CHAPTER IV
DISCUSSION AND CONCLUSIONS

Multidrug resistance (MDR) is a major problem in cancer chemotherapy. Several mechanisms responsible for MDR have been described [1, 7, 8]. Numerous clinical data revealed that the multidrug resistance phenotype in tumors is associated with the overexpression of certain ABC transporters, termed MDR proteins. The P-glycoprotein (P-gp, ABCB1) was first discovered [37, 168, 169] and probably still is the most widely observed mechanism in clinical multidrug resistance [1, 2, 35, 149]. Soon after the cloning and characterization of MDR1, it became evident that other efflux pumps may also play a significant role in transport associated drug resistance. There are two other ABC transporters, which have been definitely demonstrated to participate in the multidrug resistance of tumors: the multidrug resistance protein 1 (MRP1, ABCC1), and the mitoxantrone resistance protein (MXR, ABCG2) [2, 35, 47, 170]. Most strategies for reversing MDR have focused on the modulation of P-gp, MRP1 and MXR activity by using modifying agents called MDR reversal modulators or chemosensitizers. Many MDR modulators have been tried in clinical trials, however; mostly has been hampered by the toxic side effects that occur when non-physiological doses are used [35, 171]. This led to the search for novel and more potent MDR modulators without side effects. Recently, many studies have generated great interest in dietary natural products derived from plant compounds with the fact that they have a widespread long-term use in populations without a history of producing toxic side effects.

In this study, the research was focused on curcuminoids, which are polyphenolic pigments found in the spice turmeric. Previously [27], curcumin mixture (make up from 77 % curcumin I, 17 % curcumin II and 3 % curcumin III) was reported on its ability to reverse the multidrug resistance phenomenon in human cervical carcinoma cell lines by inhibiting both function and expression of P-gp. The experiments reported here were clarified further on the modulatory effect of curcumin mixture as well as three purified curcuminoids, curcumin I, II and III, whose structures are shown in Figure 7, on the P-gp, MRP1 and MXR mediated MDR

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phenotype. Moreover, some biological interactions between curcuminoids and the transporter(s) were also elucidated in this study.

Source of curcuminoids.

The curcuminoids used in this study were purified from turmeric (*Curcuma Longa*) which is available in the local market. The purification was accomplished by column chromatography, and analyzed by TLC (Figure 15 and 16). The purity and structure of isolated curcuminoids were determined by HPLC, IR, MS and NMR (Appendix A). The results confirmed the purity of each compound was found to be greater than 99% [28, 129]. All three pure forms of curcuminoids, curcumin I, II and III were tested for their ability to reverse the MDR mediated by P-gp, MRP1 and MXR in comparison with curcumin mixture (supplied by Sigma).

Modulation of human P-glycoprotein function by curcuminoids.

In the first set of experiments in this study, research was focused on the question of whether the curcuminoids, curcumin I, II, III and curcumin mixture, are able to reverse the P-gp mediated MDR phenotype. The experiment in this part was carried out in vinblastine–resistant human cervical carcinoma cell lines, KB-V-1 and its parent (drug sensitive) KB-3-1 cells. The KB-V-1 were selected by subjecting KB-3-1 cells in a step-wise fashion to increasing concentrations of vinblastine [172]. The P-gp expression in these cell lines was analyzed by flow cytometry and Western blot analysis. Two specific monoclonal antibodies to P-gp, MRK-16 and C219, were used. MRK-16 and C219 are both specific monoclonal antibodies of P-gp but recognize different epitopes. MRK-16 recognized an external epitope of human P-gp thereby making it easy to detect P-gp by flow cytometric analysis (without permeabilizing cells), and C219 recognized highly conserved amino acid sequences (VQEALD and VQAALD) that are internal epitopes present in P-gp, thus allowing investigation of P-gp by Western blot analysis. As shown in Figure 17, the P-gp was highly expressed in KB-V-1 and was not found in KB-3-1 cells. The result was consistent with the previous reports [172], the level of P-gp in KB-V-1 cells membranes is about 1% of total plasma membrane protein, whilst P-gp was not found in parental KB-3-1 cells [172-174]. Also, consistent with many unpublished data in the laboratory of cell

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biology (LCB), NCI, NIH; calcein AM was effluxed effectively by KB-V-1 cells not KB-3-1, and the efflux was completely inhibited by CsA, a potent modulator of P-gp (Figure 18).The results indicate that these two cell lines are suitable to use as a tool to study the efflux function mediated by P-gp transporter.

