CURCUMIN AS CHEMOSENSITIZER

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Abstract: This overview presents curcumin as a significant chemosensitizer in cancer chemotherapy. Although the review focuses on curcumin and its analogues on multidrug resistance (MDR) reversal, the relevance of curcumin as a nuclear factor (NF)-κB blocker and sensitizer of many chemoresistant cancer cell lines to chemotherapeutic agents will also be discussed. One of the major mechanisms of MDR is the enhanced ability of tumor cells to actively efflux drugs, leading to a decrease in cellular drug accumulation below toxic levels. Active drug efflux is mediated by several members of the ATP-binding cassette (ABC) superfamily of membrane transporters, which have now been subdivided into seven families designated A through G. Among these ABC families, the classical MDR is attributed to the elevated expression of ABCB1 (Pgp), ABCC1 (MRP1), and ABCG2 (MXR). The clinical importance of Pgp, MRP1, and MXR for MDR and cancer treatment has led to the investigation of the inhibiting properties of several compounds on these transporters. At present, due in part to the disappointing results associated with the many side effects of synthetic modulators that have been used in clinical trials, current research efforts are directed toward the identification of novel compounds, with attention to dietary natural products. The advantage is that they exhibit little or virtually no side effects and do not further increase the patient's medication burden.

1. INTRODUCTION

This chapter deals with the theoretical background of drug resistance in cancer chemotherapy and clinical significance of the search for chemosensitizers of multidrug resistance (MDR) in cancer. After discussing the basic features of the drug transporter proteins P-glycoprotein (Pgp), multidrug resistance protein-1(MRP1), and mitoxantrone resistance protein (MXR) responsible for this phenomenon, the possible mechanism of action of MDR chemosensitizers is reviewed. This chapter also discusses apoptosis and chemotherapy resistance.

This overview presents curcumin as a significant chemosensitizer in cancer chemotherapy. Although the review focuses on curcumin and its analogues on MDR reversal, the relevance of curcumin as a nuclear factor (NF)-κB blocker and sensitizer of many chemoresistant cancer cell lines to chemotherapeutic agents will also be discussed.

One of the major mechanisms of MDR is the enhanced ability of tumor cells to actively efflux drugs, leading to a decrease in cellular drug accumulation below toxic levels. Active drug efflux is mediated by several members of the ATP-binding cassette (ABC) superfamily of membrane transporters, which have now been subdivided into seven families designated A through G.[1] Among these ABC families, the classical MDR is attributed to the elevated expression of ABCB1 (Pgp), ABCC1 (MRP1), and ABCG2 (MXR).

The clinical importance of Pgp, MRP1, and MXR for MDR and cancer treatment has led to the investigation of the inhibiting properties of several compounds on these transporters. The calcium channel blocking agent verapamil was the first drug described as an inhibitor of the Pgp efflux mechanism.[2] After this discovery, several other compounds have been studied for their inhibitory effects (e.g., valspodar, GF120918, and LY335979).[3,4] Although these agents are effective, one of the major problems with most of them is that the *in vivo* plasma concentrations required to inhibit Pgp are too high and result in severe toxic side effects. At present, due in part to the disappointing results associated with the many side effects of modulators that have been used in clinical trials, current research efforts are directed toward the identification of novel compounds, with attention to dietary natural products. The advantage is that they exhibit little or virtually no side effects and do not further increase the patient's medication burden.

Curcumin has been described as a potent antioxidant and anti-inflammatory agent. The compound has been found to be pharmacologically safe: Human clinical trials indicated no doses-limiting toxicity when administered at doses up to 10 g/day.[5] All of these studies suggest that curcumin has enormous potential in the prevention and therapy of cancer. However, a better understanding of the mechanism would enhance the therapeutic potential of curcumin either alone or in combination with chemotherapy. The study reported by our group demonstrated that curcumin (curcumin I), demethoxycurcumin (curcumin II), and bisdemethoxycurcumin (curcumin III) are potent chemosensitizers of Pgp, MRP1, and MXR, and curcumin I was the most effective form. Tetrahydrocurcumin (THC), a major metabolite of curcumin, is also a good chemosensitizer of Pgp, MRP1, and MXR, and it is able to extend the MDR-reversing properties of curcuminoids *in vivo*.

There is increasing evidence that the inability of the cells to undergo apoptosis might critically contribute to the genesis and progression of cancer and represents an important cause of tumor drug resistance.[6] Tumor cells often evade apoptosis by overexpressing antiapoptotic proteins such as Bcl-2, NF-κB, Akt, and so forth, which give them a survival advantage. Some conventional chemotherapeutic drugs in low concentrations cause upregulation of survival signals, thereby necessitating increments of the effective dose of treatment. Activation of NF-κB has been shown to block apoptosis and promote proliferation; therefore, NF-κB activation induces resistance to chemotherapeutic agents. Thus, agents that induce apoptosis and stimulate NF-κB activity might be effective if given in combination with agents that could inhibit NF-κB. Evolving interest in recent years has focused on phytochemicals augmenting apoptosis as possible candidates for the evaluation of

their synergistic efficacy in combination with chemotherapeutic agents. Common biological modulators, including curcumin, have been researched in order to block NF-κB activation as described in this chapter.

2. MECHANISMS OF DRUG RESISTANCE IN CANCER CHEMOTHERAPY

2.1. Drug Resistance in Cancer

The presence or development of resistance to anticancer drugs is the main cause of failure of chemotherapy in the majority of the most common forms of cancer (e.g. lung, colon, breast, and cervix). Resistance to chemotherapeutic drugs has already been present at diagnosis or it can develop after chemotherapy treatment. These two forms of drug resistance are respectively called intrinsic and acquired resistance.[7,8] Intrinsic resistance or *de novo* resistance of cancer cells can be present before chemotherapy, resulting in initial treatment failure such as Hodgkin's disease, testicular cancer, and acute childhood leukemia, but acquired resistance can develop in response to chemotherapeutic intervention, eventually leading to early disease progression despite an initial treatment response (e.g., lymphoma and breast cancer).[9] In both intrinsic and acquired resistance, tumors are often found to be refractory to a variety of drugs with different structures and functions. A similar experimental phenomenon has been termed *multidrug resistance*. MDR can be the result of a variety of mechanisms that are not fully understood.[10] The most important among them are the following: (1) altered membrane transport either by decreased drug uptake or by increased drug efflux[11]; (2) perturbed expression of target enzymes or altered target enzymes[12]; (3) altered drug activation or degradation[13]; (4) enhanced DNA repair[14]; and (5) failure to undergo apoptosis.[15,16] Some of these mechanisms of drug resistance might coexist; however, the most widely implicated mechanism is that concerned with altered membrane transport in tumor cells. This mechanism is often referred to as typical or classical MDR.

