies against caspases 3, 8 and 9 were procured from Santa Cruz Biotech, California, USA. Anti-BCR-ABL Antibody [7C6] (MA1-153) was purchased from Pierce; Thermo Scientific, USA. Other reagents were of analytical grade and were purchased from local sources.

2.2 Methods

a. Cell culture

CML cells of human origin, K-562 were maintained in RPMI-1640 supplemented with 10% heat inactivated FBS and antibiotics. Cells were maintained at 37˚C in a humidified atmosphere of 5% CO_2/95% air.

b. Lymphocyte isolation

Lymphocytes isolated from blood of healthy human donors as per laboratory protocol [19] were used as study control. They were grown in the same way as CML cells were maintained.

c. Development of Drug resistant cells.

Logarithmically growing K-562 cells were exposed to gradually increasing concentrations of IM, starting from 0.1nM. The cells, isolated at 100 nM IM are named K-562^R, and are maintained in presence of 100 nM IM.

d. Treatment protocol

Exponentially growing K-562 and K-562^R cells were treated with increasing doses of IM (0.5, 1, 2.5 and 5 µM) alone and in presence of 30 µM Curcumin for 24 hours.

e. Estimation of cell cytotoxicity

MTT assay was employed to assess the cytotoxicity of IM to K-562 and K-562^R cells [20]. Cytotoxicity of adriamycin and cytarabine, two other drugs used in leukemia therapy were also checked, to verify cross-resistance.

f. Assessment of cytokine levels by Enzyme-linked immunosorbent assay

The levels of TNF-α, IL-6, IL-8, and IL-10 were detected by quantitative sandwich immunoassay using kits [19].

g. Assessment of NF-κB (p65) activity

The activity of transcription factor NF-κB was assessed using an ELISA kit [21]. The activity was determined using nuclear extracts following manufacturer's protocol.

h. Western blot analysis

Western blot (WB) analysis was employed to study the expression of Pgp, MRP1, JAK2, STAT3, ERK1/2, PI3K, AKT, Bcr-Abl, caspase 3, caspase 8 and caspase 9 in K-562 and K-562^R cells using corresponding antibodies, following protocol of Sarkar et al [22].

i. RNA Extraction and semi quantitative RT PCR

Total RNA was extracted from cells using RNAqueous 4PCR kit (Ambion) following laboratory protocol and PCR was performed [23].

j. Assessment of apoptosis by TUNEL assay

Apoptosis was studied using in situ Apoptosis Detection Kit. Harvested cells were dried on a silanized glass slide, fixed with 4% paraformaldehyde / PBS solution (pH 7.4). Removal of endogenous peroxidases was done by methanol containing 0.3% H_2O_2, followed by addition of permeabilization buffer. Cells undergoing apoptosis were labelled with fluorescein-dUTP; slides were viewed under a fluorescence microscope (Leica).

k. Statistical Analysis

Statistical analysis was done using SPSS 10.0 (one way ANOVA followed by Dunett t-test).

3 Results and Discussion

3.1 Development of drug resistance and its Confirmation

Western blot analysis was carried out to assess the constitutive level of Pgp and MRP1, the expression of these two proteins was very low in K-562 cell line, but are high in K-562^R cells, confirming development of drug resistance. Upward trend of PgP and MRP1 was observed with increasing doses of IM during the course of development of drug resistance. Corresponding band intensities are the mean of three independent experiments (Figure 1a and 1b). Unaltered GSH level during development of drug resistant cells gave an indication that GSH pathway is not involved in the process. The elevated levels of PgP and MRP1 have been found to be reduced by curcumin (Figure 1c). MTT assay was performed to assess the cytotoxicity of several anti leukemia drugs (IM, cytarabine and adriamycin) to K-562^R cells. Results revealed that K-562^R was resistant not only to IM, but also to other drugs cytarabine and adriamycin as shown in Figure1d.

