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Effect of Curcuminoids on MDR-1 Gene Expression in Multidrug Resistance *KBChR-8-5* Human Cervical Cancer Cell Line

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ABSTRACT

Drug resistance is a major problem in cancer chemotherapy. The mechanism that has been most extensively investigated is the expression of the MDR-1 gene product, Pgp. The emergence of natural products as potential anti-MDR molecules is of particular significance, since many of these are essential components of the human diet, they are expected to possess fewer side effects and may possibly represent a new generation of MDR modulators. Curcuminoids are the major component present in turmeric. Here we demonstrate that curcuminoids inhibits the growth of drug resistant cancer cell lines (KBChR8-5) in a dose dependent manner, with IC_{50} values in the 5-25μM range. In addition, MDR-1 gene expression was studied in curcuminoids treatment, it revealed that among three curcuminoids curcumin treated cells showed down-regulation and other two curcuminoids showed mild change in MDR-1 expression in comparison with control. In conclusion the Pure curcumin displayed poor anti-cancer activity in KBChR8-5 cell line, but curcumin inhibits MDR-1 gene expression which implies that it is not directly involved in cytotoxicity when compared with other two curcuminoids instead it inhibits MDR-1 gene product, P-gp (efflux pump). Curcumin in combination chemotherapy may increase accumulation of cytotoxic drugs by blocking efflux pump and significantly enhance the anti-cancer activity in the treatment of various cancers related to multidrug resistance.

Key words*: Cytotoxicity, drug resistance, doxorubicin, modulators, sensitivity.*

INTRODUCTION

Cancer is a major worldwide public health problem. Chemotherapy remains the principal mode to treat many metastatic cancers. When cancer comes back after treatment it is called cancer recurrence, in such cases patients should get regular treatments with chemotherapy, radiation therapy throughout their life time. One of the reasons for cancer recurrence may be development of drug resistance to common chemotherapeutic drugs used in cancer treatment. The development of simultaneous resistance to multiple drugs appears to be a major impediment to the successful chemotherapy of human tumors.

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Due to the problem of drug-resistance many chemotherapeutic drugs are presently placed in a predicament of reduced therapeutic effect[20].

The overexpression of MDR gene plays important role in multidrug resistant phenotype and also confers to cross resistance to a large group of lipophilic cytotoxic compounds. The human multidrug-resistance (MDR1) gene encodes a membrane transporter p-glycoprotein (p-gp) a member of ATP binding cassette family[4]. These transporter proteins harness the energy of ATP binding or hydrolysis to drive conformational changes in the transmembrane domain (TMD) and consequently transports molecules[12]. These transporters actively efflux a variety of structurally and functionally diverse chemotherapeutic drugs out of cancer cells, thereby reducing the intracellular cytotoxicity[7]. A variety of tissue culture systems have been established over the past several years to study the biochemical, physiologic and genetic bases of alterations that result in the development of multidrug-resistance. It is important to develop method to assay drug resistance and new more effective agents to overcome drug resistance. Multidrug resistance cell lines for test system have been developed either by selecting drug-resistant clones spontaneously or from mutagenized cultured mammalian cells *in vitro*[16]. To investigate the basis of drug resistance *in vitro*, drug resistance cell lines have been isolated by exposing various cancer cells to increasing amounts of chemotherapeutic agents such as adriamycin, daunomycin, colchicine, doxorubicin, actinomycin-D, taxol, vinca alkaloids etc[10]. In this study we have used a colchicines-resistant human cancer KB cell line which is cross resistance to many anticancer agents.

Modulators are that reverses drug resistance. Several generations of MDR modulators are developed in reversing p-gp mediated multidrug resistance (MDR). A large number of non- cytotoxic compounds known as chemosensitizers or MDR modulators sensitize resistant cells for the action of cytotoxic drugs. It is necessary to identify natural compounds from plant origin that reverse the MDR phenotype, sensitize cancer cells to conventional chemotherapy without undesired toxicological effects.