2.2. Multidrug Resistance and Drug Transporter Proteins

The human *MDR1* gene product, P-glycoprotein, was the first ATP-dependent system discovered that was implicated in MDR. P-Glycoprotein (also known as Pg-170, Pgp, P-170, or ABCB1) was isolated[17] and proposed to be the transporter protein that pumps out the antitumor agents.[18,19] The overexpression of P-glycoprotein is not the only cause of MDR. Another member of the ATP-binding cassette (ABC) superfamily, which is involved in MDR, is the 190-kDa multidrug resistance-associated protein (MRP1 or ABCC1), encoded by the MRP1 gene. MRP1 is simlar to P-glycoprotein in its capability of decreasing intracellular levels of drugs and is ATP-dependent.[20] The most recently discovered

ABC drug efflux transporter is breast cancer resistance protein (BCRP, MXR, or ABCG2).[21]

Among these ABC families, the classical MDR is attributed to the elevated expression of ABCB1 (Pgp), ABCC1 (MRP1), and ABCG2 (BCRP or MXR).[22,23]

2.2.1. P-Glycoprotein

In various cancer types, such as acute myeloid leukemia, various childhood tumors and locoregionally advanced breast cancer, overexpression of *MDR1*-Pgp has been found to correlate with poor outcome in patients treated with chemotherapy.[24–28] These findings have been interpreted as an indication of Pgp-mediated drug resistance. Various clinical studies have suggested that Pgp-positivity is associated with more aggressive tumor behavior. In colon cancer, Pgp was found to be expressed predominantly in the tumor cells at the invading edge of primary tumors, and Pgp-positivity in primary tumors was associated with a higher incidence of lymph node metastases.[29] In renal cell carcinoma, Pgp-positivity was found significantly more often in invasive than in noninvasive tumors.[30] In primary breast cancer, overexpression of *MDR*/Pgp seems to be more common in advanced locoregional disease than it is in small tumors.[31,32]

Increased expression of Pgp, the product of the human *MDR1* gene, is a well-characterized mechanism used by cancer cells to evade the cytotoxic action of anticancer drugs. Twenty years ago, Juliano and Ling discovered that Pgp was the most ubiquitous marker in MDR cells.[17] P-Glycoprotein (P refers to its proposed role in modulating cellular permeability to drugs) is a high-molecular-weight integral plasma membrane glycoprotein that confers MDR to mammalian cells by acting as an energy-dependent drug efflux pump. P-Glycoproteins are encoded by small gene families, with two members in humans and three in rodents.[33] Despite a high amino acid sequence identity of more than 70% among all Pgps, *MDR* gene products are subdivided into two classes: class I and class II. Overexpression of class I P-glycoprotein causes cancer cells to become resistant to a variety of anticancer drugs (e.g., vinblastine, vincristine, daunorubicin, etoposide, teniposide, and paclitaxel) as well as many other cytotoxic agents, including colchicines, emetine, ethidium bromide, puromycin, and mithramycin.[34] Class II *MDR* gene products are predominantly expressed in the liver bile canaliculi.[35–37]

2.2.1a. Structure of P-Glycoprotein

Mammalian P-glycoproteins are single-chain proteins and consist of approximately 1280 amino acid residues (170 kDa). P-Glycoproteins are composed of 43% sequence homology; between the two halves, there is a hydrophobic, membrane-associated domain (approximately 250 amino acid residues) followed by a hydrophilic nucleotide-binding fold (approximately 300 amino acid residues).[38] These two halves are connected by a linked peptide of approximately 75 amino acids defined as amino acids 633–709 in human Pgp. This peptide conjugated,

commonly called the linker region, is highly charged and contains the *in vivo* sites of phosphorylation.

Both the N-terminal membrane-associated domains and the C-terminal membrane-associated domains of human P-glycoprotein harbor six predicted trans-membrane (TM) regions. The N-terminus, the C-terminus as well as the nucleotide-binding folds are located intracellularly. The first extracellular loop is glycosylated. This 12-TM-region model of P-glycoprotein is supported experimentally by cellular epitope localization data obtained from antibodies that specifically recognize the N- or C-terminus of P-glycoprotein, its first and fourth extracellular loop, or the two ATP-binding sites. The two halves of Pgp are essential for activity of the transporter as measured by its ability to confer drug resistance or drug-stimulated ATPase activity. Both transmembrane domains 5, 6 and 11, 12 and the extracellular loops connecting them were determined by photoaffinity labeling, with the Pgp substrate analogues being the major sites of drug interaction. These transmembranes are important determinants in the drug-binding site(s), but they do not offer any insight into whether these sites are autonomous or interdependent.

2.2.1b. Mechanism of Pgp-Mediated Drug Resistance

Discovery of the molecular mechanisms by which Pgp exert its action has been one of the major tasks of research in the field of MDR. Pgp substrates can structurally be very different; however, the physical properties shared by many of them include high hydrophobicity, an amphiphilic nature, and a net positive charge, although neutral compounds, among them hydrophobic peptides, have also been described as substrates of Pgp.[39]

Whereas Pgp fulfills critical functions in transport processes involved in normal physiology, overexpression of this protein in tumor cells results in reduced intracellular accumulation of anticancer agents due to increased drug efflux. Most models of Pgp suggest that it transports drugs across cell membranes in a manner analogous to that defined for active transport proteins. This model predicts that substrates (cytotoxic drugs) bind to specific domains of the protein, which subsequently undergoes an energy-dependent conformational change. This change allows the substrate to be released on the exterior side of the membrane. Complementary models have been proposed, suggesting that (1) Pgp interacts directly with substrates in the plasma membrane (the "hydrophobic vacuum cleaner" model[38] or (2) Pgp might be involved in the transport of drugs from the inner leaflet to outer leaflet of the plasma membrane, from which they diffuse (the flippase model).[40] Identification and characterization of Pgp segments responsible for drug recognition and binding indicate that Pgp interacts directly with drug molecules. Efforts to map the drug-binding domains of Pgp by photoaffinity drug analogues and site-directed mutagenesis indicate that Pgp contains multiple nonoverlapping or partially overlapping drug-binding sites, each having different affinities for different drugs or classes of drugs.[41–43] The two nucleotide-binding domains (NBDs) are a critical feature of Pgp. Reconstitution studies with purified Pgp have shown that transport of hydrophobic substrates against a concentration gradient is coupled

to ATP hydrolysis.[44] However, the mechanism by which Pgp couples ATP energy to translocation and efflux of a diverse range of substrates is a largely unresolved debate.[45] Both NBDs can hydrolyze nucleotides, and their ATPase activity, that can be blocked by vanadate, is necessary for drug transport.[46]

Finally, Pgp is phosphorylated by protein kinase C (PKC), and PKC blockers reduce Pgp phosphorylation and increase drug accumulation. These observations suggest that phosphorylation of Pgp stimulates drug transport. However, there is evidence that PKC inhibitors directly interact with Pgp and inhibit drug transport by a mechanism independent of Pgp phosphorylation.[47,48]

2.2.2. Multidrug Resistance Protein-1

The MRP family entered drug resistance in 1992 when Susan Cole and Roger Deeley cloned the multidrug resistance-associated protein gene, now known as MRP1 and was classified to ABCC1.[20] Since then 13 genes for ABCC family have been reported and designated ABCC1 to ABCC13. In 2002, Yabuuchi et al. reported that ABCC13 is predicted to encode a nonfunction protein.[49] As a result, the ABCC family contains only 12 functional proteins. The discovery of the MRP family has considerably broadened the study of MDR in tumor cells and has led to widespread interest in the possible function(s) of the members of this family in normal metabolism.

MRP1 is broadly expressed in the epithelial cells of multiple tissues, including the digestive, urogenital, and respiratory tracts, endocrine glands, and the hematopoietic system.[50] MRP1 expression has been demonstrated in multiple tumor tissues and has been implicated as a component of the MDR phenomenon in leukemia and cancers of the lung, colon, breast, bladder, and prostate.