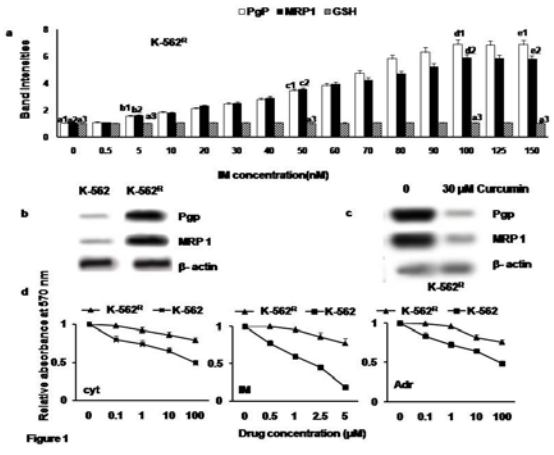


Figure 1

3.2 Curcumin in reversal of drug resistance

It was investigated whether curcumin could revert imatinib resistance. Both K-562 and K-562^R cells were treated with various concentrations of IM in presence of different doses of curcumin for 24 hours. Curcumin has been reported to increase the cytotoxicity of IM towards K-562 cells [20]. Presence of curcumin imparts cytotoxicity of IM in K-562^R, though the drug itself is non-toxic (Figure 2). EC_{50} values, (the concentration corresponding to 50% killing), in presence and absence of Curcumin are tabulated in Table 1. EC_{50} of IM in K-562^R in absence of curcumin was >> 5 and the corresponding fold resistance (EC_{50} resistant cell/ EC_{50} of parental cell) was much greater than 1.3. Treatment of cells with curcumin along with the drug helped to decrease the EC_{50} to 3.25 and the fold resistance was brought down to 1.132. Curcumin intervention thus helped to decrease the level of resistance in CML cells.

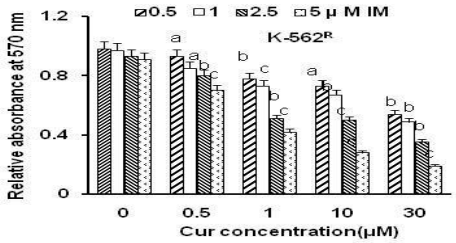


Figure 2

Table 1:

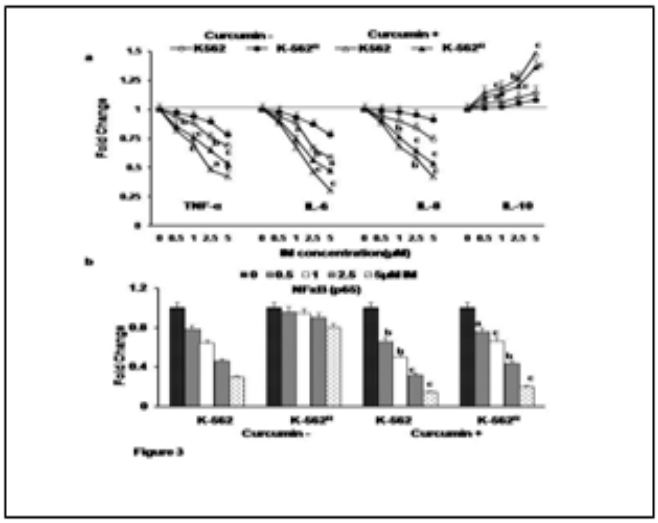
Cell	Compound	EC_{50} (µM) of IM	Fold resistance (EC_{50} resistant cell/ EC_{50} of parental cell)
K-562^R	Cur-	>>5	>>1.3
	Cur+	3.25	1.132

3.2 Modulation of cytokines and other markers by IM in K-562 and K-562^R cells, in absence and presence of curcumin.

K-562 and K-562^R cells were treated with increasing concentrations of IM (0, 0.5, 1, 2.5, 5 µM) for 24 h, in combination with Curcumin (0, 30 µM). Results from ELISA reveal that in K-562 cells, TNF-α, IL-6, IL-8 were down-regulated by IM [19], but not in K-562^R cells.

In presence of curcumin, these cytokines were negatively influenced both in K-562 and K-562^R cells. However, IM displayed no effect on IL-10, the anti-inflammatory cytokine which is low in K-562, as well as K-562^R. It is interesting to find that curcumin along with IM greatly influences the IL-10 level, irrespective of their sensitivity to the drug (Figure 3a).