Phytochemicals can synergize with cytotoxic drugs by increasing their efficacy and lowering the toxic side effects on normal cells. Combined treatment with phytochemicals can delay the onset of resistance to cancer cells[17]. Dietary phytochemicals have been found to be very promising in reversing the resistance to anticancer. A number of uses of phytochemicals suggest that a daily intake of phytochemicals can reduce the incidence of several types of cancers[8,22]. They are present in the herbal constituent's fruits, berries and spices. In spices are mint, rosemary, garlic, piper nigrum, curcumin, ginseng, onion[3]. Curcuminoids have broad spectrum of pharmacological properties.

Curcuma longa L. (turmeric) is typical of the herbaceous plant that characterizes the family Zingiberaceae. Curcumin (C), main colouring substance in Curcuma longa L. and two related compounds, demethoxycurcumin (DMC) and bisdemethoxycurcumin (BDMC), were altogether known as curcuminoids[11]. Since the curcuminoid pigments vary in chemical structures, it is possible that the physico- chemical characteristics as well as the functional properties and biological role of curcuminoids would vary among them. A number of studies are undertaken to separate curcuminoid pigments by thin layer chromatography (TLC), high-performance thin-layer chromatography (HPTLC), and column chromatography (CC). HPLC method was sensitive, precise, and accurate for detection and quantification of curcuminoids in the extract of rhizome Curcuma longa L[23].

The basic strategy to circumvent MDR is to co-administration of an anticancer drug with a chemosensitizer that impairs p-gp function resulting in enhanced intracellular anticancer drug accumulation to increase the sensitivity of cytotoxic drugs[15]. The present study was undertaken to assess the chemotherapeutic activity of curcuminoids and the effect on MDR-1 gene expression against the multidrug resistant cervical carcinoma cell lines.

MATERIALS AND METHODS

Extraction of curcuminoids

Curcuma longa L. (Turmeric) rhizome was collected from Assam - Lakhadong variety. Fresh rhizomes were cleaned, washed, dried and blended to powder. About 20g of turmeric powder was taken into a thimble and placed in a soxhlet apparatus and extracted using acetone as solvent for 6 hours. The dark brown extract yielded was then cooled, filtered, concentrated using rotary evaporator. Extracts were analysed for curcuminoids by HPLC analysis. The curcuminoids were further fractionated by silica gel (60-120 mesh) and eluted with chloroform followed by chloroform:methanol with increasing polarity. All the collected fractions were subjected to TLC silica gel 60 F254 plate using chloroform:methanol (95:5) as the developing solvent system and detected as yellow spots. And fractions with similar Rf values were pooled and the organic solvent was removed by rotary evaporator. The individual Curcuminoids collected from the column chromatography was purified and precipitated with petroleum ether. The curcuminoids were characterized by GC-MS and purity were analysed in HPLC.

Cell culture

The KB, KB-ChR-8-5 human cervical carcinoma cell lines were purchased from NCCS Pune, Maharashtra, India. The cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) with 2mM L-glutamine, 10% fetal calf serum (FCS), 1.5g/L sodium bicarbonate, 1mM non-essential amino acid and 1.0mM sodium pyruvate. Only the KBChR8-5 cell line was routinely maintained in 10ng/ml colchicines and subsequently grown in colchicine-free medium for one week prior to drug treatment. The cell lines were maintained in a humidified incubator with an atmosphere of 5% air and 5% CO_2 at 37°C.