2.2.2a. Structure of MRP1

The MDR-associated protein (MRP1) is a 190-kDa protein encoded by the *mrp1* gene and is constituted by 1531 amino acids presenting N-linked glycosylation sites.[51] Although the human genome encodes only two Pgps, it contains many genes related to MRP.[52] The protein is predominantly localized to the plasma membrane in drug-resistant cells, with detectable levels present in intracellular membrane compartments of some cell types.[53] Whereas Pgp transports neutral and positively charged molecules in their unmodified form, MRP1 overexpression is associated with an increased ATP-dependent glutathione (GSH) *S*-conjugate transport activity. MRP1 is able to transport a range of substrates as such or conjugated to GSH, glucuronide, and sulfate.[54–56] The predicted topology of MRP1 and several related ABCC proteins differs from that of most eukaryotic ABC transporters, which are composed of two membrane-spanning domains (MSDs), each containing six transmembrane (TM) domains with two NBD sites. MRP1 has an additional NH_2-terminal domain, MSD1, with five TMs and an extracellular NH_2-terminus. Thus, MRP1 is predicted to contain three MSDs with $5 + 6 + 6$ TM helices.[57,58]

2.2.2b. Mechanism of MRP1-Mediated Drug Resistance

It has been proposed that a physiological function of MRP1 is the extrusion of endogenously formed GSH-dependent detoxification products to prevent cellular damage.[55] The generation of *Mrp1* −/− knockout mice has significantly contributed to the understanding of the physiological role of MRP1. Similar to most ABC transporters, MRP1 requires ATP hydrolysis for its transport; the interaction of ATP with MRP1 was studied by photoaffinity labeling and vanadate-induced trapping experiments using ^{32}P-labeled 8-azido-ATP. The two NBDs show cooperativity in the binding and trapping of the nucleotide.[59,60] Experiments with membrane vesicles from MRP1-overexpressing cells demonstrated that MRP1 is a transporter for the unmodified anticancer drugs vincristine and daunorubicin, but only in the presence of physiological amounts of GSH.[54,61] These results extend the earlier observations that GSH is a critical factor in MRP1-mediated drug resistance. MRP1 transports a wide variety of subtrates that include drugs conjugate with GSH (GS-X pump), glucuronide, and sulfate and some anticancer drugs such as anthracyclines, vinca alkaloids, and epipodophyllotoxin. It has been proposed recently that MRP1 might interact with GSH by at least four different mechanisms.[62] First, GSH might be a direct low-affinity substrate for MRP1 ($K_m \sim 10$ mM). Second, GSH is required for the cotransport of certain MRP1 substrates (e.g., in the case of daunorubicin, vincristine, and aflatoxin) ($K_m \sim 0.1$ mM). Third, GSH stimulates the transport of certain compounds on MRP1, but it is not transported; finally, the transport of GSH is accelerated by certain compounds that are not themselves a substrate for MRP1.

2.2.3. Mitoxantrone Resistance Protein

ABCG2 was first cloned and sequenced from mitoxantrone-resistant S1-M1-80 human colon carcinoma cells and from MCF-7 AdrVp human breast cancer cells selected in doxorubicin (adriamycin).[63,64] This gene is designated ABCG2 by the new nomenclature system but is also referred to as BCRP (breast cancer resistance protein),[64] MXR (mitoxantrone resistance protein),[63] or ABCP (placenta-specific ABC transporter).[65]

2.2.3a. Structure of MXR

The human *ABCG2* gene is located on chromosome 4q22 and encodes a 655-amino-acid polypeptide with a predicted molecular weight of 72 kDa. Therefore, ABCG2 is proposed to be a half-transporter, containing only one set of six TM domains and one NBD site.[65]

Recently, it has been reported that amino acid 482 is an important determinant of substrate recognition by ABCG2.[66] For example, wild-type MXR with an Arg at position 482 does not transport daunorubicin, rhodamine123, and lyso-tracker green; however, these compounds can be transported by mutants with a Thr (T) or Gly (G) at this position.[67] On the other hand, substances such as mitoxantrone, bodipy-prazosin, and Hoechst 33342 are substrates of both wild-type MXR and the two mutants.[67,68] Recently, Miwa et al. generated a large number of mutants

in the TM segments and examined the effect of these amino acid substitutions on drug resistance conferred by ABCG2.[69] They found that amino acid substitutions of Glu at position 446, which is predicted to be located within or proximal to the TM2 of ABCG2, resulted in a complete loss of drug resistance to SN-38 and mitoxantrone. Cells transfected with mutant ABCG2 cDNA with substitution of Asn residue at position 557 to Asp (N557D) exhibited comparable resistance to mitoxantrone but significantly reduced resistance to SN-38 relative to wild-type protein. Position 557 is predicted to be located within or proximal to the TM5 segment. These data again provided strong evidence that the drug-binding sites are likely located in the MSD; therefore, amino acids in or proximal to the TM segments are important for substrate recognition by ABCG2. Alternatively, amino acid substitutions in the TM segments might alter the substrate recognition and/or translocation pathway of the protein. Position 557 is a putative N-glycosylation site of ABCG2. Whether glycosylation is important for ABCG2 function is not known at the present time.

2.2.3b. Mechanism of ABCG2-Mediated Drug Resistance

ABCG2 is endogenously expressed at high levels in human placenta and to a lesser extent in the liver, small intestine and colon, ovary, vein and capillary endothelia, kidney, adrenal, and lung, with little to no expression in the brain, heart, stomach, prostate, spleen, and cervix.[23,64,65] Based on its localization, it has been suggested that the physiological roles of ABCG2 might be to protect cells from potentially toxic substances and to prevent absorption of xenobiotics ingested in our diet by actively transporting compounds from cells.

ABCG2 confers resistance to several Pgp substrates such as mitoxantrone, the anthracyclines such as daunorubicin and doxorubicin, the camptothecins, bisantrene, topotecan, rhodamine123, prazosin, and SN-38.[23,63,64,70] In contrast, ABCG2 does not efflux other known Pgp substrates such as taxol, colchicine, verapamil, vinblastine, and calcein-AM, nor the MRP substrates calcein and GSH-conjugated monochlorobimane.[23] Substrates of ABCG2 are reviewed and summarized[71] as follows: I(1). anthracyclines (e.g., daunorubicin, epirubicin, anthracene, mitoxantrone, bisantrane), (2). camptothecin (e.g., SN-38, 9-aminocamtothecin, irrenotecan, diflomotecan, topotecan), (3). nucleoside analogs (AZT, AZT 5′ monophosphate, lamivudine), (4). fluorophores (bodipy-prazosin, Hoechst 33342, rhodamine 123, lyso-tracker green), and (5) polyglutamates (e.g., methotrexate)

2.3. Apoptosis and Chemotherapy Resistance

The determinants of cell survival and cell dealth are both extrinsic and intrinsic to the cell. All cells are in the default position of being able to undergo apoptosis but are prevented from doing so by extracellular signals within a multicellular organism.[72] These signals arise through cell-to-cell contacts, from the extracellular matrix to which cells are attached[73,74] and from circulating survival factors, such as insulin-like growth factor (IGF)-I and nerve growth factor.[75] Many of these

survival components and their downstream effectors, such as BCR-ABL, RAS, and the IGF-1 receptor, are altered in malignancy. In a metastatic tumor cell, survival must be independent of the normal topological context of a tissue. This implies that to become metastatic, intrinsic mechanisms of survival must be initiated to allow survival away from normal controls. The implications of this for cytotoxic drug therapy are that tumor cells might be intrinsically more resistant to undergoing cell death than many normal cell types. Alterations in apoptosis pathways have been shown to be involved in resistance to a variety of cytotoxic agents. Thus, it seems appropriate to refer to apoptosis-related chemotherapy resistance as a type of MDR.