Curcuminoids inhibit P-gp-mediated MDR phenotype in KB-V-1 cells

Initially, curcuminoids were tested for their toxicity in KB-3-1 and KB-V1 cells, the results shown in Figure 19. The IC_{50} of curcumin mixture and curcumin I, II and III is in the range of 24.0 -85.0 μM in KB-3-1 and 22.8-93 μM in KB-V-1 cells, and is not statistically different ($P>0.05$) compared to KB-V1 and KB-3-1 cells (Table 14), suggesting that P-gp does not confer resistance to curcumin I, II or III or curcumin mixture. Similar effect was observed in the model of human gastric carcinoma cell line SGC7901/VCR and its parental SGC7901 [175]. SGC7901/VCR is P-gp overexpressing cell line that 45 times more resistant than the parental SGC7901, the IC_{50} values of curcumin mixture in these cell lines were not significant different [175], confirm the finding that curcuminoids may not be transported by P-gp or P-gp does not confer resistance curcuminoids.

The non-cytotoxicity dose (≥ 80% survival) of the curcuminoids are used to verify their effects on P-gp mediated MDR phenotype in vinblastine –resistant KB-V-1 cell lines and its wild type, KB-3-1. The results in Figure 20 showed that curcumin I, II and III increased the sensitivity of vinblastine in KB-V1 cells, but not wild type, KB-3-1 cells. These results showed clearly that curcumin I strongly increased the sensitivity or decreased the IC_{50} in KB-V1 cells but curcumin II and III only slightly changed the sensitivity to vinblastine (Table 15). In agreement with the previous study [26], this demonstrates that curcumin I is an active ingredient in turmeric that can partially reverse MDR in cells expressing P-gp. Taken together, this data indicates that curcumin I is the most active form in increasing the intracellular drug levels and vinblastine sensitivity in KB-V1 cells by modulating P-gp function. In this study verapamil had been used as positive modulator, the verapamil at 20 μM showed significantly increases ($P<0.05$) the vinblastine sensitivity about 11 fold or about 3 fold higher activity than curcumin I (Table 15).

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Curcuminoids not only increased the sensitivity of vinblastine but the similar effect also was detected when combined the curcuminoids with paclitaxel (Figure 21), an anticancer drug transported by P-gp with a very high affinity [176]. Paclitaxel binds to tubilin and prevent depolymerization leading causes of cell arrest in the G2/M phase of the cell cycle, resulting in sensitization of cells to drug or radiation treatment, by photoaffinity labeling using BzDC-paclitaxel indicating that the binding site of taxol on P-gp is on TM7-TM8 [176]. The IC_{50} of paclitaxel (shown in Table 15) significantly decreased ($P<0.05$) when treated the cells in the presence of curcuminoids at 10 μM, the relative resistance is substantially reduced from 4516.1 to 2500.5, 2666.7, 2645.2 and 2677.4 for curcumin mixture, curcumin I, II.and III respectively. The result confirmed the reversal effect of curcuminoids on the P-gp mediated MDR in KB-V-1 cells. Moreover, the modulatory effect of curcuminoids is not cell type specific, for instance, it has been reported previously that curcumin also inhibit the function of P-gp in primary cultures of rat hepatocytes[177], human gastric carcinoma [175], melanoma [178], HL-60 [179], human CCRF-CEM leukemic cells and human carcinoma cells (Hep2A) [180].

Curcuminoids inhibit drug transport function of P-gp

Efflux function of P-gp was determined by measuring the influence of curcuminoids on cell ability to efflux the fluorescent substrate(s). Consistent with the effect of curcuminoids on vinblastine or paclitaxel accumulation in intact cells, the curcuminoids able blocked the efflux of three fluorescent substrates- calcein-AM (Figure 22), rhodamine123 (Figure 23) and bodipy-FL-vinblastin (Figure 24) in a concentration dependent manner. Curcuminoids caused a substantial increase in the accumulation of these substrates in KB-V1 cells but had no effect on drug sensitive (KB-3-1) cells, which did not overexpress P-gp. Consistent with the effect on the MDR phenotype, curcumin I showed it was the most potent curcuminoid compound found in turmeric. Moreover, these results demonstrated that this effect is not specific to a particular substrate; curcuminoids affected the accumulation of all three substrates in the same manner. Cyclosporin A was included in this study as positive modulator of P-gp (Figure 25). In addition, the experiment reported previously [28] demonstrated that this effect was found not only in KB-V1 but also in NIH 3T3-

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MDR1-G185, which is a mouse fibroblast cell line transfected with the human *MDR*1 gene, suggesting that the modulation of P-gp by curcumin is not cell type-dependent.

Biochemical interaction of curcuminoids and P-gp.

