

Chapter 2

Metastasis and Drug Resistance

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Abstract Multidrug resistance (MDR) phenotype emerging from chemotherapy is a major problem in managing patients with metastatic cancers. The discovery that a cardiovascular drug, verapamil, can bind to P-glycoprotein and reverse MDR initiated serious research efforts in MDR-reversal by various compounds and modes of pharmacological modifiers. Those include major calcium channel blockers such as bepridil, diltiazem, felodipine, isradipine, nicardipine, nifedipine and nimodipine, verapamil and analogs; calmodulin antagonists; antibiotics and analogs; indole alkaloids; cyclosporins and analogs; hormones and antihormones; pharmaceutical emulsifying surfactants; liposomal encapsulation; etc. The majority of the studies targeted one of the MDR mechanisms, P-glycoprotein. These studies have been successful under in vitro and limited in vivo animal conditions; the correlations for clinical trails are still lacking. Therefore, an effective MDR-reversing chemotherapy is not available. It is the purpose of this chapter to review the past and current experimental reversal of MDR and, in particular, the importance in targeting drug resistance in relevant cancer metastasis models.

Keywords Metastasis · MDR · Apoptosis · Animal models

Introduction

Despite improvements in diagnosis, surgical techniques, patient care, and adjuvant therapies, most deaths from cancer are due to metastases that are resistant to conventional therapies (Fidler 1990). The major obstacle to effective treatment is tumor cell biologic heterogeneity. Moreover, the metastases can be located in different organs, and the specific organ environment can influence the biologic behavior of metastatic

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cells, including their response to systemic therapy. Only a better understanding of the molecular mechanisms that regulate the process of metastasis and the interactions between the metastatic cells with the organ microenvironment can provide a foundation for the design of more effective therapy.

The Pathogenesis of Metastasis

The process of metastasis is highly selective and consists of a series of sequential, interrelated steps. To produce clinically relevant lesions, metastatic cells must complete all steps of this process. After the initial transformation and growth of cells, vascularization must occur if a tumor mass is to exceed 1 mm in diameter. The synthesis and secretion of several proangiogenic factors by tumor and host cells and the absence of antiangiogenic factors play a key role in establishing a capillary network from the surrounding host tissues. Next, local invasion of the host stroma occurs as a consequence of the enhanced expression of a series of enzymes (e.g., collagenase). Once tumor cells penetrate lymphatic or vascular channels, they may grow at the invasion site or detach and be transported within the circulatory system. The tumor emboli must survive immune and nonimmune defenses and the turbulence of the circulation, then arrest in the capillary bed of receptive organs, extravasate into the organ parenchyma, proliferate, and establish a micrometastasis. Growth of these microscopic lesions requires development of a vascular supply and evasion of host defense cells. When the metastases grow, they can shed tumor cells into the circulation to produce metastasis of metastases (Fidler 1990).

The outcome of the metastatic process depends on multiple and complex interactions of metastatic cells with host homeostatic mechanisms (Fidler 1997). More than a century ago, Stephen Paget researched the mechanisms that regulate organ-specific metastasis, i.e., pattern of metastasis by different cancers, and questioned whether the organ distribution of metastases produced by different human neoplasms was due to chance and analyzed more than 700 autopsy records of women with breast cancer. His research documented a nonrandom pattern of visceral (and bone) metastasis. This finding suggested to Paget that the process was not due to chance but, rather, that certain tumor cells (the “seed”) had a specific affinity for the milieu of certain organs (the “soil”). Metastases resulted only when the seed and soil were compatible (Paget 1889).

A current definition of the “seed and soil” hypothesis consists of three principles. First, neoplasms are biologically heterogeneous and contain subpopulations of cells with different angiogenic, invasive, and metastatic properties (Fidler 2003; Langley and Fidler 2007). Second, the process of metastasis is selective for cells that succeed in invasion, embolization, survival in the circulation, arrest in a distant capillary bed, and extravasation into and multiplication within the organ parenchyma. Although some of the steps in this process contain stochastic elements, as a whole, metastasis favors the survival and growth of a few subpopulations of cells that pre-exist within the parent neoplasm (Fidler and Kripke 1977; Talmadge et al. 1982).

Thus, metastases can have a clonal origin, and different metastases can originate from the proliferation of different single cells (Fidler and Talmadge 1986; Hu et al. 1987; Talmadge et al. 1982).

Third, and perhaps the most important principle for the design of new cancer therapies, is that the outcome of metastasis depends on multiple interactions (“cross-talk”) of metastatic cells with homeostatic mechanisms, which the tumor cells can usurp (Fidler 1995). Therapy of metastasis, therefore, can be targeted not only against tumor cells but also against the homeostatic factors that promote tumor cell growth, survival, angiogenesis, invasion, and metastasis.

Multidrug Resistance

One of the most depressing and predictable facts of cancer management is the development of the multidrug resistance (MDR) phenotype in patients treated chronically with certain natural chemotherapeutic drugs. This clinical phenomenon accounts for the unsatisfactory low incidence of response rate for the majority of solid tumors to chemotherapy – one of the few conventional treatments for metastatic diseases in past few decades.

Since the heterogeneous nature of tumor and cancer metastasis was conceptualized (Paget 1889; Fidler 1973; Fidler and Kripke 1977; Fidler 1978; Poste and Fidler 1979; Fidler and Poste 1985; Fidler 2001), it is clear that MDR is a resultant clinical outcome manifested by successful cancer cells endowed with multiple mechanisms for survival. Like other vital traits of metastatic cancer cells, MDR should be conceived as a phenotype marked by a collection of independent or collateral modifications, overexpressions, and/or amplifications of endogenous molecules that interplay with distinct normal cellular pathways (Fig. 2.1) that include

MDR

P-glycoprotein
PKC Expression
ABC Transporters
Tubulin Mutation
Episomes Amplification
Topoisomerase II Mutation
Altered Cellular Calcium Levels
Enhanced Sodium Pump Activity
Altered Reduction-Oxidation Pathways
Formation of Double Minute Chromosomes
Overexpression of Cytoplasmic 22 kDa Sorcin