There are external signals that engage apoptosis: Ligation of the APO-1/fas receptor initiates a discrete cell death signaling cascade, presumably by removing the action of internal inhibitors of the default position of cell death.[76] The expression levels of both the death-promoting ligand and its receptor will again determine a hierarchy among different cells as to whether they might readily engage apoptosis. The intrinsic determinants of a survival/death hierarchy are epitomized by members of the BCL-2 family or antiapoptotic genes. It is important for malignant tumors arising from some epithelia that the cell has a relatively high survival potential determined by the expression of antiapototic genes, sufficient for it to survive DNA damage without deletion by apoptosis. These common, high-death-threshold tumors would be resistant to chemotherapy, whereas those rarer tumors arising from hematopoietic cells might be more amenable to the engagement of cell death following cytotoxic therapy. The genes that determine survival and death thresholds might determine intrinsic drug sensitivity and resistance.[77,78]

2.3.1. The BCL-2 Family of Proteins

The BCL-2 gene was identified as a translocated product in follicular lymphoma. Expression of BCL-2 suppressed the apoptosis stimulated by the withdrawal of serum survival factors.[79] A number of gene homologues of BCL-2 have now been discovered that encode both suppressors of apoptosis and accelerators of the process (see the review in Ref. 80). Bcl-2 is the archetypal member of a family of proteins that undergo homodimerizations and heterodimerizations to each other via binding through conserved BH1, BH2, and BH3 domains.[81] The isolation of the Bcl-2 homologue Bax as a protein that immunoprecipitated with Bcl-2 and the finding that its expression accelerated apoptosis suggested a model whereby Bax-Bax homodimers promote apoptosis, whereas the Bcl-2-Bax heterodimer inhibits apoptosis by limiting Bax-Bax homodimerization.[80,81] Knowledge of the family of BCL-2-like genes has been expanding with recent discoveries of sequence-related promoters of apoptosis (bad, bak, bcl-X_s) and inhibitors of apoptosis (bcl-X_L).[82–85] In a variety of cellular backgrounds, BCL-2 and BCL-X_L expression has been shown to delay the onset of apoptosis induced by almost all classes of cytotoxic drugs. It could be claimed that the expression of BCL-2 or bcl-X_L provides a genuine multidrug or pleotropic resistance, because its inhibition of drug-induced apoptosis crosses the entire spectrum of the pharmacopoeia. Whether ectopic

expression of BCL-2 universally provides pleiotropic drug resistance, associated with the long-term survival of cells, is complicated by findings that certain types do not appear to be protected by BCL-2 apoptosis, but are, instead, protected by the expression of its homologue BCL-X_L.[86,87] On the other hand, ectopic expression of the death-promoting Bcl-Xs protein in BCL-2-expressing MCF-7 human breast carcinoma cells sensitized them to the cytotoxicity of both etoposide and paclitaxel[88] - Strategies like this, delivering apoptotic accelerators such as bax and bcl-X_s or inhibitors of bcl-2 or bcl-X_L by expression of mimetics, which prevent proapoptotic homologues from binding to bcl-2 protein, would seem to offer an important route for pleotropic drug resistance.

2.3.2. The Role of TP53 in Determining Drug Sensitivity and Resistance

Many anticancer drugs damage DNA, either directly or indirectly. This damage per se is not lethal but has to be "sensed" by the cell, and, coupled with the execution of apoptosis, this suggests that the failure of sensors could lead to drug resistance. The tumor suppressor TP53 has been suggested to be a direct sensor of DNA damage. Loss of functional p53 might promote pleiotropic drug resistance to DNA-damaging agents. The importance of p53 in promoting DNA damage-induced apoptosis was demonstrated by studies of immature thymocytes *in vitro* or intestinal epithelia *in vivo* from homozygous TP53 null animals.[89–91] Cells that had been γ-irradiated did not undego apoptosis in comparison with those that were homozygously TP53 positive. TP53-null thymocytes also failed to undergo apoptosis after treatment with the topoisomerase II inhibitor etoposide treatment with the non-DNA-damaging corticosteroid dexamethasone, suggesting that the non-DNA-damage-induced pathway was discrete and p53 independent.

3. CHEMOSENSITIZERS FOR CANCER CHEMOTHERAPY

As soon as Pgp and sister proteins were recognized as the main reason of MDR, blocking the efflux of drugs by inhibition of the functions of these transporters has become a realistic way to circumvent MDR.[92] Several chemicals, already known or used as drugs for other purposes, have been tested *in vitro* and *in vivo* on resistant tumor cells. Verapamil, a calcium channel antagonist, was the first compound found active in reversing MDR,[2] and after it, many other compounds have been found effective in the resensitization of resistant malignant cells (see the review in Ref. 93). The compounds are called chemosensitizers, MDR modulators, or MDR-reversing agents.

3.1. Chemosensitizers of Pgp

The process of chemosensitization involves administering a Pgp inhibitor (MDR modulator) and an anticancer drug to cause enhanced intracellular anticancer drug accumulation by impairing the Pgp function. Numerous compounds have been shown to inhibit the drug efflux function of Pgp and, therefore, reverse cellular

resistance. In general, they have been classified as MDR modulators belonging to the first, second, or third generation.[94]

The history on the studies of MDR modulators began more than two decades ago with the discovery by Tsuruo and co-workers that the calcium channel blocker verapamil can reverse MDR.[2] Later, it was reported that verapamil inhibits Pgp activity via direct competition with Pgp substrates.[95] Other first-generation MDR modulators include the antimalarial drug quinidine, the calmodulin antagonist trifluoperazine, and the immunosuppressant cyclosporin A.[96] The Cyclosporin A is proved to compete with Pgp substrates for binding to a common drug-binding site of Pgp[96] There were promising results in phase I clinical trials with some of the firstgeneration MDR modulators, but most required high doses,[97] and nonspecific side effects were noted. As a result, their clinical applications in cancer patients have been limited, and this has led to the discovery of so-called second- and third-generation MDR chemosensitizers.

The second-generation MDR modulators include dexverapamil, PSC 833, dexniguldipine, and VX-710. Among these, most of the studies are with PSC833 and VX-710.

PSC833 (valspodar) is an analogue of cyclosporin D, and the results to date suggest that PSC 833 acts as a noncompetitive inhibitor by binding to site(s) other than the substrate-binding site to alter the conformation of Pgp.[98] Numerous studies have been reported for its clinical trials, including phase III clinical trials. Although PSC833 exhibited increased potency, and thus required lower doses to achieve effective *in vivo* plasma concentrations to modulate MDR, it retained some properties that limited its clinical usefulness. VX-710 (biricodar) is an amido-ketopipecolinate derivative that has been shown to block both Pgp and MRP activity.[99] However, similar to PSC833, the use of VX-710 is limited by its unpredictable pharmacokinetic interactions with cytotoxic agents. Most of the third-generation MDR modulators have been developed based on structure–activity relationships and combinatorial chemistry, in the hope of overcoming limitations exhibited by the second-generation molecules.[100] The third-generation MDR modulators, which are currently in clinical development, are LY335979, XR9576, laniquidar (R101933), GF120918, and ONT-093.[100] Both LY335979[101] and XR9576[102] are among the most studied in this group of modulators.