MTT assay

The MTT [3-(4,5-dimethy-2-thiazoly)-2, 5-diphenyl-2H-tetrazolium bromide] assay was used to determine drug sensitivity[13]. KB and KBChR8-5 cells in the exponential growth phase were harvested by trypsinization and seeded into 96-well plate at a concentration of 3500 cells per well. The cells were treated in triplicate with gradient concentration of curcuminoids ranging from 0.01µg, 0.1µg, 1.0, 10 and 100µg and incubated at 37°C for 24 hours. MTT reaction was performed, the development of purple formazan crystals was visualized and the absorbance was read at 595nm. The experiments were conducted in triplicates and the values represent mean ±S.D of three independent experiments. Measurements were performed and the concentration required for a 50% inhibition of viability (IC_{50}) was determined. The IC_{50} value defined as the drug concentration required to reduce cell survival to 50% as determined by the relative absorbance of MTT. IC_{50} value was calculated by (mean absorbance in test wells) / (mean absorbance in control wells) x 100.

Real-time PCR

MDR-1 mRNA expression was examined on treatment of three curcuminoids at 5µM in KB and KBChR8-5 cells and normalized with expression by GAPDH gene. Total cellular RNA from KB and KBChR8-5 cells and curcuminoids treated KB and KBChR8-5 cells were extracted by TRIzol Reagent[5] (Chomvzynski et al., 1987). (Invitrogen, Carlsbad, CA, USA) and treated with RQ1 RNase-free DNase. Total RNA was reverse-transcribed to generate cDNA using RT-PCR kit (Promega Corporation, Madison, USA). The cDNA pool was subjected to Quantitative Real-Time PCR (QRT-PCR) by using SYBR green PCR Master Mix for MDR1 gene expression using gene specific primers on the AB 7500 fast real time PCR system (Applied Biosystems), Primers for MDR1 gene forward primer sequence CAGAGCAAGAGAGGCATCCT and the reverse primer sequence TGAAGGTCTCAAACATGAT and GAPDH were used as internal control. The data are the mean value ±SEM of three independent experiments

Polymerase chain reaction (PCR): The following conditions were used in QPCR: 2 min at 50^0C, 10 min at 95^0C, and 40 cycles of

15 s at 95^{0}C and 1 min at 60^{0}C. Total 10 µL of each PCR product as well as DNA Ladder was electrophoresed in 1X Tris/acetate/EDTA (TAE) electrophoresis buffer on a 1% agarose gel at 100 volts for 20-30 minutes, visualized using UV transillumiator and photographed after staining with Ethidium bromide (Huang *et al*., 2006).

RESULTS

Extraction of curcuminoids

In our study the total extract contained 40.1% of curcuminoids were found in Assam variety turmeric among that 23.9% curcumin, 10% DMC 6.1% BDMC by HPLC analysis. Each curcuminoids were separated by column chromatography and the purity profile of isolated individual curcuminoids were analysed by HPLC. C, DMC, BDMC showed single peaks at retention times of 10.81, 12.79 and 13.03 min respectively. The identity of each peak was confirmed by determination of retention times and by spiking with standards. Purity profile for each curcuminoids were calculated as 99%, 98%, 95% purity for C, DMC, BDMC respectively. The full scan mass spectra of each curcuminoids isolated were detected shows molecular weight of each individual compounds of C, DMC, BDMC was found to be 368.31, 337.77, and 307.95 respectively.

MTT assay

To examine cytotoxicity of each curcuminoids the KB and KBChR8-5 cells were exposed to various concentrations of curcuminoids and the cytotoxicity was determined by MTT assay. Dose response cytotoxicity profiles for three curcuminoids C, DMC, and BDMC were established for KB and KBChR8-5 cell lines were shown in figure 1 A, B; The percent of viable cells was calculated to determine the IC_{50}. The IC_{50} value of C, DMC, and BDMC in KB cells were 18.3, 22.8, and 7.8µM respectively, whereas in KBChR8-5 cell lines the IC_{50} values were 19.2, 26.7, and 5.2 respectively. This suggests that IC_{50} values of resistant and parental cell lines were nearly same.