TNF-α plays a dual role; on one hand it may induce apoptosis and on the other it may act as an inducer of survival signals, which is influenced by NF-κB. Hence it is worthwhile to study the modulation of NF-κB activity under similar conditions. Combination of IM and curcumin was able to bring down the activity of NF-κB more effectively than the drug alone not only in K-562, but also in K-562^R cells (Figure 3b).



Several proteins (JAK2, STAT3, ERK1/2, PI3K, AKT) implicated in CML have been studied by WB analysis. All these proteins are over-expressed in K-562 cells compared to normal control, and even higher in K-562^R (Figure 4a). IM down-regulated these markers dose-dependently in K-562 cells, but there is hardly any influence in K-562^R cells; curcumin aids in down-regulation of these markers in both the cells (Figure 4b).

Bcr-Abl, a signature marker of CML, has been followed at the protein and the genetic level. Elevated expression of this fusion protein was reported in K-562 cells from our laboratory [19]. WB and RT-PCR results reveal that treatment of cells with IM results in down-regulation of Bcr-Abl dose-dependently, effect being more in K-562 cells than K-562^R. The decrease in Bcr-Abl, is more pronounced in presence of 30 μM curcumin (Figure 4c).

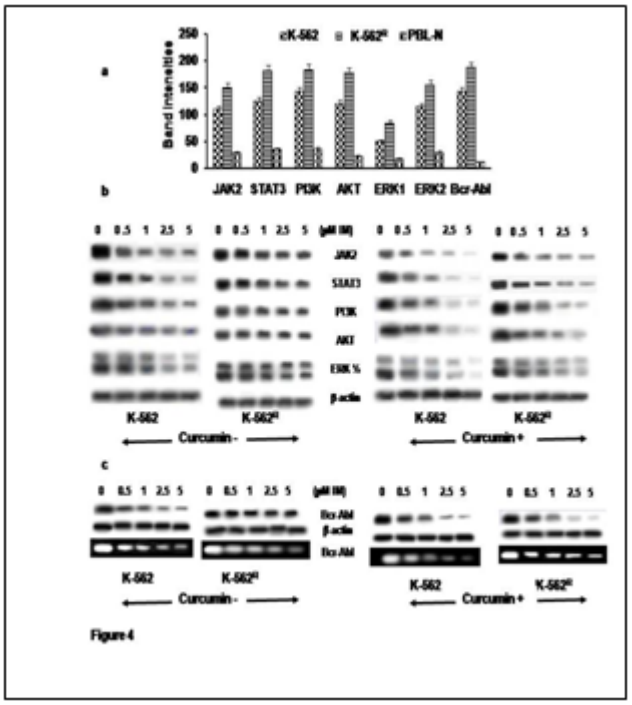


Figure 4

3.2 Induction of apoptosis as assessed by TUNEL assay

Induction of apoptosis was followed employing TUNEL assay. Cells were visualized under fluorescence microscope using FITC. Representative micrographs are shown in Figure 5a. The number of apoptotic cells was counted, corresponding Apoptotic index (AI) is shown in Figure 5b. It was observed that IM could induce apoptosis in K-562 cells, but not in K-562^R cells. Administration of curcumin along with IM, increases the AI value not only in K-562 cells, but facilitated induction of apoptosis in K-562^R cells, where IM alone shows no effect.

3.5 Activation of caspases

Western Blot results reveal that IM induces caspases 3, 8 and 9 in K-562 cells only, not in the drug resistant counterpart. Presence of curcumin leads to further up-regulation of caspases in K-562 cells and most interestingly a combination treatment results in caspase induction in the drug-resistant cells as well. These results are in agreement with our finding that a combinatorial treatment aids in induction of apoptosis in both the sensitive and resistant CML cells (Figure 5c). Results also reveal that expression of caspase 3 and 9 was more prominent than that of caspase 8, implying involvement of the intrinsic apoptotic pathway in IM induced programmed cell death.