3.2. Chemosensitizers of MRP1

Most MRP1 substrates, as well as inhibitors, are anionic compounds that enter cells poorly, thus making it difficult to obtain a good inhibitor for MRP1 compared with Pgp. A variety of MRP1 inhibitors have been reported.[103] For instance, general inhibitors of organic transport are probenecid, sulfinpyrazone, and indomethacin; inhibitors of MRP-related transporters are the LTD_4 analogue MK571, ONO-1078, glibenclamide,[104] and some GSH conjugates[105]; inhibitors of MRP1 and Pgp are VX-710, agosterol A, PAK-104, verapamil, cyclosporin A, genistein, and quercetin; and GSH-dependent inhibitors of MRP1 are LY 475776 and LY 402913. These compounds are mostly not specific to MRP1 and they need to be used at relatively high concentrations to overcome the MDR mediated by MRP1.[58]

3.3. Chemosensitizers of MXR

A variety of MXR inhibitors have been identified.[71,106] It has been reported recently that GF120918, a third-generation Pgp inhibitor, is also a potent inhibitor of MXR.[107] Various studies showed that GF120918 can be tolerated in humans and animals at concentrations sufficient to inhibit MXR.[106,108] The natural product fumitremorgin C (FTC) secreted from the fungi *Aspergillus fumigatus* was another potent modulator of ABCG2 that was able to completely reverse mitoxantrone resistance and topotecan resistance in ABCG2-overexpressing cells at 1–5-μM concentrations[109] Many studies showed that this compound is highly specific to ABCG2 and did not reverse Pgp- or MRP1-mediated drug resistance. Recently, several FTC analogues such as Ko132 and Ko134 have been developed.[106] These compounds could potentially be further developed as clinically useful ABCG2 inhibitors because they were more potent than FTC; the IC_{50}s are in the range of 85–270 nM. Several of the tyrosine kinase inhibitors (e.g., CI1033) have also been shown to be potent inhibitors of MXR that inhibit the MXR-mediated efflux of topotecan and SN-38 at low micromolar concentrations. Recently, HIV protease inhibitors ritonavir, saquinavir, and nelfinavir also have been found to be effective inhibitors of MXR.[110] Collectively, although a large number of MXR inhibitors has been described, whether any of these compounds are clinically useful in reversing MXR-mediated MDR has yet to be determined.

3.4. Mechanism of Action of MDR Chemosensitizers

Multidrug resistance chemosensitizers might function in two major ways: They can modify either the function or the expression of the proteins involved in MDR.

3.4.1. Modulation of the Function of MDR1/MRP

The idea of finding chemosensitizers that inhibit the function of the drug transporters and thereby reverse MDR has grown in parallel with the biochemical and clinical investigations of the molecular mechanism and regulation of these proteins. The compounds that inhibit MDR might be categorized according to their mode of action on the targeted transporter proteins. The first category involves analogues of the transported (drug) substrates that either competitively or noncompetitively inhibit drug extrusion through MDR1 or MRP. These agents interact with the transporters on their drug-binding sites with significantly higher affinity than any cytotoxic drugs and might be either efficiently transported or not transported by the pumps. In the former case (which is probably true, e.g., for verapamil and for several calmodulin inhibitors), stimulation of the pump turnover might greatly increase ATP consumption in the MDR tumor cells.[111] This might result in an advantageous collateral sensitivity of the tumor cells to the modulating agent. In the latter form (this is probably the case with PSC833), the transporter becomes locked by a substrate analogue that cannot be pumped, thus cannot be cleared from the binding sites.[112]

In the case of MRP, which transports various glutathione *S*-conjugates, agents inducing cellular GSH-depletion might be good candidates for substrate-dependent reversal of drug resistance.[113,114] Moreover, certain prostaglandins (PGA1), tyrosine kinase inhibitors (genistein), and inhibitors of uric acid transport (e.g., benzbromarone) seem to be effective substrate-analogue MRP chemosensitizers.[115,116] The second category of MDR chemosensitizers includes inhibitors of ATP binding, or ATP utilization in the drug pumps. Various non-hydrolyzable or covalently reacting ATP analogues (such as azido-ATP) or compounds like NBD chloride react with crucial lysines at or near the ATP-binding sites.[117,118] Certainly, very little specificity against MDR can be expected from such compounds, as most ATP-binding proteins, including, for example, ion pumps or protein kinases, will be affected as well. The MDR1 protein has two cysteines located in the two highly conserved ATP-binding regions, which can be modified by alkylating agents (e.g., NEM). Alkylation of these cysteine residues irreversibly blocks the function of MDR1, whereas the presence of ATP protects these sites from NEM.[117,118] Recent reports indicate that flavonoids like quercetin might inhibit drug pumps by reacting preferentially with their ATP-binding domains.[119] A dream compound of this kind would be specifically recognized by MDR1 or MRP as a toxic product to be eliminated, and then the compound would irreversibly modify ATP binding or hydrolysis in the same proteins.

The third category of MDR chemosensitizers includes specific antibodies interfering with the function of drug transporters. There are several monoclonal antibodies that react with intracellular functional domains of MDR1 or MRP, but their *in vivo* application is not considered, as they do not enter tumor cells. However, some of the antibodies thatt recognize extracellular epitopes block the conformational changes required for drug transport function and might be good candidates for medical application. The frist such anti-MDR1 monoclonal antibody, MRK16, was developed by Hamada and Tsuruo[14] and shown to inhibit ATP-dependent drug extrusion and to modulate drug resistance.

The last category of mode of action of MDR chemosensitizers would include all other possible drug pump inhibitors that cannot be easily separated by their mode of action. Oligomycin, an effective inhibitor of both MDR1 and MRP, does not seem to be a substrate analogue but might directly block ATP hydrolysis, although its action is certainly not selective.[117] Various detergents seem to inhibit MDR pumps at the site(s) of hydrophobic interactions in or near the membrane lipid bilayer[120,121] with little selectivity.

3.4.2. Modulation of the Expression of MDR1/MRP

Chemosensitizers of the drug transporter transcription might become useful inhibitors, and potential promoter regions of these proteins were identified and characterized in detail.[122,123] Most previous studies on the regulation of *MDR1* gene expression have concentrated on identifying transcription factors involved in the induction of *MDR1* gene promoter activity in drug-resistant cancer cell lines.

The human *MDR1* gene promoter contains a number of regulation sites for SP1, NF-Y, and YB-1 transcription factors.[123–125] These transcription factors have been shown to upregulate *MDR1* gene promoter activity. Recently, it was reported that *MDR1* gene promoter activity might be linked to the cyclic AMP-dependent protein kinase signal pathway, which plays a key role in activating SP1.[126] Activation of Ras and PKC has also been shown to stimulate Jun and Fos families, forming the activator protein-1 (AP-1). AP-1-responsive genes are important in DNA synthesis, DNA repair, and drug detoxification. The promoter/enhancer element of the *MDR1* gene contains the AP-1-binding-site sequence. Because the transcription efficiency of the *MDR1* gene appears to be regulated by AP1,[122] the activation of Fos and Jun might lead to increased expression of the *MDR1* gene. Fos is thought to mediate its effects through transcriptional activation, after it interacts with the Jun protein to form AP-1. Therefore, overexpression of Fos might cause the MDR phenotype by modulation of *MDR1* gene expression.

Overall, it is important to note that the MDR1 promoter is responsive to cellular stress triggered by anticancer drugs, carcinogens, heavy metals, ultraviolet light (UV), heat shock, serum stravation, phosphatase inhibitors, and phorbol esters.[127–129] These regulations occur probably in a species- and cell-specific fashion[130] thus, any effort for their clinical modulation seems to be a long shot. Rather, the expression of the drug pumps MDR1 or MRP might be efficiently modulated by chemically stabilized antisense oligonucleotides[131] or synthetic catalytic RNAs (ribozymes).[132] The most critical issues for their therapeutic use will be increased stability and effective delivery to the target cancer cells.