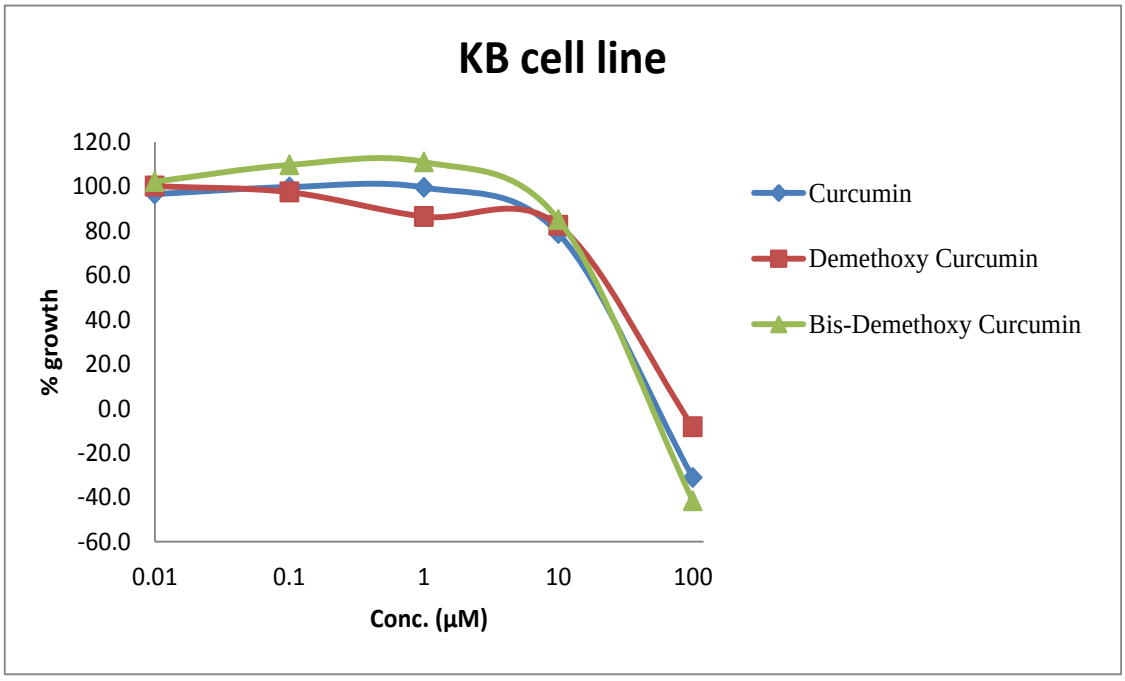

Fig.1A Percentage growth against KB cells

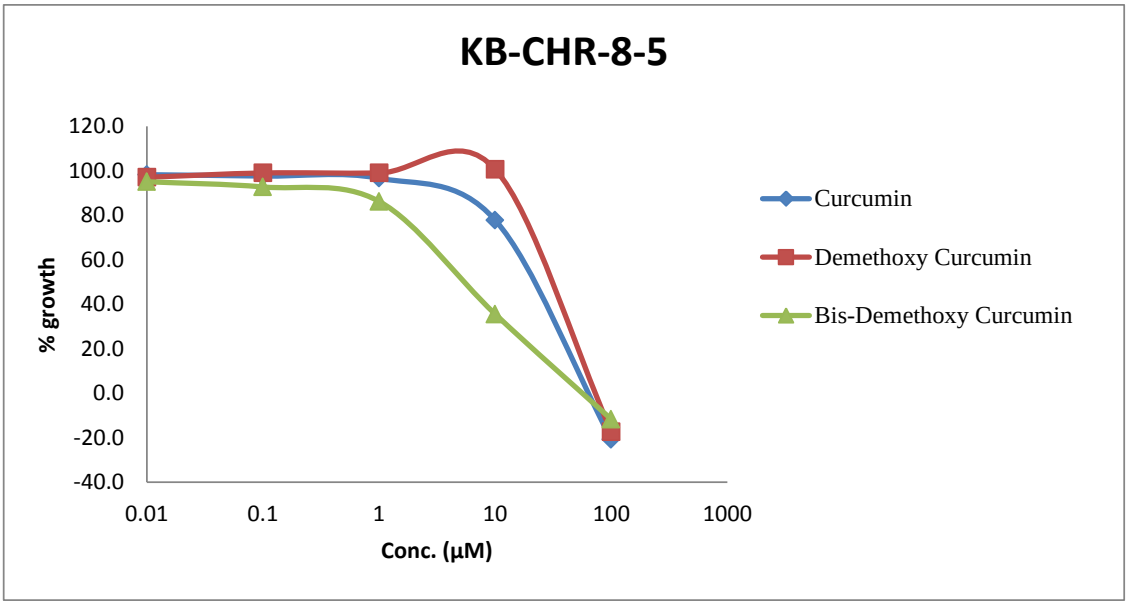

Fig.1B Percentage growth against KBChR8-5 cells

Fig.1: Cytotoxicity of C, DMC, and BDMC in KB (1A), KBChR8-5 (1B) cells

Real time PCR

MDR-1 mRNA expression was examined in KB (drug sensitive) cells on treatment of three curcuminoids at 5µM for three days; the expression level varies among the three curcuminoids. The mean normalized expression of MDR-1 gene in KB with and without treatment is shown in figure 2. The result implies that treatment with curcuminoids reduced MDR1 expression, but there was significant difference showed a low level of expression in BDMC treated cells. Treatment of KB cells with BDMC showed 2.4 fold decreased expression of MDR-1 gene.