Fig. 2.1 MDR-associated mechanisms

P-glycoprotein (Juliano and Ling 1976; Kartner et al. 1983), protein kinase-C (PKC) overexpression (Fan et al. 1992a, b; Aftab et al. 1994), ABC transporters (Adachi et al. 2007), tubulin mutation (Inaba et al. 1987), episomes amplification (Ruiz et al. 1989), formation of double minute chromosomes (Von Hoff et al. 1990), altered cellular calcium levels (Nair et al. 1986), topoisomerase II mutation and altered reduction–oxidation (Deffie et al. 1988), and the overexpression of cytoplasmic 22-kDa sorcin (Hamada et al. 1988). Those cancer cells employing single and especially unique oncogenic mechanism, presumably exist, would likely be eliminated by the host defense mechanisms during the progression of cancer or by conventional cancer therapy and would be without further clinical manifestation. Therefore, initial treatment with chemotherapeutic drugs and subsequent reversal of MDR should be combined as a standard protocol for effective chemotherapy at the onset of cancer management, rather than resolving to salvage therapies – when the patients return with compromised performance status and growth of refractory tumors.

Unfortunately, an effective MDR-reversing chemotherapy is not available. Since the inception of an organized drug-screening program (DeVita et al. 1979), research efforts have been largely compound-oriented (sensitive drug-screens) (Frei 1982; Venditti 1981) rather than disease-oriented (tumor panels) (Alley et al. 1988), metastasis-oriented (orthotopic animal models) (Wilmanns et al. 1992; Singh et al. 1994; Killion et al. 1999; Langley and Fidler 2007) or MDR-oriented (relevant resistant drug-screens) (Mickisch et al. 1991a, b, Dong et al. 1994). In the following sections, we review the past and current experimental reversal of MDR and, in particular, the importance in targeting drug resistance in cancer metastasis.

Reversal of Experimental MDR

The majority of the MDR-reversing studies were in vitro assays that cannot address the complexity of physiology and pathology in cancer patients, and in particular metastasis of the cancer. It is physiology and pathology that modulate the progression of cancer and metastasis and the pharmacokinetics (what the host and cells do to the drugs) and pharmacodynamics (what the drugs do to the host and cells) (Ford and Hait 1990). Decisively, the validity of an in vitro assay is governed by its ability to derive an acceptable level of sensitivity (the prediction of true positives) and specificity (the prediction of true negatives) (Fan et al. 1985).

Calcium Channel Blockers

Verapamil and Other Clinically Approved Agents

As the elements of time and costs stacking up against the development of new anticancer drugs, the initial observation of Tsuruo et al. (1981) of a reversal by verapamil (a coronary vasodilator) on an MDR phenotype in P388 leukemia cells (Tsuruo et al. 1981, 1982) inscribed the beginning of an explosive search for

MDR-reversing anticancer therapeutics. Several major calcium channel blockers approved for clinical use in the United States were candidates for such a search: bepridil (a pyrrolidylamine), diltiazem (a benzothiazepine), felodipine, isradipine, nifedipine, nicardipine, nifedipine and nimodipine (dihydropyridines), and verapamil (a benzeneacetonitrile). Although one of the major biological effects of verapamil is the blockage of the slow-channel-mediated calcium entry into cardiac cells (Kohlhardt et al. 1972) and drug-resistant cancer cells (Bucana et al. 1990), its MDR-reversing mechanism is not clearly understood and may be quite apart from its physiologic action, in which a trial depolarization plays an important role and is related to the fast-channel effect for sodium influx (Rougier et al. 1969). Verapamil-mediated enhancement of intracellular accumulation of MDR-linked anticancer drugs is universally observed and attributed to an effect on P-glycoprotein-mediated efflux in a variety of cancer cell lines (Tsuruo et al. 1982; Inaba et al. 1979; Harker et al. 1986). Extensive efforts were made to identifying and translating the unique MDR-reversing properties of verapamil and other calcium channel blockers into clinical terms (Slater et al. 1982; Tsuruo et al. 1983a, b; Fojo et al. 1985; Fine et al. 1987; Ford et al. 1990; Fan et al. 1994a, b). Unfortunately, the adverse hemodynamic effects of verapamil have limited its clinical potential for routine use in adjunct chemotherapy (Ozols et al. 1987). In addition to verapamil, many clinically approved calcium channel blocker were shown to affect intracellular accumulation of MDR-linked anticancer drugs and to reverse MDR phenotype in vitro (Tsuruo et al. 1983a, b; Schuurhuis et al. 1987; Holtt et al. 1992; Fan et al. 1994a, b). However, with the exception of the bepridil studies, most preclinical studies employed concentrations of calcium channel blockers much higher (to achieve experimental MDR-reversing activity) than the peak plasma levels derived from patients whose performance status was less compromised than those of cancer patients entering clinical trials with advanced disease. Therefore, it was not surprising that difficulties were encountered in clinical trials using verapamil (Ozols et al. 1987) and with other calcium channel blockers for reversing drug resistance to standard chemotherapeutics in advanced cancer patients. At a micromolar dose range of verapamil, the cytotoxic effects to normal cells are remarkably severe (Lampidis et al. 1986). Furthermore, the high-dose requirement for reversal of MDR in vitro suggested additional effects (mechanisms of action) other than a simple physiologic blockage of the calcium channels (Huet and Robert 1988). Several groups have shown that verapamil can bind to P-glycoprotein and compete for binding sites for MDR-related agents to P-glycoprotein (Cornwell et al. 1987; Safa et al. 1987; Akiyama et al. 1988; Beck et al. 1988). It was shown that in the process of reversing an MDR phenotype, verapamil also stimulated marked ultrastructural changes (an MDR-associated twofold increase in the number of intramembrane particles) of drug-resistant P388 cells (Garcia-Segura et al. 1992). Moreover, under certain experimental conditions, treatments of the drug-resistant human colon LS 180 with verapamil, nifedipine, nicardipine, or diltiazem could increase *mdr-1* mRNA expression and induce cell differentiation (Herzog et al. 1993).