Biochemical interaction of curcuminoids with P-gp was assessed by ATPase assay. It has been proposed previously [1] that the profile of substrate stimulated ATPase activity reflects the nature of P-gp interaction with the substrate, which was categorized previously into three distinct classes based on their effect on human P-gp ATPase activity. Class I, refers to the agents that stimulate ATPase activity at low concentrations but inhibit the activity at high concentrations. The agents in this class include vinblastine, verapamil and paclitaxel. Class II is the group of agents that enhance ATPase activity in a dose dependent manner without inhibition and the agents of this group include bisantrene, valinomycin, and tetraphenylphosphonium. Finally Class III, in contrast with Class II, the agents in this group inhibit both basal and verapamil-stimulated ATPase activity. This group included cyclosporin A, rapamycin and gramicidin D.

In this study, the interaction of curcuminoids with P-gp was demonstrated by ATPase assay, the results showed clearly that curcuminoids could stimulate ATPase activity at a low concentration (0.5-1 μM) and inhibit its activity at higher concentrations (Figure 26-27). Although the stimulation of ATP hydrolysis by curcuminoids can be taken as evidence that the curcuminoids involved are substrates for transport linked to ATP hydrolysis. However, the previous results by MTT assay showing that P-gp does not confer resistance to curcuminoids, or in other words, curcuminoids may not be a substrate for P-gp, has lead researcher suggesting that the interaction of curcuminoids on the P-gp may change the conformation of P-gp (allosteric binding) or hinder the binding of other drug substrates of P-gp.

An alternative strategy to demonstrate interaction of the curcuminoids with the substrate binding site is to look for inhibition of the clear stimulation of ATPase activity by another substrate [138]. Verapamil has already been shown to produce such stimulation of ATPase association with P-gp [181]. In this study, by ATPase assay, it demonstrated that curcuminoids inhibit verapamil-stimulated ATPase activity significantly (Figure 26-27), suggesting competitive interaction at the

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substrate binding sites of the P-gp or, the binding site for the three curcuminoids overlap with that of verapamil. The binding for curcuminoids with the substrate binding sites of P-gp were further supported by the effect of curcuminoids on the photoaffinity labeling analog, IAAP which is an analog of prazosin [182]. Data in Figure 28-31 clearly showed that curcuminoids effectively inhibited photoaffinity labeling of P-gp with IAAP in a concentration–dependent manner, and again the IC_{50} (Table 16, 17) from both assays (ATPase and IAAP labeling) indicated that curcumin I was the most effective form. Therefore, these results demonstrate that curcuminoids interacts directly with P-gp and possibly binds to the same binding sites as other agents such as prazosin and verapamil.

In conclusion, the results in the first part show that all three forms of curcuminoids, curcumin I, II and III can inhibit P-gp function; however, curcumin I is the most potent MDR modulator compared with other forms of curcuminoids. The effect of curcuminoids on the P-gp expression has been determined previously [27, 128], it has been proposed that curcuminoids lower the expression of *MDR1* gene by transducing the signal through protein kinase A (PKA) and regulate the function of transcription factor CREB which is believed to control to transcription process of *MDR1* gene [26].

Modulation of human MRP1 function by curcuminoids.

In the second part of the study, the research had focused the interest on the ABCC1 (or MRP1) transporter. Specifically efforts were focused to compare the effects of the individual curcumins and the curcumin mixture with regard to their ability to inhibit MRP1 function. The research was also probed the mechanism to clarify the nature of the modulatory effects of the curcuminoids on MRP1.

In this study curcuminoids were tested for their ability to modulate the function and expression of MRP1 by using Human Embryonic Kidney, HEK 293 cells transfected with MRP1-pcDNA3.1 and pcDNA3.1vector alone. The expression of MRP1 in these cell lines were determined by Western blot analysis. In agreement with the previous report [133], MRP1 was detected as a 190 kDa protein with the MRP1- specific monoclonal antibody MRPm6 in MRP1-HEK 293 cells, and not in pcDNA 3.1 HEK 293 cells (Figure 32), indicating these cell lines is appropriated to

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use as a model to study MRP1 function and expression. The functional status of MRP1 in MRP1-HEK 293 cells was assessed by using calcein-AM, the result showed that MRP1-HEK 293 cells effectively efflux calcein- AM, and this efflux of calcein AM is blocked by the MRP1-specific inhibitor, MK-571 at 20 μM.

Curcuminoids inhibit MRP1-mediated MDR phenotype in MRP1 transfected HEK 293 cells.