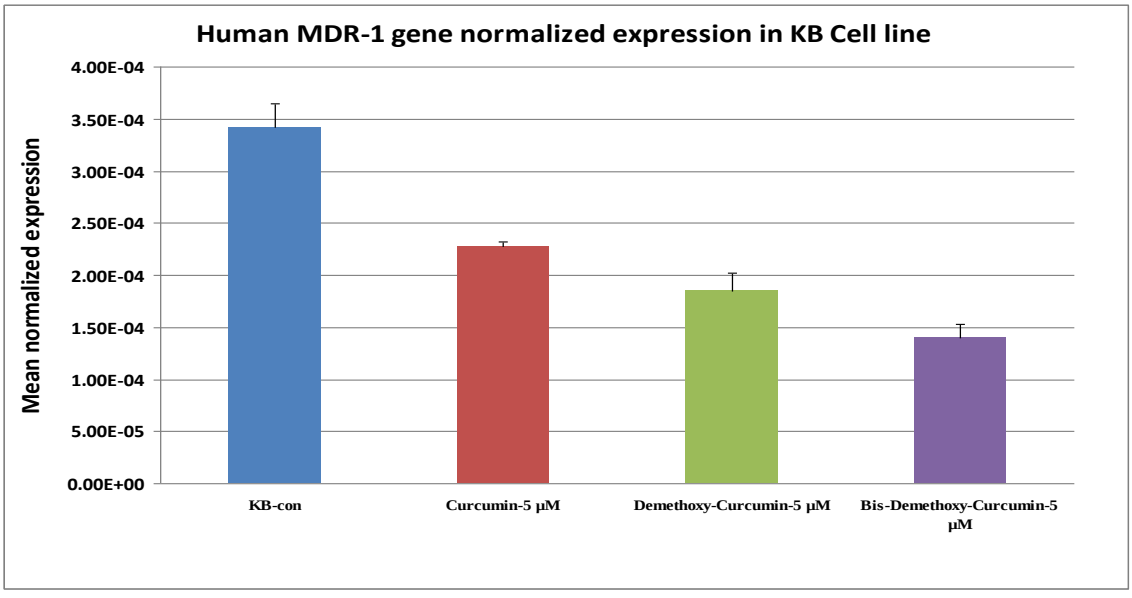

Fig 2: Effect of C, DMC, BDMC on MDR-1 mRNA expression in KB cells

We examined the influence of curcuminoids on MDR-1 mRNA expression in KBChR8-5 cells using real time PCR. Expression of MDR-1 mRNA level in drug resistant KBChR8-5 cells were examined on treatment with C, DMC, BDMC at 5µM for three days

and normalized with expression by GAPDH gene were shown in figure 3. Among the three curcuminoids curcumin showed down-regulation and reduced level of MDR -1 mRNA expression, but there is no much significant expression found in DMC and BDMC.