If one examines the bulk of in vitro literature and the clinical pharmacokinetic information, one finds in general a lack of consideration for controlled

pharmacokinetic parameters (e.g., plasma elimination half-life of chemosensitizers and of standard anticancer drugs) to simulate relevant pharmacodynamic effects in vitro. As nonphysiologic as in vitro assays are, chemical–cell interactions do follow the law of concentration and time; this kind of pharmacologic consideration may help in reducing the frequency and costs in deriving false-positive experimental new drugs and MDR-reversing agents. Therefore, while it may be feasible to seek enhancement for the efficacy of standard anticancer drugs by verapamil and other calcium channel blockers, the selectivity, scheduling, and dose intensity of the chemosensitizers must be taken into consideration inasmuch as these parameters may influence MDR, tumor spread, and clinical outcome of therapy.

Verapamil Derivatives and Other Experimental Calcium Channel Blockers

The disappointment of the initial clinical trials with verapamil (Ozols et al. 1987) stimulated an intense effort to develop chemosensitizers that were less cytotoxic to normal cells: one of the critical parameters in systemic cancer therapeutics (Lampidis et al. 1986; Fan et al. 1988). A number of structural analogs to verapamil (e.g., devapamil, emopamil, gallopamil, D528, D595, D792) have been implicated in the reversal of MDR in vitro (Pirker et al. 1990) with marginal toxicity in animal models (Nawrath and Raschack 1987; Pirker et al. 1989). The less calcium antagonistic and less cardiotoxic R-enantiomer of verapamil (versus that of clinically approved racemic verapamil) could reverse an MDR phenotype in vitro (Mickisch et al. 1990a, b). R-enantiomer of verapamil decreased the expression of P-glycoprotein, resistance to tamoxifen, and experimental pulmonary metastases of the R3230AC rat mammary adenocarcinoma in vivo (Kellen et al. 1991). Therefore, the potential for clinical enhancement of standard chemotherapeutic drugs mediated by verapamil and its derivatives remains on the horizon (Chatterjee et al. 1990; Mickisch et al. 1991a; Holtt et al. 1992; Kroemer et al. 1992; Teodori et al. 2005; Shen et al. 2008).

Calmodulin Antagonists

Calmodulin is an intracellular calcium-binding protein that plays critical roles in a wide range of cellular activities (Ramakrishnan et al. 1989). Although the lack of down-regulation of calmodulin was found to produce higher levels of this protein in transformed cells (Jaffrézou and Laurent 1993), such difference was not found between the drug-sensitive and MDR P388 leukemia cells (Nair et al. 1986). However, its calcium-sequestering regulatory roles prompted investigation on the MDR-reversing effects of the potent calmodulin antagonist trifluoperazine (Tsuruo et al. 1982, 1983b; Klohs et al. 1986), and many antipsychotic phenothiazines marketed in the United States for clinical use and investigational compounds were found to reverse experimental MDR (Ganapathi et al. 1984; Ford et al. 1989; Ford et al. 1990; Fan et al. 1994b; Zhu et al. 2005).

Antibiotics and Analogs

New drug development is time consuming and costly, hindering the availability of effective anticancer drug for the treatment of metastatic cancer. Similar to the circumvention of clinical side effects of anticancer drugs (Tsuruo et al. 1983a, b), one approach to overcome drug resistance of cancer cells would be the development of derivatives amongst clinically proven chemotherapeutic compounds. Several antibiotics such as the third-generation broad-spectrum cephalosporins (cefoperazone and ceftriaxone) (Gosland et al. 1989), protein synthesis inhibitor antibacterial erythromycin (Hofsli and Nissen-Meyer 1989), veterinary antimicrobial monensin (Ling et al. 1995), a variety of vinca alkaloid derivatives (Ruiz et al. 1989; Nasioulas et al. 1990) were found to reverse experimental MDR phenotypes. Of particular interest is the MDR-reversing effect of an anticancer benzyloquinoline plant alkaloid thaliblastine that binds to P-glycoprotein and reverses doxorubicin resistance of the P388 MDR cells (Chen et al. 1993). Its low toxicity (Todorov 1988) and structural similarity to other compounds that have a photoaffinity for P-glycoprotein (Beck and Qian 1992) make it a potential chemosensitizer with a promising prospect (Pajeva et al. 2004).

Indole Alkaloids

Mdr-like genes exist across the entire phylogenetic spectrum. The reversal of MDR phenotypes in mammalian cells by calcium channel antagonists has functional analogies with the effects of agents circumventing chloroquine resistance in parasite protozoa (Bitonti et al. 1988), in which the drug-resistant phenotype was conferred by a protein coded by a gene closely related to mammalian *mdr1* (Wilson et al. 1989). The indole-containing antimalarial quinine and structurally related compounds such as its anti-arrhythmic stereoisomer sodium channel blocker quinidine have been found to produce an MDR-reversing activity (Tsuruo et al. 1984; Lehnert et al. 1991; Sato et al. 1991). Many of those compounds are neurohumoral antagonists that include reserpine (antihypertensive and antipsychotic) and yohimbine (α -adrenergic blocker chemically similar to reserpine) (Fan et al. 1994a). Although it is clear that the functionality of the human *mdr1* gene product is distinct from the malarial counterpart (Ginsburg and Krugliak 1992), the reversal of drug resistance in both systems by similar compounds implies the possibility of similar responses for action (Vezmar and Georges 2000).

Cyclosporins and Analogs

Cyclosporin A, the complex hydrophobic fungal cyclic undecapeptide, is commonly used as an immunosuppressant for organ transplantation. At concentrations achievable clinically, it is considered one of the most effective MDR-reversing agents.

The original reports of Slater et al. (1986a, b) initiated extensive studies on MDR-reversal, mediated by cyclosporins and related compounds (Twentyman et al. 1987; Chao et al. 1990; Dorr and Liddil 1991; Spoelstra et al. 1991; Loor et al. 1992; Arceci et al. 1992). Although cyclosporins have high affinity for P-glycoprotein (Goldberg et al. 1988; Foxwell et al. 1989), the reversal effects of cyclosporins did not correlate consistently with either drug accumulation (Slater et al. 1986a, b; Chambers et al. 1989; Hait et al. 1989) or direct interaction with P-glycoprotein (Hait et al. 1989). Nevertheless, cyclosporins and related compounds continue to produce reversal of experimental MDR phenotypes (Shen et al. 2008). Its derivative SDZ PSC-833 (Boesch et al. 1991; Ludwig et al. 2006; Shen et al. 2008) and the semi-synthetic cyclic peptolide derivative SDZ 280–446 (Loor et al. 1992; Lehne et al. 2000) showed MDR-reversing effects superior even to those of cyclosporin A, which was about one order of magnitude more active than other known chemosensitizers such as verapamil.