Initially MTT assays were used to determine the relative cytotoxicities of the curcuminoids in MRP1-HEK293 and pcDNA3.1-HEK293 cells. Curcuminoids have different IC_{50} values in the range of 14.5 -39.33 μM as shown in Figure 33. The results indicated that curcuminoids might not be MRP1 substrates since the IC_{50} values were almost identical in both parental and MRP1-transfected cells (Table 18). As suggested in the P-gp study; additional experiments using radiolabeled or curcuminoids conjugated with a fluorescent probe will have to be carried out to resolve this issue. Concentrations with minimal toxicities were then chosen to investigate using curcuminoids on its ability to sensitize etoposide drug. MK-571, indomethacine and vinblastine at non toxic concentratons (Figure 34, Table 18) were included in the experiment as positive modulators. Curcuminoids at 5 μM and 10 μM substantially enhanced the sensitivity towards etoposide of MRP1 transfected cells, while they had no effect in the control cells (Figure 36). Curcumin I showed the most significant potentiation effect while the rest of the curcuminoids had mild to strong reversing effects (Figure 36, Table 19). It is likely that curcuminoids either directly inhibited MRP1-mediated etoposide efflux in MRP1-transfected cells or they reduced the expression level of MRP1 protein, thus increasing the etoposide sensitivity. The latter was found not to be the case since curcuminoids had no effect on MRP1 protein level in MRP1-HEK293 cells (Figure 38). This suggested that the potentiation effect of curcuminoids is by interfering with the function of MRP1.

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Direct evidence of curcuminoids inhibit drug transport by MRP1 and the binding sites of curcuminoids on MRP1

The direct inhibition of curcuminoids on MRP1-mediated transport was confirmed by flow cytometry studies where the curcuminoids increased the

accumulation of MRP1 substrates fluo4-AM (Figure 38) and calcein-AM (Figure 39) in a concentration-dependent manner. The potency of curcumin I was comparable to MK-571, which is known to inhibit MRP1-mediated transport with high affinity (Figure 40). Moreover, curcuminoids were able to stimulate the ATPase activity of MRP1 at the low concentrations and slightly inhibit the activity at high concentrations (Figure 41, Table 20). This stimulation is most likely due to the interactions between curcuminoids at the MRP1 substrates binding site(s) and not the nucleotides binding sites since curcuminoids had no effect on 8 azido [α-^{32}P]-ATP binding (Figure 43). This is further supported by the fact that curcuminoids inhibited the quercetin-stimulated ATP hydrolysis by MRP1 (Figure 42).

The structure of curcuminoid (s) contains electrophilic α, β- unsaturated carbonyl groups, and as a result of this structure, may affect MRP1 in either interaction with substrate binding sites as observed in this study or through interaction with GSH. GSH was reported to play an important roles in the transport function of MRP1 in both unconjugated and conjugated compounds [183], thus the depletion of intracellular GSH levels can decrease the transport of several substrates in MRP1-mediated transport. Curcumin, was reported to deplete intracellular GSH levels in human melanoma cells [184], thereby this effect of curcuminoids could be a beneficial effect of curcuminoids to circumvent MDR phenotype in MRP1 overexpressing cells.

Furthermore, as mentioned in the Literature review – high affinity substrates for MRP1 are conjugated compounds. Curcuminoids were reported to biotransform monoglucuronide conjugates in the cells [154] as conjugate forms of curcuminoids may act as competitive inhibitors for MRP1 transport, as was reported for the glutathionyl conjugates of ethacrynic acid in the vesicle model [185]. Taken together, curcuminoids may exhibit inhibitory effects on MRP1 via the following mechanisms: (i) direct inhibition of the MRP1 mediated transport process through interaction of the parent compound with the MRP molecule (as shown in this study), (ii) formation of GSH conjugates which can competitively inhibit MRP1, or (iii) depletion of GSH.

This study found that both curcumin II and curcumin III appeared to act as MRP1 inhibitors, but were less effective than curcumin I. Whether the difference in activity in intact cells is due to the differences in metabolism and/or to a better

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membrane passage or passive diffusion across the membrane due to higher lipophilicity remains to be established. As for the ATPase activity which was studied in isolated membrane of MRP1-High Five insect cells (in which the metabolism is not an issue), the results indicate that curcumin I is the most active form of curcuminoids found in turmeric, suggesting that the methoxyl group in curcuminoid structure is somehow responsible for the potency of the MRP1 inhibition.

In addition, curcumin mixture appears to affect the trafficking of ΔF508 mutant of cystic fibrosis transmembrane regulator (CFTR) [186], which also belongs to ABCC subfamily (ABCC7), similar to MRP1 (ABCC1) and MRP2 (ABCC2). Curcumin mixture has been reported to stimulate the chloride channel activity of wild-type CFTR [187-190]. However, this is the first report to our knowledge in which the purified curcumin forms I, II, and III rather than a mixture, have been used to assess their effect on the function of MRP1. It remains to be seen whether the function of MRP2 or CFTR is also modulated to the same extent by curcumin I alone.

Modulation of human MXR function by curcumin I, II and III.