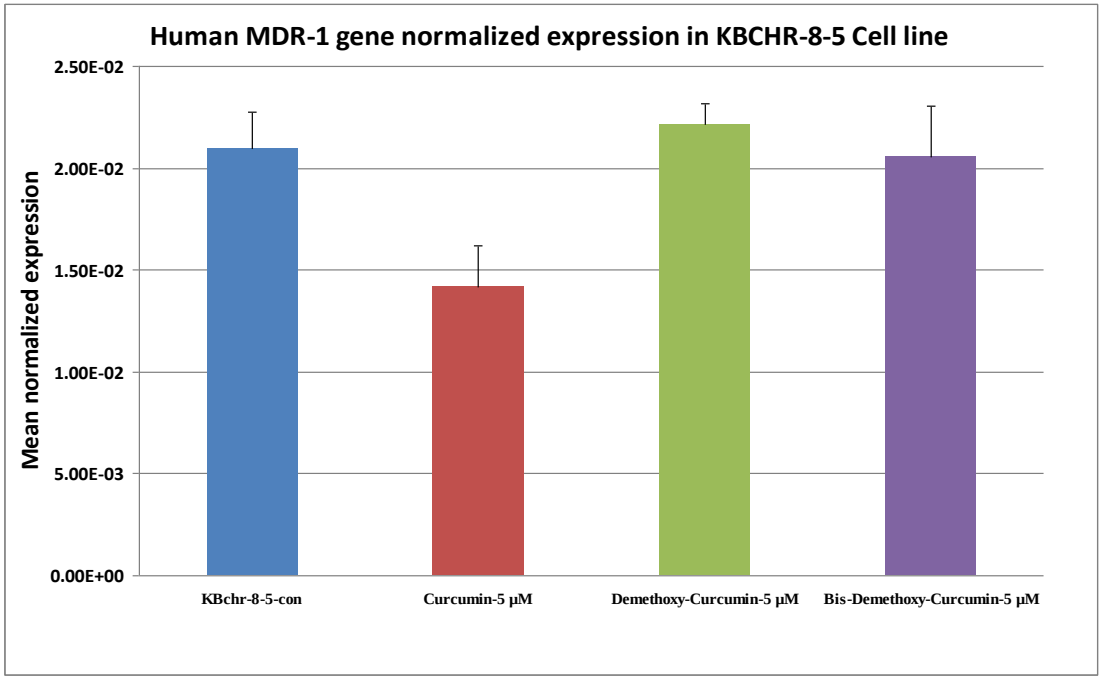

Fig. 3*:* Effect of C, DMC, BDMC on MDR-1 mRNA expression in KBChR8-5 cells

DISCUSSION

Multidrug resistance (MDR) is one of the major causes of chemotherapy failure in cancer treatment. Various types of cancers have been observed to exhibit this phenomenon, a strategy that involves cellular and non-cellular mechanisms employed by cancer cells to survive the cytotoxic actions of various structurally and functionally unrelated drugs[18].

The overexpression of MDR transporter occurs in 40-50% of cancer patients and is associated with poor clinical outcome[14]. The mechanism of Overexpression of p-gp has been well established as the cause of the MDR phenotype in many *in vitro* selected drug resistant cell lines. KB cell lines have been used to catalog the changes in specific protein synthesis associated with the development of multidrug resistance. KB-V1 cells were selected by subjecting KB cells in a step-wise fashion to increasing concentration of vinblastine. KB-V1 cells have been shown to express only p-gp at a higher level on their plasma membrane. The level of p-gp in KB-V1 cell membrane is about 1% of total plasma membrane of drug resistant cell lines[2].