Hormones and Antihormones

The induction, in pregnant murine uterus, of high levels of *mdr1* mRNA, mediated by estrogen and progesterone (Arceci et al. 1988), and the cross-resistance of MDR breast carcinoma cells to antiestrogens with concomitant loss of estrogen receptors (Vickers et al. 1988) and progesterone receptors (Kacinski et al. 1989) initiated extensive studies on the role of steroid hormones in MDR-reversal (Berman et al. 1991; Hu et al. 1991; Fleming et al. 1992; Stuart et al. 1992; Mutoh et al. 2006). Although the effects of antiestrogens tamoxifen, toremifene, and 4-hydroxy tamoxifen may be influenced by serum protein binding (Wurz et al. 1993; Chatterjee and Harris 1990), the reversal effects by the most active hormone progesterone have been shown to interact directly with P-glycoprotein (Yang et al. 1989; Naito et al. 1989; Safa et al. 1990). Subsequently, it was demonstrated that progesterone distinguishes two *mdr* gene products (Yang et al. 1990) and specifically regulates the activity of the *mdr1b* promoter via the A form of the progesterone receptor (Piekarz et al. 1993). However, results from clinical trials with vinblastine and high-dose megestrol acetate were unremarkable (Matin et al. 2002).

Pharmaceutical Emulsifying Surfactants

Woodcock et al. (1990) found that Cremophor EL, a relatively inert formula of polyethoxylated castor oil commonly used as pharmaceutical emulsifier (e.g., for preparations of water-insoluble compounds such as cyclosporins and taxol), could reverse experimental MDR at attainable clinical concentrations. This reversal effect has been since confirmed by using various MDR cells and pharmaceutical surfactants such as Solutol HS15 (Coon et al. 1991), Triton X-100, and Thesit (Friche et al. 1990; Spoelstra et al. 1991; Woodcock et al. 1992). There was also evidence

that nontoxic amounts of Cremophor EL and Tween 80 could effectively compete for P-glycoprotein binding with photoaffinity azidopine (Friche et al. 1990). The reformulation of conventional anticancer agents to include sufficient but nontoxic concentrations of these surfactants may enhance their clinical efficacy and overcome MDR.

Liposomal Encapsulation

A major limitation to the use of anticancer drugs is their nonspecific clinical toxicities that impair the therapeutic efficacy of these agents. Liposomes are biodegradable, nonimmunogenic, and relatively nontoxic, and they can be safely used to modify pharmacokinetic properties such as distribution, circulatory transit time, and drug metabolism, to target drugs and biologicals to most of the major organs in animals and humans (Lopez-Berestein et al. 1984; Fogler et al. 1985), to avert systemic clinical toxicities (Forssen and Tokes 1981), and to improve therapeutic efficacy of antimicrobials (Lopez-Berestein et al. 1985), anticancer drugs (Huang et al. 1992; Ahmad et al. 1993), immunomodulators (Fidler 1988), and growth factors (Schackert et al. 1989; Fan et al. 1989). Other studies have shown that liposomes composed of various phospholipids can enhance the cytotoxicities of MDR-linked drugs such as doxorubicin, vinblastine, vincristine, and annamycin (Fan et al. 1990; Seid et al. 1991; Rahman et al. 1992; Thierry et al. 1993). Although the mode of action for MDR-reversal by liposomes is not clearly understood, the experimental reversal of drug resistance by liposomes containing specific phospholipids has been attributed to perturbation of the plasma membranes (Fan et al. 1990), to increasing drug incorporation and intracellular redistribution (Thierry et al. 1993), or to direct interaction with P-glycoprotein (Thierry et al. 1993). The practical utility of liposome encapsulation in cancer treatment is obvious, but its mechanism remains to be defined (Zalipsky et al. 2007).

Other Molecules

The studies using calcium channel blockers and calmodulin antagonists to overcome MDR phenotypes continued. Dexniguldipine (B-859-35), the (–) isomer of antihypertensive niguldipine, was found better than verapamil in reversing MDR (Hofmann et al. 1991; Reymann et al. 1993; Dietel et al. 1996; He and Liu 2002). Various less toxic derivatives of verapamil (e.g., Ro11-2933) (Abderrabi et al. 1996), dihydropyridine (e.g., S16324, S16317) (Saponara et al. 2007), benzothiazepines (MDL 201,307) (Newman et al. 1996), and isoquinolinesulfonamides (e.g., W-77, CKA-1083) (Maeda et al. 1993), have been found potential agents for overcoming MDR. In the past years, many innovative molecules have also been investigated for their ability to circumvent MDR. Those findings included the studies of employing MRK16 anti-P-glycoprotein antibody to reverse bone marrow drug resistance

of MDR transgenic mice (Mickisch et al. 1992a, b). Another anti-P-glycoprotein antibody, UIC2, was also effective in reversing experimental MDR (Mechetner and Roninson 1992). Other important compounds capable of reversing MDR include those of the adenylyl cyclase inhibitor forskolin (Wadler and Wiernik 1988; Morris et al. 1991; Yin et al. 2000), potassium-sparing diuretic amilorides (Epand et al. 1991; Miraglia et al. 2005), α - or β -adrenoceptor antagonists amiodarone (Lehnert et al. 1996) and SKB 105854 (Fan et al. 1994b), antidepressant trazodone (Fan et al. 1994a), antipsychotic benzquinamide (Mazzanti et al. 1992), triazine S 9788 (Dhainaut et al. 1992; Moins et al. 2000), and the hydrophobic platelet anticoagulant dipyridamole (Verstuyft et al. 2003) and its derivative BIBW22BS (Schröder et al. 1996). The antihistaminic terfenadine (Seldane) restored the sensitivity of MDR cells to doxorubicin (Hait et al. 1993). FB642 is a systemic benzimidazole fungicide with antitumor activity against a broad spectrum of tumors and drug-resistant and MDR cell lines (Hammond et al. 2001). Furthermore, it was found that introduction of an MDR1-targeted small interfering RNA duplex into drug-resistant cancer cells markedly inhibited the expression of MDR1 mRNA and P-gp and restored sensitivity to multidrug-resistant cancer cells (Wu et al. 2003).