Inhibitors of protein processing and posttranslational modifications, in principle, might also be used to block the expression of a functional form of MDR1 and MRP drug pumps, as both proteins are posttranslationally modified by N-glycosylation and phosphorylation. However, the inhibitors of their processing are basically unaffected or is not an efficient way to modulate drug transport.[133–135]

4. CHEMOSENSITIZING ACTIVITIES OF CURCUMIN AND ITS ANALOGUES

Curcuminoids are natural phenolic coloring compounds found in the rhizomes of *Curcuma longa* Linn., commonly known as turmeric. The rhizomes contain three major pigments of curcuminoids: curcumin I (diferuloylmethane), curcumin II (demethoxycurcumin), and curcumin III (bisdemethoxycurcumin).[136,137] their chemical structures are illustrated elsewhere.[138,139] All three impart the hallmark yellow pigmentation to the *Curcuma longa* plant and particularly to its rhizome. Ongoing experimental and clinical studies indicate that turmeric and its curcuminoid components exhibit unique antioxidant,[140] anti-inflammatory,[141] and antitumorigenesis properties.[142–144] Their potential use in the prevention of cancer and in the treatment of human immunodeficiency virus (HIV) infection is also a subject of intensive research.[136]

Curcumin has been found to be safe, with no dose-limiting toxicity, when administered at doses up to 10 g/day in humans.[145] However, curcumin undergoes rapid and extensive metabolism in the liver and intestine[146] and demonstrates poor bioavailability, thereby limiting its usefulness as a potent chemopreventive agent. To date, curcumin-glucuronide, dihydrocurcumin-glucuronoside, THC-glucuronoside, and THC (tetrahydrocurcumin) have been demonstrated as the major curcumin metabolites *in vivo*.[144,147,148]

4.1. Effect on Pgp

Due to its wide range of biological and pharmacological effects, lack of toxicity, cyclicity, and lipophilicity, curcumin was examined to determine possible interactions with Pgp expression and function.[149] The commercial grade of curcumin, which contain approximately 77%, 17%, and 3% curcumin I, II, and III, respectively, was used in this study. Curcumin (1–10 μM) downregulated Pgp expression and reduced Pgp-mediated efflux in drug-resistant human cervical carcinoma cells (KB-V1). Curcumin increased rhodamine 123 accumulation in a concentration-dependent manner (1–55 μM) and inhibited the efflux of rhodamine 123 from these cells but had no effect on the wild-type drug-sensitive KB-3-1 cells, which do not express Pgp. Because the time of exposure of cells to curcumin in these experiments was short (1–2 h), it is unlikely that curcumin acted by downregulating MDR1 gene expression, resulting in a reduced level of cellular Pgp. However, the effect of curcumin on the expression of Pgp at the protein (Western blotting) and mRNA [reverse transcription–polymerase chain reaction (RT-PCR)] levels was examined. There was no difference in Pgp expression in KB-V1 cells when treated with curcumin for 1–2 h. Treatment of drug-resistant KB-V1 cells with curcumin increased their sensitivity to vinblastine, but not in wild-type KB-3-1 cells. In addition, curcumin inhibited verapamil-stimulated ATPase activity and the photoaffinity labeling of Pgp with the prazosin analogue iodoarylazidoprazosin in a concentration-dependent manner, indicating direct interaction of curcumin with Pgp and possible binding to the same site as other agents such as prazosin and verapamil. These findings suggest that curcumin might represent a new reversal agent for the chemosensitization of cancer cells.

In another study, curcumin inhibited vinblastine induced Pgp level in a dose- and time-dependent manner in the vinblastine-resistant subline KB-V0.1.[150] Another report from the same group demonstrated that three major curcuminoids modulated Pgp function using human MDR KB-V1 cells and crude membranes of Pgp-overexpressing HighFive insect cells.[138] The IC_{50} of curcumin I, II, and III is not statistically different compared to KB-V1 (expressing high levels of Pgp) and KB-3-1 cells (parental drug sensitive), suggesting that Pgp does not confer resistance to curcumin I, II, or III; in other words, these curcuminoids most likely are not transported by Pgp. Treating the cells with nontoxic doses of curcuminoids increased their sensitivity to vinblastine only in the Pgp-expressing drug-resistant cell line KB-V1, and curcumin I retained the drug in KB-V1 cells more effectively than curcumin II and III. Effects of curcumin I, II, and III on rhodamine 123,

calcein AM, and bodipy- FL vinblastine accumulation confirmed these findings. Curcumin I, II and III increased the accumulation of fluorescent substrates in a dose-dependent manner, and at 15 μM, curcumin I was the most effective. 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. The inhibitory effect in a concentration-dependent manner of curcuminoids on verapamil-stimulated ATPase activity and photoaffinity labeling of Pgp with the [^{125}I}-iodoarylazidoprazosin offered additional support that curcumin I was the most potent modulator. Thus, these biochemical results demonstrate that curcuminoids interact directly with Pgp and possibly bind to the same binding sites as other agents such as prazosin, vinblastine, and verapamil. Chemical structure of curcumin I might make it more suitable for binding to the drug-binding site of Pgp than that of curcumin II and III, because curcumin I has a balance of two hydroxyl and methoxyl groups on each side, and the presence of two methoxyl groups in the curcumin I molecule might help its inhibitory activity on the Pgp function.

In another study bisdemethoxycurcumin has been demonstrated to be the most active form of the curcuminoids present in turmeric for modulation of MDR1 gene expression in MDR KB-V1 cells by Western blot and RT-PCR analysis.[151] The nuclear protein was identified by competitive electrophoretic mobility shift assay (EMSA) using unlabeled SP1, AP1, AP2, OCT1, NF-κB, and cAMP-responsive element binding (CREB) oligomers (200 M excess). The result demonstrated that the CREB consensus sequence can compete more completely with the nuclear factor that binds to the labeled probe (MDR1 gene promoter –84 to –65 DNA fragment) than other unlabeled probes[149] This result indicates that CREB is the transcription factor that binds to the MDR1 gene promoter in residues –84 to –65, and this result was confirmed by supershift assay using an anti-CREB antibody. In additional studies, pretreatment of KB-V1 cells with curcuminoids significantly decreased the activity of the MDR1 gene promoter, and bisdemethoxycurcumin produced the maximum inhibitory effect.[149] As tetrahydrocurcumin is the ultimate metabolite of the curcumins *in vivo*, we recently extended our investigation to assess whether THC is able to retain the MDR-reversing activity (manuscript in preparation). Two types of cell line were used for Pgp study: human cervical carcinoma KB-3-1 (wild type) and KB-V-1 and human breast cancer MCF-7 (wild type) and MCF-7 MDR, respectively. The results by flow-cytometry assay indicated that THC is able to inhibit the function of Pgp and thereby significantly increase the accumulation of rhodamine and calcein AM in KB-V-1 cells. The result was confirmed by the effect of THC on [^{3}H]-vinblastine accumulation and efflux in MCF-7 and MCF-7MDR. THC significantly increased the accumulation and inhibited the efflux of [^{3}H]-vinblastine in MCF-7 MDR in a concentration-dependent manner. This effect was not found in the wild type MCF-7 cell line. The interaction of THC with the Pgp molecule was clearly indicated by ATPase assay and photoaffinity labeling of Pgp with the transport substrate. THC stimulated Pgp ATPase activity and inhibited the incorporation of [^{125}I]-iodoarylazidoprazosin (IAAP) into Pgp in a concentration-dependent manner. The MDR-reversing properties of THC on Pgp was determined by 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium

bromide (MTT) assay. THC at 25 μM significantly increased the sensitivity of vinblastine in drug-resistant KB-V-1 cells. This effect was not found in respective drug-sensitive parental cell lines. Taken together, the present study clearly showed that THC inhibits the efflux function of Pgp and it is able to extend the MDR-reversing activity of curcuminoids *in vivo*. Additional *in vivo* studies are required to determine if curcumin has potential as an effective and safe chemosensitizer for treating cancers expressing Pgp.