In the two previous parts the results clearly showed that curcumin mixture, curcumin I, II, and III are able to modulate the function of P-gp and MRP1. The effects of curcuminoids on MXR function and expression were further determined by using HEK stably transfected with MXR –pcDNA3.1 and pcDNA3.1 vector alone. The expression and transport ability of MXR in these cell lines were evaluated. High level of MXR protein was detected in MXR-HEK 293 cells and not in the empty vector control, pcDNA3.1-HEK 293 cells (Figure 44). Representative histogram of bodipy-prazosin accumulation in the presence and absence of MXR inhibitor fumitremorgin C, FTC (Figure 45) revealed that the reduced accumulation of bodipy-prazosin was increased by FTC in MXR transfected HEK293 cells and not the empty vector control cells. It can be suggested that MXR was expressed only in MXR-HEK 293 cells and not in the empty vector control cells. The MXR transporter in this cell line exhibited the efflux function effectively. The results consistence with the previous study by Robey et al., [65] (the original source of the MXR-HEK 293 cells used in this study) in which the expression of MXR was detected by Northern blot, Western blot and cell surface staining assay. The result showed that the HEK 293

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stably transfected with MXR had enforced expression of the MXR gene at the RNA level, whereas in the cell line transfected with empty vector, no MXR RNA was detected. In addition, by Western blot and cell surface staining assay, high level of MXR protein was detected in MXR-HEK 293 cells and not in pcDNA3.1-HEK 293 cells.

Curcuminoids inhibit MXR (wild type, 482R)-mediated MDR phenotype

To attain the non-toxic concentrations of curcuminoids for the MDR phenotype study. The cytotoxicity of curcuminoids was determined by MTT assay. The IC_{20} values in pcDNA3.1- and MXR-HEK 293 cells are in the range of 10-18 μM, and similar to P-gp and MRP1, the curcuminoids themselves may not be substrates for MXR since the IC_{50} values in the drug resistant and drug sensitive cell lines were not significant different (Figure 46, Table 21). When compared the drug resistant profile, it was clearly showed that the non toxic concentrations of curcuminoids at 3, 5 and 10 μM increased the drug sensitivity of mitoxantrone, topotecan and SN-38 (Figure 47-49, Table 22-24) in a concentration dependent manner. The modulatory effect of curcuminoids on MXR function was directly confirmed by the bodipy- prazosin and mitoxantrone accumulation assay. It was clearly demonstrated that each pure form of curcuminoids and curcumin mixture were able to increase the accumulation of bodipy-prazosin (Figure 50) and mitoxantrone (Figure 51) in a dose-dependent manner. Additionally, curcuminoids displayed the reversing activity as strong as 10 μM of FTC, which is a very potent modulator of ABCG2. This is the first report to show the potent modulatory effect of curcuminoids on MXR function. For the rhodamine123 accumulation, it was consistence with the earlier study [65], no decreasing of the rhodamine123 accumulation was observed in MXR (wild type, 482R) transfected cells indicating that the rhodamine123 is not a substrate for MXR wild type. It was proposed that the bulky positively charge amino acid of arginine at the position 482 may hinder the transport of rhodamine123 which is a cationic fluorescent dye [61].

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Curcuminoids inhibit MXR (RR482T mutant)-mediated MDR phenotype

Amino acid at position 482 of MXR has a crucial role in MXR function [64, 65, 158, 159]. The mutation of MXR at amino acid position 482 from arginine to threonine (R482T) has altered drug resistance profiles and substrate specificity of MXR [65]. As a result, the research was expanded to evaluate the effect of curcuminoids on the function of MXR mutant. In this part of the study, the MDR cell line encoded “Thr (T)” at amino acid position 482 of MXR, MCF-7AdrVp and its parental cell, MCF-7 were used as a model. By Western blot analysis, high level of MXR protein was detected in this cell lines. In contrast to HEK 293 transfected with MXR wild type, 482R, rhodamine123 was effective effluxed by MCF-7AdrVp and not the parental MCF-7 cells (Figure 55).

Similar effects of curcuminoids were observed in MCF-7AdrVp, mutant MXR (R482T); curcuminoids increased the sensitivity of mitoxantrone and doxorubicin in MCF-7AdrVp but not in MCF-7 (Figure 57, Table 24). Curcumin I showed the most potent MDR reversing capacity when combined with mitoxantrone; however it is likely that in the presence of doxorubicin all three forms of curcuminoids exhibited in the similar activity. Additional experiments at lower concentrations of curcuminoids may be required to distinguish the MDR reversing activity of each form.

Similar with MRP1, curcuminoids had no effect on MXR protein level in MCF-7AdrVp cells (Figure 62).

The inhibitory effect of curcuminoids has been confirmed by the accumulation assay of bodipy-prazosin and rhodamine123. The result showed clearly that curcuminoids significantly increased the accumulation of the fluorescence substrates in a concentrations dependent manner (Figure 58-61).