Clinical Reversal of MDR

Extensive clinical trials have been conducted in the past decade. As the first of such reversing agents entering clinical trials, calcium channel blocker verapamil was met with mixed outcomes that were discouraging in some studies (no response) (Rougier et al. 1969; Benson et al. 1985; Saltz et al. 1994) but were exceptionally promising in others (>50–70% response rate) (Cairo et al. 1989; Holmes et al. 1989; Figueredo et al. 1990; Miller et al. 1991; Salmon et al. 1991). Lymphoma was consistently more responsive to the chemosensitizing effects of verapamil (Holmes et al. 1989; Miller et al. 1991; Chabner et al. 1994). Although the number of patients was small, the combination trial of chloroquine with conventional chemotherapy and radiotherapy was clinically effective in improving mid-term survival for glioblastoma multiforme (Sotelo et al. 2006). Unfortunately, the number of clinical studies of phenothiazines such as the calmodulin-inhibitor trifluoperazine (Miller et al. 1988; Murren et al. 1996) and antiemetic prochlorperazine (Sridhar et al. 1993; Raschko et al. 2000) was small, and the outcome was marginal. Likewise, trials of doxorubicin derivative 4'-iodo-4'-deoxydoxorubicin (Sessa et al. 1992), antiestrogen tamoxifen (Stuart et al. 1992; Trump et al. 1992; Millward et al. 1992), and calcium channel blocker nifedipine (Philip et al. 1992) were not highly remarkable. However, isolated trials of the benzothiazepine calcium channel blocker diltiazem and antimalarial quinine showed enhancement of leukemia responses to cytarabine, mitoxantrone, and vincristine (Bessho et al. 1985; Solary et al. 1992). Although cyclosporin A may not be effective in enhancing the efficacy of epidoxorubicin in colon cancer patients (Verweij et al. 1992), a Southwest Oncology Group study showed responses in poor-risk acute myeloid leukemia patients

receiving sequential treatments with cytarabine and daunomycin with concurrent infusion of cyclosporin A (List et al. 1992). Although clinical correlations are wanting (Holzmayer et al. 1992; Hait and Yang 2005), the use of biochemical modulation of clinical MDR remains a viable approach to improve treatments of cancer with conventional chemotherapy (Fojo and Bates 2003).

Overview of Experimental MDR-Reversal

Many compounds, whose primary mechanism of action is blocking calcium channels, have been found to have MDR-reversing activities. It is not clear why chemicals that affect the flux of calcium in cells can elevate the accumulation of MDR-associated natural chemotherapeutic drugs. Many MDR-reversing agents are cationic amphiphiles (contains both hydrophobic and polar regions) that are highly lysosomotropic (Jaffrézou et al. 1991; Akiyama et al. 1984; Ramakrishnan et al. 1989) and could modulate intracellular turnover and trafficking of specific phospholipids that may affect the MDR phenotype (Jaffrézou et al. 1992; Jaffrézou and Laurent 1993). These implications may be unorthodox because of the seeming departure from the functionality of P-glycoprotein. It must be noted that proteins are rigid in nature and their functionality (mechanisms of action) and efficiency (kinetics) are often conformationally regulated by phospholipid matrix. This was shown by restoration of calcium accumulation across lipid bilayers by reconstitutive sarcoplasmic reticulum phospholipid-mediated, calcium- and magnesium-activated ATPase activity (Racker 1972) and by alterations in lipid fluidity that modulates P-glycoprotein-mediated drug transport in rat liver canalicular membrane vesicles (Sinicrope et al. 1992). Amplification of *mdr1* and overexpression of its messenger underline that typical MDR phenotypes are probably by way of induction via plasma membrane with specific chemotherapeutic drugs.

Unlike proteins, phospholipid compositions are remarkably different in transformed and tumor cells (Bergelson et al. 1970; Hatten et al. 1977), and the lipolytic activity of neoplasms is accentuated (Elwood and Morris 1968; Fran-son Patriaria and Elsbach 1974). The negative headgroup of the commonly internal phosphatidylserine (externalized in undifferentiated and neoplastic cells) has high affinity for calcium via coordination-chelation bonds (Paphadjopoulous 1968) that could initiate a localized drop in pH and a lateral phase separation of phosphatidylserine in the plasma membrane (Ohnishi and Ito 1973; Träuble and Eibl 1974). Acidic pH can produce a reversible nonbilayer inverted micelle type in membranes containing phosphatidylserine (Hope and Cullis 1980). In addition to the regulatory effects of calcium and pH, phosphatidylserine was found deacylated by a unique membrane-associated phospholipase-A in SV40-transformed 3T3 fibroblasts and in human gastric carcinoma cells (Fan and Voelz 1977, 1980, 1984) to generate short-lived but fusogenic lysophosphatidylserine (Stein Y and Stein O 1966; Ahkong et al. 1973). Therefore, the unique properties of plasma membrane lipids such as phosphatidylserine allow them to participate in biological phenomena by

means of a transient rearrangement of the membrane structure similar to that seen in MDR P388 leukemic cells (Garcia-Segura et al. 1992), and it regulates the efficiency (conformational changes and kinetic enhancements) of the efflux pump. It has been demonstrated that MDR phenotype of the human KB-V1 cell line (Ambudkar et al. 1992) and expression of the human *mdr1* in Sf9 insect cells can generate a high-capacity drug-stimulated membrane ATPase (Sarkadi et al. 1992), and alterations in lipid fluidity can modulate P-glycoprotein-mediated drug transport in rat liver canalicular membrane vesicles (Sinicrope et al. 1992) and partially in the purified MDR CHRC5 Chinese hamster ovary cell plasma membrane P-glycoprotein (Doige et al. 1993). Therefore, the ATP-dependent calcium channel P-glycoprotein in cardiac and tumor cells could be retarded by blocking agents via a “nonspecific” perturbation (evidenced by the manifold variety of effectors) of the plasma membrane phospholipids to conformationally hinder ATPase activity (energy supply) and consequentially the functions of calcium channel and P-glycoprotein.