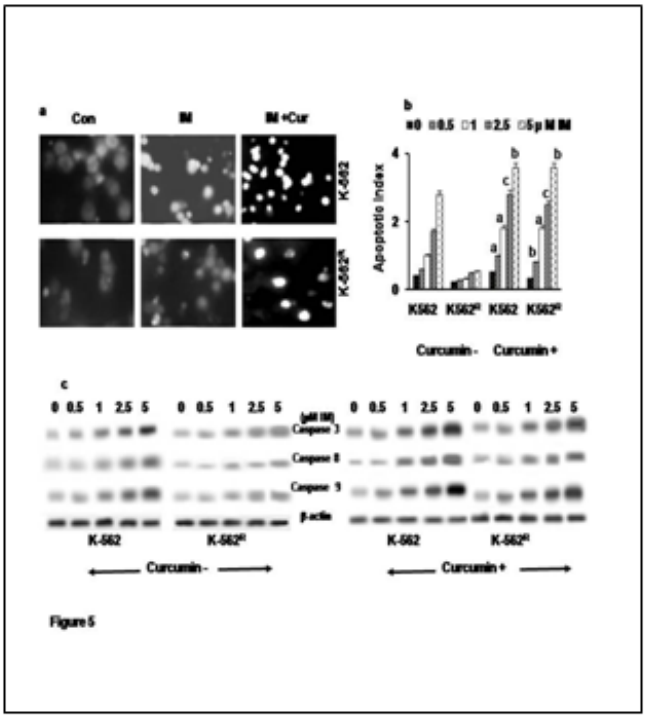


Figure 5

The trait of CML is the Philadelphia chromosome, which is a reciprocal translocation between chromosome 9 and 22, leading to the formation of the BCR/ABL fusion gene. Therapy of chronic myeloid leukemia therefore, involves targeting the fusion protein Bcr-Abl by the tyrosine kinase inhibitor IM. Bcr-Abl helps in CML progression by activating signalling pathways like JAK-STAT, PI3K-AKT and MAPK that contribute to the survival and proliferation of leukemic cells [24]. This drug is highly effective in management of CML and has helped to improve prognosis. However, administration of this drug to CML patients is often associated with severe adverse toxic effects and development of resistance to this drug is a common problem, thereby compromising the prognostic outcome [25]. Phytochemicals, active ingredients of plants, are well-known due to their anti-cancer activity; they preferentially target the cancer cells, sparing the normal cells. They are also known to revert drug resistance in cancer [26]. Present study explored the potential of non-toxic phytochemical curcumin in reversing drug resistance in CML cells. Development of drug resistance was confirmed by assessing the expression of multi drug resistant proteins Pgp and MRP1, which were found to be reduced by curcumin. This is in concurrence with the research findings from other laboratories [27]. The drug resistance was developed by exposing K-562 cells to increasing concentration of IM; however, the isolated cells were resistant not only to IM, but, also to cytarabine and adriamycin, two other drugs commonly used in leukemia therapy. Previous reports from our laboratory showed that curcumin increased the cytotoxicity of K-562 cells towards IM [20]. Present study unveils that curcumin helped to bring down the level of resistance in K-562^{R} cells, thereby rendering them sensitive to the drug IM.

Several proteins are implicated in progression of CML. Cytokines like TNF-α, interleukins (IL-6, IL-8, IL-10) are known to be aberrantly expressed in CML. Previous finding from our laboratory indicated that IM could modulate the levels of these cytokines alone, whereas, in conjunction with curcumin the extent of modulation is more [19]. It was observed in this study that in K-562^{R} cells, IM hardly showed any effect on the levels of these cytokines and other signalling proteins. Presence of curcumin reverses this aberrant level of biomarkers, contributing to sensitivity of the cells to the drug IM. Similar effect has been observed in case of Bcr-Abl, which was highly expressed in K-562^{R} cells. Non-toxic dose of curcumin along with the drug IM, brings down the level of Bcr-Abl at the protein as well as at the genetic level. Most of the drugs implicated in cancer therapy works by induction of apoptosis. Present study also showed that modulation of the above-mentioned markers by the combination of IM and curcumin was found to induce apoptosis in both the drug sensitive as well as the drug resistant cells. Apoptosis or programmed cell death involves various morphological changes in cells and as a consequence fragmentation of DNA is apparent, which is a hallmark of apoptosis. All these changes are mediated by various molecules. An important player in this regard is the family of cysteine proteases or caspases. IM induced caspases in K-562 cells, but, not in the resistant ones. However, drug along with curcumin efficiently induced caspases in both the cells. Among the three caspases studied, caspase 9 was found to be activated the most, suggesting involvement of the intrinsic pathway of apoptosis. This study highlights the promising role of curcumin in tackling the problem of drug resistance in CML, when used in conjunction with conventional anti-leukemia drug IM. Curcumin thus may aid in the reversal of drug resistance and betterment of CML therapy.