The interaction of curcuminoids with MXR was indicated by the stimulatory effect of curcuminoids on the ATP hydrolysis of MXR. The unpublished result at LCB/NCI/NIH indicated that curcuminoids were able to inhibit the IAAP incorporation into the purified membrane of MXR suggesting that the binding site of curcuminoids on MXR maybe at the same binding site with prazosin

Collectively, the results clearly indicated that curcuminoids are able to inhibit the efflux function of MXR both in wild type (R) and mutant (T) probably by competitive interactions at the substrate binding sites of the transporter.

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Tetrahydrocurcumin, an ultimate metabolite form of curcuminoids exhibited the MDR reversing capacity to P-gp, MRP1 and MXR

Finally, the research raised the question as to whether an ultimate metabolite form of curcuminoids is able to extend the MDR reversing capacity of the curcuminoids. Tetrahydrocurcumin is acknowledged as an active metabolite form of curcuminoids [24, 25, 121, 191] and it has been reported widely on its potential antioxidant activity as well as some other biological properties for instance anti-inflammatory and anticarcinogenesis [192-197] (see review for tetrahydrocurcumin at http://www.tetrahydrocurcuminoids.com). As indicated in Figure 65, tetrahydrocurcumin significantly increased the accumulation of calcein in KB-V-1, in a concentration dependent manner. This effect was not found in KB-3-1, suggesting that tetrahydrocurcumin significantly inhibit the efflux function by P-gp. The inhibitory effect of tetrahydrocurcumin was not cell type dependent since it was also able to increase the accumulation and inhibited the efflux of ^{3}[H]-vinblastine in MCF-7MDR, in a concentration dependent manner, and not in its parental MCF-7 (Figure 66). MCF-7MDR cell line is the MDR1 transfected cell that had been maintained in the anticancer drug colchocine [198]. The direct interaction of tetrahydrocurcumin with the P-gp was assessed directly by photoaffinity labeling of IAAP. Similar to the effect with curcuminoids, the incorporation of IAAP into P-gp was significantly inhibited by tetrahydrocurcumin in a concentration manner. The effect of tetrahydrocurcumin on MRP1 and MXR mediated MDR phenotype was evaluated by MTT (MDR reversing activity) assay. The results in Figure 70 and Figure 71 showed indubitably that tetrahydrocurcumin was able to reverse MDR in MRP1 or MXR overexpressing cells. A consistence finding was reported in MDCKII cells transfected with MRP1, tetrahydrocurcumin showed significantly increased ^{3}[H]-EGCG in MDCKII/MRP1 overexpressing cells [199]. It is premature to explain the mechanisms of action of tetahydrocurcumin on the three ABC transporters. However, evidence reported in the present study reveals that tetrahydrocurcumin is able to extend the MDR reversing capacity of curcuminoids, and these findings support the development of curcuminoids purified from turmeric as a modulator using in combination with conventional chemotherapy.

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CONCLUSIONS

Taken together, the effects of curcuminoids on P-gp, MRP1 and MXR can be summarized as follows:

1. Curcuminoids can be good modulators of P-gp, MRP1 and MXR
2. Curcuminoids bind directly to P-gp, MRP1 or MXR; however, they are not transported by these transporters
3. The most potent form of curcuminoids to exhibit the modulatory effect on P-gp, MRP1 and MXR is curcumin I
4. Curcuminoids exert their inhibitory effects on P-gp, MRP1 and MXR by the following mechanisms:

P-gp: Curcuminoids either directly inhibit the efflux function or reduce the expression of P-gp.

- By MTT assay, curcuminoids significantly increase the sensitivity of P-gp overexpressing cells, such as KB-V-1 (but not that of the parental cells) to anticancer drugs (vinblastine, paclitaxel).
- By flow cytometry studies, curcuminoids were shown to increase the accumulation of P-gp fluorescent substrates (rhodamine 123, calcein-AM and bodipy-vinblastine) in a concentration dependent manner. The results clearly confirmed the MDR reversing activity of curcuminoids from the MTT assay .
- The ATPase assays suggest that curcuminoids have a biphasic effect on P-gp. Curcuminoids stimulated the P-gp ATPase activity at low concentrations and inhibited at high concentrations. Such an effect suggests that at higher concentrations, curcuminoids may reduce the net rate of substrate dissociation after the translocation step of the transport cycle.
- The inhibitory effect of curcuminoids on verapamil stimulated P-gp ATPase activity, or the inhibitory effect on the binding of IAAP to P-gp, made it more evident that curcuminoids exert their inhibitory effect by

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competitive binding to the substrate binding sites and probably at the same binding site with verapamil or prazosin.