Metastasis and Drug Resistance

The various modes of MDR-reversal put into effect that cancer is biologically heterogeneous and metastatic cells are the champions of survival. The process of tumor metastasis is highly selective and consists of a series of sequential and unified steps (Fidler 1990). Despite improvements in diagnosis, surgical techniques, patient care, and adjuvant therapies, most deaths from cancer are due to metastases that are resistant to conventional therapies. The major obstacle to effective treatment is the biologic heterogeneity of tumor cells. Moreover, metastases can be located in lymph nodes and different organs, and the specific organ microenvironment influences the biologic behavior of metastatic cells, including their response to systemic therapy (Fidler 2002). One of the factors is the development of drug resistance phenotype in metastatic cancer cells (Dutour et al. 2007; La Porta 2007). While cancer metastases are of clonal origin (Talmadge et al. 1982), variant clones with diverse phenotypes can form and rapidly result in the generation of significant cellular diversity within individual metastases (Fidler and Hart 1982). The outcome of the metastatic process depends on multiple and complex interactions of the metastatic cells with the host homeostatic mechanisms (Liotta et al. 1991; Fidler 1997).

We determined whether the expression level of metastasis-related genes is regulated by specific organ microenvironments. Highly metastatic clones of human prostate cancer were implanted into the prostate (orthotopic site) and subcutis (ectopic site). Tumors were harvested and processed for in situ hybridization (ISH) analysis. Spontaneous metastases in the lymph nodes were also evaluated. Tumors growing in the prostate exhibited higher levels of epidermal growth factor-receptor (EGF-R), basic fibroblast growth factor (bFGF), interleukin (IL)-8, type IV collagenase, and the multidrug resistance (*mdr-1*) gene than those growing in the subcutis (Greene et al. 1997).

The orthotopic implantation of human cancer cells was mandatory for analysis of metastasis-related genes. Specifically, highly metastatic cells expressed higher mRNA levels of type IV collagenase (which affects invasion), bFGF and IL-8 (which affect angiogenesis), and *mdr-1* compared with cells of low metastatic potential. No difference in EGF-R expression (which affects growth) was found between the cells, but the expression of E-cadherin (which affects cell cohesion) was decreased in the metastatic cells. Vascular endothelial growth factor/vascular permeability factor (VEGF/VPF), which affects tumor angiogenesis, has also been found to be overexpressed in prostate cancer in comparison with normal epithelium or benign prostatic hyperplasia. We found that VEGF/VPF levels correlated with microvessel density and metastatic potential of human prostate cancer cells growing in the prostate of nude mice (Balbay et al. 1999). Furthermore, there is an intratumoral heterogeneity of expression of tyrosine kinase growth receptors found in human colon cancer surgical specimens and in orthotopic tumors (Kuwai et al. 2008). Collectively, these data suggest that the expression level of metastasis-regulating genes by metastatic cells can be induced by factors in the organ microenvironment and can influence the drug resistant phenotype of metastases.

Since the expression of various cytokines, growth factors, and their receptors on metastatic tumor cells and their microenvironment (e.g., endothelial cells) can interact to provide survival advantage and mediate MDR phenotype, combining chemotherapy and targeting the receptors of specific tyrosine protein kinases may be effective in treating metastatic diseases. Our group has been successful in such combination therapies against several metastatic cancers in orthotopic animal models. These included inhibiting the EGFR signaling by PKI166 in human renal cell carcinoma growing orthotopically in nude mice (Kedar et al. 2002); targeting the expression of platelet-derived growth factor receptor (PDGF-R) by STI571 (Kim et al. 2006); targeting EGF-R by PKI166 and VEGF-R by AEE788 with irinotecan in orthotopic colon carcinoma (Kitadai et al. 2006; Sasaki et al. 2007); simultaneously inhibiting EGF-R/VEGF-R by AEE788 and PDGF-R by STI571 with gemcitabine against human pancreatic carcinoma (Yokoi et al. 2005); targeting tumor cells and tumor-associated endothelial cells in human prostate cancer cells growing in the bone of nude mice by inhibiting EGF-R using PKI166 (Kim et al. 2003); inhibiting EGF-R by PKI166 and PDGF-R by STI571 with taxol (Kim et al. 2004); or STI571 with zoledronate and paclitaxel (Kim et al. 2005).

Tumor Angiogenesis

The survival and growth of cells is dependent on an adequate supply of oxygen and nutrients and on the removal of toxic molecules. Oxygen can diffuse from capillaries for only 150–200 μm . When distances of cells from a blood supply exceed this, cell death follows (Gimbrone et al. 1974; Folkman and Klagsbrun 1987; Kerbel and Folkman 2002; Fidler and Ellis 2004). Thus, the expansion of tumor masses beyond 1 mm in diameter depends on neovascularization, i.e., angiogenesis

(Folkman 1986). The formation of new vasculature consists of multiple, interdependent steps. It begins with local degradation of the basement membrane surrounding capillaries, followed by invasion of the surrounding stroma and migration of endothelial cells in the direction of the angiogenic stimulus (Fidler et al. 2005). Proliferation of endothelial cells occurs at the leading edge of the migrating column, and the endothelial cells begin to organize into three-dimensional structures to form new capillary tubes (Auerbach and Auerbach 1994). Differences in cellular composition, vascular permeability, blood vessel stability, and growth regulation distinguish vessels in neoplasms from those in normal tissue (Fidler and Ellis 1994).

The onset of angiogenesis involves a change in the local equilibrium between proangiogenic and antiangiogenic molecules (Fidler 2001; Kerbel and Folkman 2002). Some of the common proangiogenic factors include bFGF, which induces proliferation in a variety of cells and has also been shown to stimulate endothelial cells to migrate, to increase production of proteases, and to undergo morphogenesis (Folkman and Klagsbrun 1987). Likewise, VEGF/VPF has been shown to induce the proliferation of endothelial cells, to increase vascular permeability, and to induce production of urokinase plasminogen activator by endothelial cells (Dvorak 1986; Dvorak et al. 1995). Additional proangiogenic factors include IL-8, a cytokine produced by a variety of tissues and blood cells (Singh et al. 1994; Yoneda et al. 1998), platelet-derived endothelial cell growth factor, which has been shown to stimulate endothelial cell DNA synthesis and to induce production of FGF (Kim et al. 2005, 2006), hepatocyte growth factor (HGF), or scatter factor, that increases endothelial cell migration, invasion, and the production of proteases (Bussolino et al. 1992), and platelet-derived growth factor (PDGF) (Risau et al. 1992).