In another study by Romiti et al., using primary cultures of rat hepatocytes expressing high levels of Pgp after 72 h culture, curcumin (commercial grade) inhibited rhodamine 123 efflux in a dose dependent manner.[152] Western blot analysis indicated that curcumin decreased the protein levels of Pgp in cultures. In photoaffinity labeling studies, curcumin competed with azidopine for binding to Pgp, suggesting a direct interaction with glycoprotein. These results suggest that curcumin is able to modulate *in vitro* both expression and function of hepatic Pgp.

4.2. Effect on MRP-1

The inhibitory effects of a mixture of curcumin I, II, and III on MRP1-mediated transport using isolated membrane vesicles of MRP1-expressing Sf9 cells was recently reported.[153] However, the mechanism of inhibition remains unknown. Moreover, it is unknown whether each curcumin form in the curcumin mixture exhibits the same effect. In another study by Chearwae et al., curcumin mixture and three major curcuminoids purified from turmeric (curcumin I, II, and III) were tested for their ability to modulate the function of MRP1 using HEK293 cells stably transfected with MRP1-pcDNA3.1 and pcDNA3.1 vector alone.[139] The IC_{50} of curcuminoids in these cell lines ranged from 14.5 to 39.3 μM. Results indicated that curcuminoids might not be MRP1 substrate because the IC_{50} values were almost identical in both parental and MRP1-transfected cells. Upon treating the cells with etoposide, in the presence of 10 μM curcuminoids the sensitivity of etoposide was increased several-fold only in MRP1-expressing and not in pcDNA3.1-HEK 293 cells. Western blot analysis showed that the total cellular level of the MRP1 protein level was not affected by treatment with 10 μM curcuminoids for 3 days. The modulatory effect of curcuminoids on MRP1 function was confirmed by the inhibition of efflux of two fluorescent substrates: calcein-AM and fluo4-AM. Although all three curcuminoids increased the accumulation of fluorescent substrates in a concentration-dependent manner, curcumin I was the most effective inhibitor. The potency of curcumin I was comparable to MK-571, which is known to inhibit MRP1-mediated transport with high affinity. In addition, curcuminoids did not affect 8azido[α-^{32}P]ATP binding; however, they did stimulate the basal ATPase activity and inhibited the quercetin-stimulated ATP hydrolysis of MRP1, demonstrating the interaction of curcuminoids most likely at the substrate-binding site(s) on this multidrug transporter. In summary, these results demonstrate that curcuminoids effectively inhibit MRP1-mediated transport, and among curcuminoids, curcumin I, a major constituent of curcumin mixture, is the best modulator.

Recently, other workers have reported the modulation of MRP1 and MRP2 function by curcumin mixture.[153,154] In addition, the curcumin mixture appears to affect the trafficking of ΔF508 mutant of cystic fibrosis transmembrane regulator (CFTR),[155] which also belongs to the ABCC subfamily (ABCC7), similar to MRP1 (ABCC1) and MRP2 (ABCC2). The curcumin mixture has been reported to stimulate the chloride channel activity of wild-type CFTR.[156] Further extensive work of interest is whether curcumin I concentrations achieved *in vivo* are sufficient to inhibit MRP1 function and or expression, and extensive pharmacokinetics studies with curcumin I will be required to know the steady-state levels of phytochemical reached in blood and tissue after its administration at pharmacological doses. However, recent work suggested that the curcumin mixture, and all three pure forms of curcumin (I, II, and III) inhibit the function of MRP1.[139] Curcumin I was the most effective form as an inhibitor of MRP1, similar to previous results with Pgp.[138] These agents thus might have a beneficial effect on cancer chemotherapy with respect to the possibility of long-term use without concerns regarding MRP1 or MDR1 activation.

We recently extended our investigation to assess whether THC, a major metabolite of curcumin, is able to modulate MRP1 function using pcDNA 3.1 and pcDNA3.1-MRP1 transfected HEK293 cells.[157] The efflux of a fluorescent substrate calcein AM was inhibited effectively by THC; thereby, the accumulation of calcein was increased in MRP1-HEK 293 and not its parental pcDNA3.1-HEK 293 cells. The MDR-reversing properties of THC on MRP1 were determined by MTT assay. THC (20–25 μM) significantly increased the sensitivity of etoposide in MRP1-HEK 293 cells. This effect was not found in respective drug-sensitive parental cell lines. A consistent finding was reported in MDCKII cells transfected with MRP1; THC significantly increased 3[H]-EGCG in MDCKII/MRP1-overexpressing cells.[154] Taken together, these studies clearly showed that THC inhibits the efflux function of MRP1 and it is able to extend the MDR-reversing activity of curcuminoids *in vivo*.

4.3. Effect on MXR or ABCG2

As reported earlier, the curcumin mixture and purified curcuminoids (curcumin I, II, and III) could reverse the MDR in cells expressing Pgp and MRP1 by inhibiting the functions mediated by these transporters.[138,139,149] It was also shown that curcumin I, which is a major constituent (70–75%) of a curcumin mixture, was most potent among the purified curcuminoids in inhibiting the activity of both of the transporters. Purified curcuminoids were further evaluated for their modulating effects on the function of either the wild-type 482R or mutant 482T ABCG2 transporter, stably expressed in human embryonic kidney 293 cells and drug-selected MCF7FLV1000 and MCF7AdrVp3000 cells.[158]

It has been reported previously that the amino acid at position 482 has a crucial role in the substrate and inhibitor specificity of ABCG2 and that mutants R482→T/G exhibit altered drug resistance profiles and substrate specificity of MXR.[67,68] Therefore, we decided to investigate the modulating effects

of curcuminoids on ABCG2 activity in both wild-type R482 stably expressed in HEK 293 cells, and the mutant 482T overexpressed in MCF7AdrVp3000 cells. The drug-selected MCF7FLV1000 and MCF7AdrVp3000, which overexpressed the wild-type R482 and the mutant 482T ABCG2, respectively, were chosen because the protein was overexpressed in these breast cancer cell lines under its own promoter at higher levels[159] in sufficient quantity for biochemical characterization. Curcumin I, II, III and the curcumin mixture inhibited the efflux of ABCG2 substrates and the presence of nontoxic concentrations of curcuminoids (10 μM) increased (threefold to eightfold) the sensitivity of ABCG2-expressing cells to anticancer drugs, including mitoxantrone, topotecan, SN-38, and doxorubicin. This reversal was not due to reduced expression, becauseABCG2 protein levels were unaltered by treatment with 10 μM of curcuminoids for 3 days. In addition, [^{3}H]-curcumin-I transport assays demonstrate that the curcuminoids are not transported by ABCG2. Curcuminoids stimulated (2.4- to 3.3-fold) ATPase activity of ABCG2 at very low concentrations (7–18 nM) and inhibited both the photolabeling of ABCG2 with two photoaffinity analogues, [^{125}I]-IAAP and [^{3}H]-azidopine, and also the transport of these agents.

Curcuminoids interact at the drug–substrate binding sites on drug transporters with very high affinity and inhibit ABCG2-mediated drug resistance. Taken together, our previous work with Pgp and MRP1 and this study with ABCG2 suggest that curcumin I is a very effective modulator, which should be considered as a potential compound for development of reversal agents designed to overcome MDR mediated by these three major ABC drug transporters.