- For the effect of curcuminoids on P-gp expression, (although it has not been shown in the present study), it was proved previously by our lab [26, 27] that curcuminoids decreased the expression of P-gp in a dose (0-10 μM) and time (0-4 days) dependent manner. One of the proposed mechanisms was that curcuminoids intervene *MDR1* gene expression by inhibiting transcription factor CREB probably via the protein kinase A (PKA) pathway [26].

MRP1: Unlike with P-gp, curcuminoids exhibit their inhibitory effect on MRP1 only by intervention at the efflux function and not at the expression level.

- After treatment of MRP1 transfected cells with 10 μM of curcuminoids, the level of MRP1 protein was not significantly different from the control DMSO treated cells, whereas at this concentration, curcuminoids significantly increased the sensitivity of etoposide drug in the MRP1 transfected cells with no effect in the empty vector cells.
- The result was directly confirmed by the accumulation assay (FACS). Curcuminoids increased the accumulation of MRP1 fluorescent substrate (calcein AM, fluo-4AM) in a concentration dependent manner.
- Similar with P-gp, curcuminoids were found to interact directly with MRP1 and probably at the substrate binding site rather than the ATP binding sites since curcuminoids inhibit the quercetin stimulated MRP1 ATPase activity with no effect on 8-azido [α-^{32}P]-ATP binding.

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MXR: Similar to MRP1, curcuminoids act as MXR modulators by restraint only at the functional and not the expression level

- Upon treatment of MXR overexpressing cells (MCF-7AdrVp) with 3 and 10 μM of curcuminoids, the level of MXR protein was not significantly different from the control DMSO treated cells, whereas at this concentration curcuminoids significantly increased the sensitivity of anticancer drugs in

both MXR wild type, 482R transfected HEK 293 cells and mutant R482T, MCF-AdrVp cells.

- Their inhibitory effect was assured by the accumulation assay analysed by FACS. Curcuminoids significantly increased the accumulation of MXR fluorescent substrates in a concentration dependent manner.
- ATPase and IAAP labeling assay clearly demonstrated the interaction of curcuminoids to the substrate binding site of MXR, suggesting that curcuminoids may retain the anticancer drug inside the MDR cells by competitive binding to the substrate binding site on the transporter. However, curcuminoids themselves may not be transported by the transporter.

5. Obviously, the inhibitory effect of curcuminoids is not selective to P-gp or MRP1 or MXR. This can be viewed in two ways:

First, in a positive way, the multiple inhibition by curcuminoids maybe useful in light of the increasing awareness that tumors are likely to exhibit multiple mechanisms of drug resistance. And second, in a negative way, multiple inhibition of curcuminoids could lessen the ability of normal cells and tissues to protect themselves from cytotoxic agents, as most of these transporters are expected to play a physiological role in the elimination of foreign compounds [82].

The latter case could be relieved since recently [200], a study in mice with disruptions of *Mdr1a, Mdr1b* or both *Mdr1a and Mdr1b* genes found that each strain is viable, healthy and normally fertile under laboratory conditions [200], similar to what was observed from two independent laboratories in the case of MRP1 [201, 202] and MXR [112, 203]. The knock-out mice of MRP1 and MXR are viable, healthy and fertile as normal condition. This indicates that although those transporters have been interrupted, the human body system would have other mechanisms to maintain those physiological functions of P-gp, MRP1 and MXR .

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6. Curcuminoids not only increase the sensitivity of anticancer drugs by exerting their inhibitory effect on the drug efflux pumps (P-gp, MRP1 , MXR) but also by exhibiting other synergistic mechanisms in killing the cancer cells e.g.

- Curcumin has been reported extensively to inhibit or down regulate NF-κB [204-209], a transcription factor that is involved in the regulation of the mdr1 gene, thereby also contributing to the MDR phenotype in cancer cells. A recent study [208] showed that doxorubicin or 5-FU or cisplatin - induced NF-κB activation was attenuated significantly when pretreating the carcinoma cells with curcumin. A study in multiple myeloma cells supported such evidence. The result indicated that curcumin down regulates NF-κB and makes these cells more sensitive to the chemotherapy drugs vincristine and melphalan [210]. Another study in prostate cancer [211], also showed that curcumin enhances cytotoxicity of doxorubicin, 5-FU and paclitaxel in a dose dependent manner by suppressing NF-kappa B activation.
- Recently a study in MDR human gastric carcinoma cell lines,SGC7901/VCR [175] showed that curcumin could promote the vincristine mediated apoptosis of MDR SGC7901/VCR cells through induction of caspase-3 activation [179]. Apoptosis in tumor cells plays a critical role in chemotherapy induced tumor cell killing, and suggests that blocking the apoptosis-inducing pathway could be another mechanism for MDR to chemotherapy [212]. Thus the induction of apoptosis by curcuminoids in cancer cells would be a synergistic benefit in killing cancer cells for chemotherapeutic agents. In addition, curcumin has been reported to deplete the glutathione (GSH) and inhibit the glutathione-S-transferase (GST) [178, 184, 213-218] thereby reducing detoxification and efflux of anticancer agents. in cancer cells.