Moreover, the structure and architecture of tumor vasculature can dramatically differ from those found in normal organs (Ebhard et al. 2000; Nor and Pulverini 1999; Nels et al. 1992). Indeed, blood vessels in tumors are different than those found in wound healing, and inflamed tissues. The blood flow through tumors can be tortuous and is characterized by regions of necrosis, rapid cell division, and presence of infiltrate cells. Receptors for VEGF (KDR in humans, Flt-1 in mice) are expressed specifically by tumor endothelium as well as the angiopoietin tyrosine kinase receptor, Tie-2 (reviewed in Liu et al. 2000). In addition, receptors for PDGF and EGF are found on tumor endothelial cells (Uehara et al. 2003; Suhardja and Hoffman 2003). The endothelium is fragile, and upregulation of survival factors (such as Bcl-2 and survivin) by molecules found in abundance within the tumor microenvironment such as VEGF and bFGF helps to prevent apoptosis of new endothelium (Wang et al. 2002; Karsan et al. 1997; Gerber et al. 1998). There is increased leakiness to macromolecules (perhaps due to the presence of VEGF) (Jain 1987; Dvorak 1990), and vessels often lose distinct features of arteriole, capillary, and venule formation. Modern techniques, such as phage-display targeting, have defined "vascular addresses" that may be distinct for different organs as well as tumors in those organs and perhaps offer attractive targets for antivascularity therapy (Pasqualini et al. 2002).

Angiogenic heterogeneity exists within a single tumor (zonal or intralesional) between different metastases even in a single organ, and different neoplasms of the

same histologic type are also documented (Kumar et al. 1998; Yu et al. 2001). For example, the expression of proangiogenic molecules (and, therefore, blood vessel density) in murine or human tumors growing at orthotopic sites in athymic mice is zonal, i.e., demonstrates intralesional heterogeneity. Small tumors (3–4 mm in diameters) expressed more bFGF and IL-8 than large tumors (>10 mm in diameters), whereas more VEGF is expressed in large tumors. Immunostaining showed a heterogeneous distribution of these angiogenic factors within the tumor; expression of bFGF and IL-8 was highest on the periphery of a large tumor, where cell division was maximal. VEGF expression was higher in the center of the tumor (Kumar et al. 1998). Similarly, heterogeneous dependence on angiogenesis was reported for cell subpopulations isolated from human melanoma xenografts having differential expression of hypoxia-inducing factor-1 (Yu et al. 2001).

Heterogeneity of blood vessel distribution in surgical specimens of human cancers is well documented (Weidner et al. 1992). Benign neoplasms are sparsely vascularized and tend to grow slowly in contrast to highly vascularized and rapidly growing malignant tumors (Weidner et al. 1992). The distribution of vessels in a tumor, however, is not uniform, and Weidner et al. cautioned that to predict the aggressive nature of human cancers, one must determine the mean vessel density (MVD) in the “areas of most intense neovascularization”, i.e., tumors exhibit intralesional and zonal heterogeneity for MVD (Weidner et al. 1992; Jain 1987, 2008). Similarly, the expression of proangiogenic molecules in surgical specimens of human colon carcinoma was determined by *in situ* hybridization technique. Matrix metalloproteinase-9 and bFGF were overexpressed at the periphery of the tumor where cells were rapidly dividing, whereas VEGF expression was higher in the center of the lesions (Kitadai et al. 1995).

The extent of angiogenic heterogeneity in malignant neoplasms is also regulated by the organ microenvironment. For example, human renal carcinoma cells implanted into the kidney of athymic mice produced a high incidence of lung metastasis, whereas those implanted subcutaneously did not (Singh et al. 1994). Histopathologic examination of the tissues revealed that the tumors grown in the subcutis of nude mice had few blood vessels, as compared to tumors in the kidney. The subcutaneous tumors also had a significantly lower level of mRNA transcripts for bFGF than tumor in the kidney, and the expression of the naturally occurring angiogenic inhibitor, IFN- β (which downregulates bFGF) was high in epithelial cells and fibroblasts surrounding the subcutaneous tumors. This was not detected in or around tumors grown in the kidney (Singh et al. 1995). The production of IL-8 by melanoma cells is regulated by complex interactions with skin keratinocytes (Herylyn 1990). IL-8 expression can be increased by co-culture of melanoma cells with skin keratinocytes, and this expression is inhibited by coinubation of melanoma cells with hepatocytes from the liver (Gutman et al. 1995). The organ microenvironment also influences the expression of VEGF. Human gastric cancer cells implanted into the stomach were highly vascularized and expressed high levels of VEGF, as compared to implantation into an ectopic (subcutaneous) site, such as the skin. In addition, metastasis only occurred from the tumor implanted in the stomach (Takahashi et al. 1996).

The molecular cross-talk that occurs with tumor cells and endothelium within the tumor microenvironment results in sufficient recruitment of a vascular supply that has physiological properties that allow migration and eventual escape of subpopulations of tumor cells able to complete a cascade of events necessary for metastasis.

Antivascular Therapy of MDR Prostate Carcinoma

Cancer of the prostate is the most common cancer affecting men in North America and is the second leading cause of cancer-related deaths. Mortality from prostate cancer usually results from the metastasis of hormone-refractory cancer cells. Reports examining the pattern of metastasis in advanced prostate cancer indicate that dissemination to bone and lymph nodes occurs in over 80% of the cases (Garnick and Fair 1996). The pathophysiology of prostate cancer bone metastases is complex and involves the interaction of tumor cells with osteoclasts, osteoblasts, endothelial cells and an assortment of regulatory proteins (e.g., steroid hormones, cytokines, and growth factors).