In another study by our group using MXR-overexpressing MCF7AdrVp3000 or MCF7FL1000 and its parental MCF-7, we assessed whether THC, a major metabolite of curcumin, is able to modulate MXR function.[157] The binding of [^{125}I]-IAAP to MXR was also inhibited by THC, suggesting that THC interacted with the drug-binding site of the transporter. THC dose-dependently inhibited the efflux of mitoxantrone and pheophorbide A from MXR-expressing cells (MCF7AdrVp3000 and MCF7FL1000). THC at 25 μM significantly increased the sensitivity of mitoxantrone in drug-resistance MCF7AdrVp3000 cells. This effect was not found in MCF-7 drug sensitive parental cell lines.

4.4. Effect on NF-κB and Inhibitor Apoptotic Proteins

Nuclear factor-κB has been implicated in both carcinogenesis and the development of drug resistance in cancer cells.[160,161] Most reports suggest that NF-κB mediates survival signals that counteract apoptosis.[156a,162] NF-κB-activated expression of genes that inhibit apoptosis, such as A20, IAPs (inhibitor apoptotic proteins), and TRAFs, is probably involved in the mediation.[163,164] Upon activation, NF-κB dissociates from the inhibitory IκBα and translocates from the cytoplasm to the nucleus, where it binds to the promoter elements and transactivates gene expression.[165] This general activation of NF-κB by anticancer drugs can be attenuated by pretreatment with common biologic modulators. Chuang et al. demonstrated that NF-κB can be activated by all four of the anticancer drugs

in three cancer cell lines (liver, uterine cervix, and urinary bladder) examined.[166] Each of the four anticancer drugs used (doxorubicin, 5-FU, cisplatin, and paclitaxel) possesses distinct modes of action that cause different types of damage to cancer cells. However, universal NF-κB activation was observed. These results suggest the existence of a common set of cellular elements that sense the challenge by these drugs as a type of stress and transmits this signal to NF-κB. When cells were pretreated with common biologic modulators such as tamoxifen, dexamethasone, and curcumin, the doxorubicin-induced NF-κB activation was attenuated significantly. This inhibition might play a role in sensitizing cancer cells to chemotherapeutic drugs.

In another study using human hepatic cancer cells and the combination of curcumin with cisplatin or doxorubicin, the levels of NF-κB were lower than those predicted from the effects of the single agents.[6] Except for Bcl-2, the human hepatic cancer cells expressed different other genes, including the IAPs, implicated in cell proliferation and survival. Curcumin determined early changes in cyclooxygenase (COX)-2 and c-myc mRNAs, which were downregulated, and in livin mRNA, which was upregulated. Later it decreased Bcl-X_L mRNA and increased Bcl-X_s and c-IAP-2 mRNAs. Cisplatin and doxorubicin exerted distinct effects on gene expression. The cytotoxic interactions between curcumin and these agents were accompanied by synergistic or additive effects of decrease in the expression of different genes, including c-myc, Bcl-X_L, c-IAP-2, NAIP, and XIAP. The expression of XIAP and other IAPs can be upregulated by NF-κB.[167,168] Thus, the inhibition of NF-κB by curcumin might be of help to antagonize the IAPs as well as other NF-κB target genes (e.g., COX-2, Bcl-X_L and c-myc) involved in the adverse biology of cancer. Singh and Aggarwal showed that curcumin could suppress NF-κB activation induced by TNF, phorbol ester, and H_2O_2 through suppression of IκBα degradation.[169] Recently, Aggarwal et al. demonstrated that curcumin inhibits the tumor necrosis factor (TNF)-induced IκBα kinase complex and Akt activation, which blocks phosphorylation of IκBα and p65, leading to suppression of events required for NF-κB gene expression.[170]

In human cervical carcinoma cells, curcumin sensitizes tumor cells more efficiently to the therapeutic effect of paclitaxel.[171] Paclitaxel is the best anticancer agent that has ever been isolated from plants, but its major disadvantage is its dose-limiting toxicity. Furthermore, tumors tend to acquire resistance to cytotoxic chemotherapeutic agents, including paclitaxel. A combination of 5 nM paclitaxel with 5 μM curcumin augments anticancer effects more efficiently than paclitaxel alone, as evidenced by increased cytotoxicity and reduced DNA synthesis in HeLa cells. This synergistic effect was not observed in normal cervical cells in which paclitaxel downregulates NF-κB. Evaluation of signaling pathways common to paclitaxel and curcumin reveals that this synergism was in part related to downregulation of NF-κB and serine/threonine kinase Akt pathways by curcumin. An electrophoretic mobility shift assay revealed that activation of NF-κB induced by paclitaxel is downregulated by curcumin. Curcumin-downregulated paclitaxel induced phosphorylation of the serine/threonine kinase Akt, a survival signal regulated by NF-κB. Tubulin polymerization and cyclin-dependent kinase

Cdc2 activation induced by paclitaxel was not affected by curcumin. These results lead to the conclusion that the synergistic effect of Taxol and curcumin in inducing apoptosis in cervical cancer cells follows a pathway that is independent of tubulin polymerization and cell cycle arrest, at least at lower concentrations of curcumin.

5. CONCLUSIONS AND FUTURE PERSPECTIVES

Many studies have been performed with the aim of developing effective resistance modulators to overcome the MDR of human cancers. Potent MDR modulators are being investigated in clinical trials. Many current studies are focused on herbal constituents because these have been used for centuries without producing any harmful side effects. Curcuminoids (curcumin, demethoxycurcumin, and bisdemethoxycurcumin) purified from turmeric are able to modulate the efflux function of Pgp, MRP1 and mitoxantrone resistance protein (MXR), and curcumin I, a major constituent of curcumin mixture, was the most effective. Tetrahydrocurcumin, a major metabolite form of curcuminoids in humans, inhibits the efflux function of these three major ABC drug transporters and it is able to extend the MDR-reversing activity of curcuminoids *in vivo*.

Curcuminoids were reported to modulate Pgp, MRP1, and MXR by interacting directly with drug- or substrate-binding site(s). This might involve competitive binding to the substrate-binding site or binding to other drug-binding sites and altering molecular conformation, as indicated by the altered photoaffinity labeling. Curcuminoids did not affect the ATP-binding site; however, they did stimulate the basal ATPase activity and inhibited verapamil-stimulated ATP hydrolysis of Pgp or quercetin hydrolysis of MRP1. Curcuminoids also stimulated ATPase activity of MXR or ABCG2 at very low concentrations (7–18 nM). Curcumin is the most potent inhibitor for all three drug transporters, as the chemical structure of curcumin I might make it more suitable for binding to the drug-binding site of Pgp than that of curcumin II and III, because curcumin I has a balance of two hydroxyl and methoxyl groups on each side, and the presence of two methoxyl groups in the curcumin I molecule might help its inhibitory activity on the Pgp function.

Bisdemethoxycurcumin, or curcumin III, can upregulate MDR1 gene expression. Western blot and RT-PCR analysis indicated that bisdemethoxycurcumin decreased the protein and mRNA levels of Pgp in cultures. The EMSA demonstrated that CREB is the transcription factor that binds to the MDR1 gene promoter in residues –84 to –65. However, curcuminoids do not change protein and RNA levels of MRP1 and MXR drug transporters. The mechanism for herbal modulation of the *MDR1* gene is largely undetermined.

The inhibition of Pgp, MRP1, and MXR by curcumin might provide a novel approach for reversing MDR in tumor cells. Additional *in vivo* studies are required to determine if curcumin has potential as an effective and safe chemosensitizer for treating cancers expressing Pgp. Phase II and III clinical trials of many known MDR modulators might soon yield informative results that should help to decide whether the chemosensitizer works in clinical oncology. In addition, many ABC

transporters have not yet been identified and characterized. As more information on these proteins becomes available, we might be able to more effectively design drug combinations that will provide increased selectivity of action at the desired tissue site.

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