Taken together, curcuminoids not only inhibit MDR efflux pump but also exhibit other synergistic effect that help to kill cancer cells or inhibit the MDR phenomenon by other possible mechanism.

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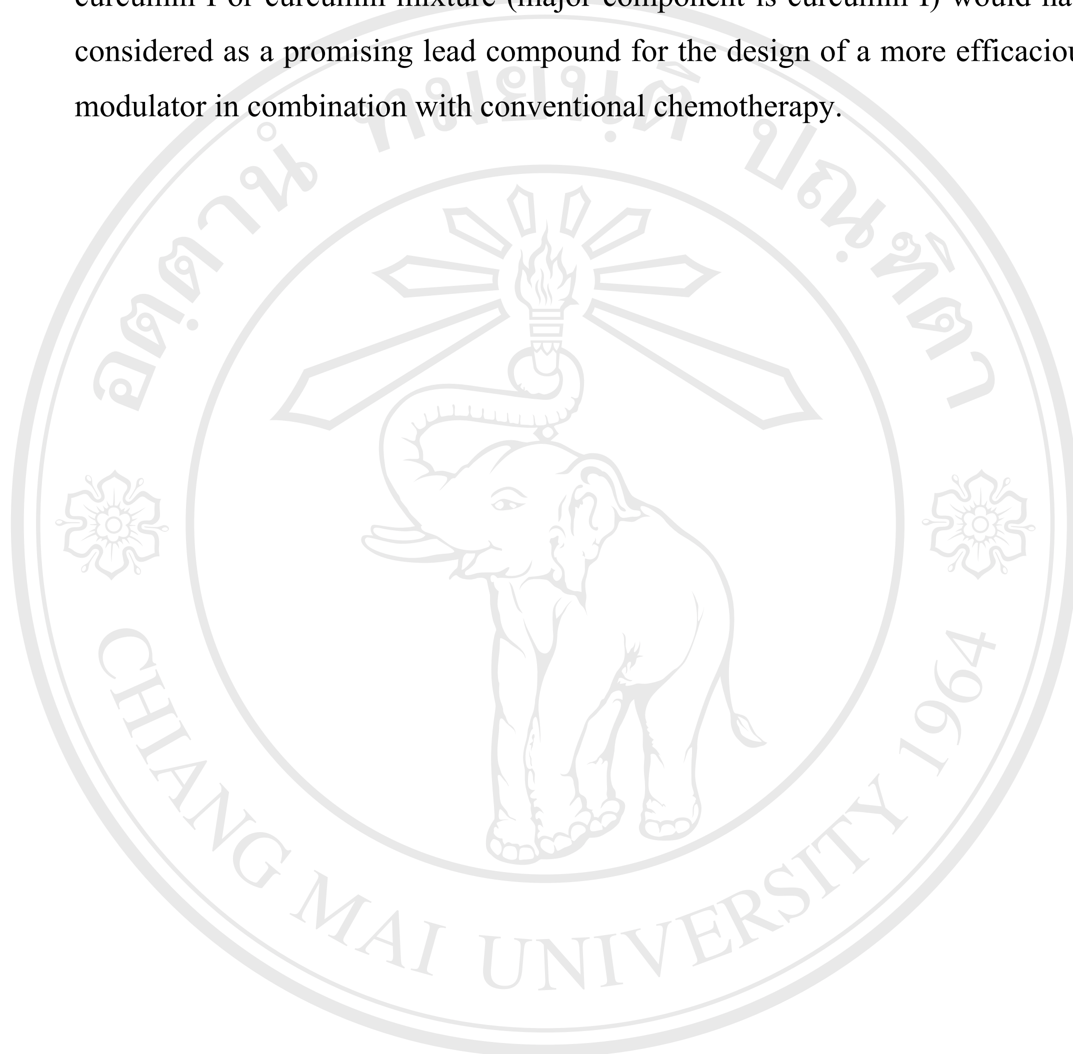
In summary, with all those beneficial effects of curcuminoids, although another subject of interest is whether curcuminoid concentrations achieved in vivo are sufficient to inhibit P-gp, MRP1 or MXR function, and extensive pharmacokinetic studies with curcuminoids will be required to know the steady-state level of

phytochemical reached in blood and tissue after its administration at pharmacological doses. However, the results of the present study reveal that curcuminoids, particularly curcumin I or curcumin mixture (major component is curcumin I) would have to be considered as a promising lead compound for the design of a more efficacious MDR modulator in combination with conventional chemotherapy.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