To study the factors that are critical for growth of prostate cancer cells in the bone, we established a murine model of hormone-refractory prostate cancer bone metastasis. To generate prostate cancer growth in the bone, we performed a percutaneous intraosseal injection on nude mice by inserting a 27-gauge needle into the tibia immediately proximal to the tuberositas tibia (Uehara et al. 2003). After penetrating the cortical bone, we deposited 20 μ l of tumor cell suspension (2×10^5 androgen-independent PC3-MM2 cells) in the bone cortex with the use of a calibrated, push button-controlled dispensing device. Five weeks later, we resected the tumor-bearing leg and performed an extensive immunohistochemical survey of the bone lesions in an effort to identify potential factors that may be involved in the regulation of prostate tumor cell growth. A preliminary immunohistochemical evaluation revealed robust tumor cell expression of bFGF, VEGF, IL-8, PDGF BB, and its receptor PDGFR- β . Expression of these proteins was most pronounced in tumors that were growing adjacent to the bone. In contrast, in those tumors that had lyzed the bone and extended their growth to include the surrounding muscle, we detected only minimal levels of the angiogenic proteins, suggesting that factors within the bone environment were influencing the phenotype of the tumor cells.

A more comprehensive examination of distribution pattern of PDGFR β revealed that PDGFR- β was present on both prostate tumor cells and on tumor-associated endothelium and that, moreover, this receptor tyrosine kinase was activated. Phosphorylated PDGFR- β was not found in either the contralateral nontumor leg or in tumor cells growing away from the bone, i.e., in the muscle. These findings indicate that the PDGF BB produced by tumor cells was acting in an autocrine manner to stimulate tumor cells and in a paracrine fashion to convey information to tumor-associated endothelial cells. The expression pattern of activated PDGFR- β in the bone metastases suggested that it might be a target for therapy in that inhibition of

this signaling cascade could potentially affect both the malignant cell population and the tumor blood supply. To test this hypothesis, we treated mice with experimental bone metastasis using the tyrosine kinase inhibitor of PDGFR- β , STI571 (imatinib mesylate, Gleevec). In mice treated with STI571 or the combination of STI571 plus paclitaxel, we found induction of significant apoptosis of endothelial cells and tumor cells that resulted in inhibition of tumor growth, a significant decrease of lymphatic metastases, and a significant decrease of bone lysis (Uehara et al. 2003). These experiments demonstrated that tumor-associated endothelial cells express phosphorylated PDGFR when adjacent tumor cells express PDGF, and that inhibition of this activation with a PDGFR tyrosine kinase inhibitor, particularly in combination with chemotherapy, can produce significant therapy.

To determine the molecular mechanism for the antiangiogenic effects observed on the tumor-associated endothelial cells *in vivo*, we established cultures of murine bone microvascular endothelial cells and examined their response to stimulation with PDGF BB ligand and to blockade of PDGFR signaling with STI571 (Langley et al. 2004). Cultured bone endothelial cells expressed PDGFR- β , and PDGF BB-induced phosphorylation on these cells could be inhibited by STI571 in a dose-dependent manner. Stimulation of the bone endothelial cells with PDGF BB resulted in activation of Akt and ERK1/2, and this signaling cascade could be completely abrogated by STI571. In addition, we found that bone endothelial cells respond to PDGF BB by increasing their cell division and upregulating the anti-apoptotic protein Bcl-2. We then examined the response of bone endothelial cells to treatment with STI571 and taxol. Treatment of bone endothelial cells with only a single agent produced little effect. However, the combined treatment of STI571 and taxol resulted in a significant increase in the number of cells expressing activated caspase-3 and a concomitant decline in Bcl-2. Consistent with these results, we found that when bone endothelial cells were confronted with both STI571 and low levels of taxol, there was a threefold increase in their cytotoxicity.

Collectively, these data suggest that a primary target for the STI571 and paclitaxel therapy may be the tumor-associated blood vessels. To test this hypothesis, we established a multidrug resistant prostate cell line by chronically exposing PC3-MM2 cells to increasing concentrations of taxol (Kim et al. 2006). The resulting cell line, PC3-MM2-MDR, is 70 times more resistant to paclitaxel *in vitro*, and the growth of the cells is not affected by treatment with paclitaxel or the combination of paclitaxel and STI571. When the PC3-MM2-MDR cells were implanted into the bone microenvironment, they displayed the same angiogenic profile as the parental cell line. Endothelial cells in normal tissues rarely divide, whereas 2–3% of the endothelial cells in prostate cancer divide daily (Augustin et al. 2002; Eberhard et al. 2000). These dividing endothelial cells should be sensitive to anticycling drugs such as paclitaxel. Nevertheless, in the present experiment, paclitaxel did not decrease the MVD appreciably, likely because of the fact that stimulation of endothelial cells with PDGF leads to resistance to paclitaxel, and that blockade of PDGF-R phosphorylation with imatinib reverses the resistance to paclitaxel (Langley et al. 2003). As stated above, the first wave of apoptosis in bone tumors from mice treated with imatinib and paclitaxel for only 2 weeks occurred in tumor-associated endothelial cells,

followed by apoptosis of tumor cells and ultimately tumor necrosis. By the fourth week of treatment with imatinib and paclitaxel or imatinib alone, concurrent apoptosis of tumor cells and tumor-associated endothelial cells was observed. Without paclitaxel, imatinib may produce therapeutic effects by the blockade of PDGF-R, which serves as a survival factor (Langley et al. 2003).

Thus, the imatinib-induced blockade of PDGF-R combined with paclitaxel appears to target the tumor-associated endothelial cells. Whether this approach can be useful for other types of tumors is unknown. The heterogeneity of angiogenesis in human tumors and the findings that endothelial cells of different organs are phenotypically distinct (Langley and Fidler 2007) indicate that further investigations to understand the interaction of different types of tumor cells and endothelial cells in different organs are necessary for the development of optimal regimens of targeted antivasculature therapies. For this reason, we performed another series of experiments using the multidrug resistant PC-3MM2-MDR cells growing in the prostate of nude mice and treated the mice with paclitaxel and the tyrosine kinase inhibitor, AEE788, that targets phosphorylation of EGF-R/VEGF-R (Busby et al. 2006). The significant inhibition of local tumor growth and lymph node metastases again demonstrated that tumor-associated endothelial cells, rather than the tumor cells, were the primary target of the chemotherapy. Those studies provide a better understanding of the molecular mechanisms that regulate the process of metastasis and of the complex interactions between the metastatic cells and the organ microenvironment (Kim et al. in press).

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