5 Legends to figures

Figure 1- Development and confirmation of drug resistance. **a.** Band intensities from the western blot bands reveal that Pgp and MRP1 expression increases with concentrations of IM upto 100nM, beyond which band intensities remain nearly same. No such change has been observed with GSH levels. Results depict mean of three independent experiments ± SE and values are significant with respect to drug treated cells. {a($p<0.5$), b($p<0.05$), c($p<0.01$), d($p<0.005$) and e($p<0.0005$); 1 denotes Pgp, 2 denotes MRP1 and 3 denotes GSH}. **b.** shows constitutive expression of Pgp and MRP1 in parental K-562 and drug resistant cells K-562^R. Band intensities clearly show that Pgp and MRP1 are expressed much more in resistant cells indicating development of drug resistance. **c.** Elevated expressions of Pgp and MRP1 is brought down by curcumin in K-562^R cells**. d.** MTT assay results show that K-562^R shows resistance not only to IM but also to anti-leukemia drugs cytarabine and adriamycin.

Figure 2- Influence of curcumin on the cytotoxicity of resistant CML cell K-562^R. Developed K-562^R is resistant to IM upto a concentration of 5µM. In presence of curcumin, the cells become sensitive to IM; the degree of sensitivity increases with increasing concentration of curcumin. The values are significant a($p<0.05$), b($p<0.005$) and c($p<0.0005$) with respect to the cells treated with IM alone.

Figure 3- Modulation of cytokines and NFKB by IM, as influenced by curcumin. **a.** shows modulation of cytokines (TNF-α, IL-6, IL-8 and IL-10) by IM alone and in conjunction with curcumin in K-562 and K-562^R cells by ELISA. When curcumin is present, the cytokines get modulated in both the cell lines. **b.** shows that curcumin down regulates the effect of IM on NFKB activity not only in K-562 cells but also in K-562^R, where IM fails to show any effect. Values are mean of three independent experiments and are significant a($p<0.05$), b($p<0.005$) and c($p<0.0005$) with respect to only IM treated cells

Figure 4- Regulation of various CML markers by IM and its influence by curcumin. **a.** Constitutive expression of leukemia markers (JAK2, STAT3, ERK1/2, PI3K, AKT, Bcr-Abl) in K-562, K-562^R and control lymphocytes isolated from normal healthy donors. **b.** Modulation of proteins implicated in leukemogenesis by IM alone and with 30µM curcumin in K-562 and K-562^R cells. **c.** Down-regulation of fusion protein Bcr-Abl by IM as influenced by 30µM curcumin. IM fails to modulate Bcr-Abl status in K-562^R which can be achieved by curcumin.

Figure 5- IM induced induction of apoptosis, as modulated by curcumin. **a.** shows induction of apoptosis by TUNEL assay. Based on TUNEL assay results, apoptotic index was calculated and is shown in **b**. Findings from **a** and **b** show that IM induces apoptosis in K-562 cells which is enhanced in presence of curcumin. In K-562^R cells, however, induction of apoptosis by IM is minimal, which is facilitated by 30µM curcumin. **c.** shows activation of caspases 3,8 and 9 by IM alone and in combination with 30µM curcumin in K-562 and K-562^R cells. Western blot bands show that activation of caspase 9 is more prominent, indicating intrinsic pathway. Results are significant a($p<0.05$), b($p<0.005$) and c($p<0.0005$) with respect to cells treated with drug alone.

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