Colchicines resistance clone KBChR8-5 with multidrug resistance is derived from the KB cell line[9]. KBChR8-5 cells were selected by subjecting KB cell lines in a stepwise fashion to increase the concentration of colchicine[1]. The amount of purified p-glycoprotein thus obtained accounted for approximately 3 to 4% of the total plasma membrane protein in colchicine-resistant mutant cell line[21]. In our previous studies KBChR8-5 cells showed higher level of MDR-1 gene expression at 7.1 fold increase in MDR-1 when compared to KB cells.

It is important to find new more effective agents to overcome drug resistance. The traditional uses of turmeric or natural curcuminoids in folk medicine are multiple, including antioxidant, anti-cancer, anti-inflammatory, anti-venom, anti-mutagenic

activity in vitro[19]. Recently it was reported that the effect of curcuminoids was examined on the proliferation of MCF-7 human breast tumor cells that demethoxycurcumin was the best inhibition of MCF-7 cells followed by curcumin and bisdemethoxycurcumin.

The curcuminoids were found more toxic for cancer cells than normal cell line. In this regards, therapeutic index' is an important parameter to select samples for developing drugs. The result of MTT assay to determine the relative cytotoxicity of curcuminoids in KB and KBChR8-5 cell lines showed that each curcuminoids have different IC_{50} values in the range of 5-25µM. Chearwae *et al*[6]., studied cytotoxic activity of three curcuminoids on KB-V1 and its parental cell lines results in IC_{50} range 25-90µM cytotoxicity of each compound on both cell lines are nearly equal. Curcumin showed greater cytotoxic effect[6].

The cytotoxicity of curcuminoids against drug resistant cell lines showed that higher cytotoxicity at BDMC treatment on KB and KBChR8-5 but curcumin showed poor cytotoxicity. The IC_{50} value of curcuminoids on MDR subline was nearly equivalent to that of parental cell line. The effect of MDR-1 mRNA expression was examined in KB (drug sensitive) cells on treatment of three curcuminoids at 5µM for three days; the expression level varies among the three curcuminoids. The treatment of BDMC in KB cells showed 2.4 fold decreased expression of MDR-1 gene, but there is no drastic change found in MDR expression on KBChR8-5 cells.

KBChR8-5 cells on treatment 5µM of each curcuminoids results in different range of inhibition of MDR-1 gene. Curcumin showed most effective inhibitory level than other two curcuminoids, curcumin, DMC and BDMC showed 1.47, 0.94, and 1.01 fold decreases in MDR-1 expression respectively. Treatment of curcumin at 5µM showed significant inhibitory effect with level of fold change at 1.47% inhibition. Curcumin showed down-regulation and reduced level of MDR-1 mRNA expression, but there is no much significant expression found in DMC and BDMC.

This suggest that if curcuminoids enters cell through multidrug transporter there should be significant difference in IC_{50} values of resistant and parental cell lines, but IC_{50} value of KBChR8-5 and KB cells posses nearly same value, there was no much difference in cytotoxicity. The higher cytotoxic activity shown in BDMC may be due to inducing apoptosis by inhibiting proliferation of cancer cells by cell cycle arrest, it may not target the pathways involved in MDR. Eventhough curcumin showed poor cytotoxic activity it down-regulates MDR-1 mRNA expression which implies that curcuminoids may be transported by multidrug transporter and block the efflux pump for cytotoxic activity. This result suggests that curcumin inhibits MDR-1 gene expression but not directly involved in cytotoxicity when compared with other two curcuminoids.

CONCLUSION

Individual curcuminoids vary in chemical structure and the knowledge of structure-activity relationship in combination with conventional chemotherapy is useful for drug design. These results revealed that curcumin have synergistic effect that target the pathway related to multidrug resistance and reduce the production of efflux pump to increase the accumulation of anticancer drugs and induce cytotoxicity.

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