Review Article

Molecular targeting therapy of cancer: drug resistance, apoptosis and survival signal

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Recent progress in the development of molecular cancer therapeutics has revealed new types of antitumor drugs, such as Herceptin, Gleevec, and Iressa, as potent therapeutics for specific tumors. Our work has focused on molecular cancer therapeutics, mainly in the areas of drug resistance, apoptosis and apoptosis resistance, and survival-signaling, which is related to drug resistance. In this review, we describe our research on molecular cancer therapeutics, including molecular mechanisms and therapeutic approaches. Resistance to chemotherapeutic drugs is a principal problem in the treatment of cancer. P-Glycoprotein (P-gp), encoded by the *MDR1* gene, is a multidrug transporter and has a major role in multidrug resistance (MDR). Targeting of P-gp by small-molecular compounds and/or antibodies is an effective strategy to overcome MDR in cancer, especially hematologic malignancies. Several P-gp inhibitors have been developed and are currently under clinical phased studies. In addition to the multidrug transporter proteins, cancer cells have several drug resistance mechanisms. Solid tumors are often placed under stress conditions, such as glucose starvation and hypoxia. These conditions result in topo II poison resistance that is due to proteasome-mediated degradation of DNA topoisomerases. Proteasome inhibitors effectively prevent this stress-induced drug resistance. Glyoxalase I, which is often elevated in drug- and apoptosis-resistant cancers, offers another possibility for overcoming drug resistance. It plays a role in detoxification of methylglioxal, a side product of glycolysis, which is highly reactive with DNA and proteins. Inhibitors of glyoxalase I selectively kill drug-resistant tumors that express glyoxalase I. Finally, the susceptibility of tumor cells to apoptosis induced by antitumor drugs appears to depend on the balance between pro-apoptotic and survival (anti-apoptotic) signals. PI3K-Akt is an important survival signal pathway, that has been shown to be the target of various antitumor drugs, including UCN-01 and geldanamycin, new anticancer drugs under clinical evaluation. Our present studies provide novel targets for future effective molecular cancer therapeutics. (Cancer Sci 2003; 94: 15–21)

Cancer chemotherapy has gradually improved with the development of novel antitumor drugs and has profound, positive results when applied to many hematologic malignancies, and some solid tumors, especially germ cell and some childhood malignancies. While treatment of certain malignancies with chemotherapy has been successful and encouraging, the effectiveness has often been limited by drug resistance of tumors and by side effects on normal tissues and cells. In fact, many tumors are intrinsically resistant to many of the more potent cytotoxic agents used in cancer therapy. Other tumors, initially sensitive, recur and are resistant not only to the initial therapeutic agents, but also to other drugs not used in the treatment. Because of the serious problem of clinical drug resistance, much effort has been expended to advance our understanding of the mechanisms of drug resistance in cancer cells. The molecular mechanisms of drug resistance, however, are not fully understood.

Apoptosis and anti-apoptosis pathways are also deeply related to drug sensitivity and resistance. A number of tumor cells have been reported to undergo apoptotic cell death when treated with such chemotherapeutic agents as etoposide, camptothecin, cisplatin, 1-β-D-arabinofuranosyl cytosine (Ara-C), mitomycin C, adriamycin, and vincristine. The findings indicate that apoptosis in tumor cells plays a critical role in chemotherapy-induced tumor cell killing and also suggest that blockade of the apoptosis-inducing pathway could be another mechanism for multidrug resistance (MDR). Based on the drug- and apoptosis-resistance mechanisms, we can design a rational strategy to target resistant cancers. In this review, we discuss several drug resistance mechanisms, including drug transporters, cellular stress responses, apoptosis regulation and cellular survival signals that have relevance to drug resistance. These pathways could provide new targets for effective cancer therapy.

1. MDR

1-1. Mechanism of MDR by ATP-binding cassette (ABC) transporters

Resistance to a broad spectrum of chemotherapeutic agents in cancer cell lines and human tumors has been called MDR.[1)] The MDR phenotype is associated with increased drug efflux from the cells that is mediated by an energy-dependent mechanism. Studies on the MDR phenotype have led to discovery of ABC transporters, such as P-glycoprotein (P-gp), and MDR-associated protein (MRP).

Overexpression of P-gp, encoded by the *MDR1* gene, confers resistance to a variety of structurally and functionally unrelated antitumor drugs, such as vinblastine, vincristine, doxorubicin, daunorubicin, etoposide, teniposide, paclitaxel, and many others.[2)] P-gp, localized on the plasma membrane of resistant cancer cells, can bind and transport the antitumor drugs in a ATP-dependent manner (Fig. 1).[3–5)] The expression of P-gp is elevated in intrinsically drug-resistant cancers of the colon, kidney and adrenal, as well as in some tumors that acquire drug resistance after chemotherapy. The expression of MDR1 mRNA in tumors correlates well with certain kinds of clinical drug resistance. MDR1 promoter activity is up-regulated by various stimuli, such as anticancer drugs, DNA-damaging agents, heat shock, serum starvation, and ultraviolet irradiation. MDR1 expression can also be up-regulated as a consequence of tumor

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progression, such as mutation of tumor suppressor gene *p53* and activation of *ras* oncogene.

1-2. Reversal of MDR

Because P-gp appeared to be involved in both acquired and intrinsic MDR in human cancers, selective killing of tumor cells that express P-gp could be very important for cancer therapy. In 1981, we reported that the calcium channel blocker verapamil inhibited active drug efflux and restored drug sensitivity in MDR cells.[6] Various compounds, including calcium channel blockers and calmodulin inhibitors, have been shown to enhance the cytotoxic activity of various agents.[7–10] All of these agents antagonize MDR by increasing the drug accumulation in MDR cells, whereas they show little or no effect on drug-sensitive cells. Most of the reversing agents, such as verapamil, cyclosporin A, diltiazem and FK-506, are substrates for P-gp-mediated transport and competitively inhibit P-gp transport of antitumor drugs.[11] A new generation of the MDR reversal agents has been developed and is in clinical trials. These include non-immunosuppressive cyclosporine PSC-833 and a quinoline derivative, MS-209. MS-209 was shown to inhibit both P-gp and MRP. In clinical trials, MDR-reversing agents have shown promising effects on drug-resistant hematologic malignancies.

It is important to note that any P-gp inhibitor could have deleterious effects *in vivo* because P-gp is also expressed in normal tissues, such as adrenal, gravid uterus, kidney, liver, colon and capillary endothelial cells in brain.[12, 13] P-gp expressed in normal tissues could prevent xenobiotic uptake, and therefore, many P-gp inhibitors affect the pharmacokinetics of antitumor drugs. To improve combined chemotherapy, the pharmacodynamic features of anti-cancer drugs and P-gp inhibitors should be carefully investigated.

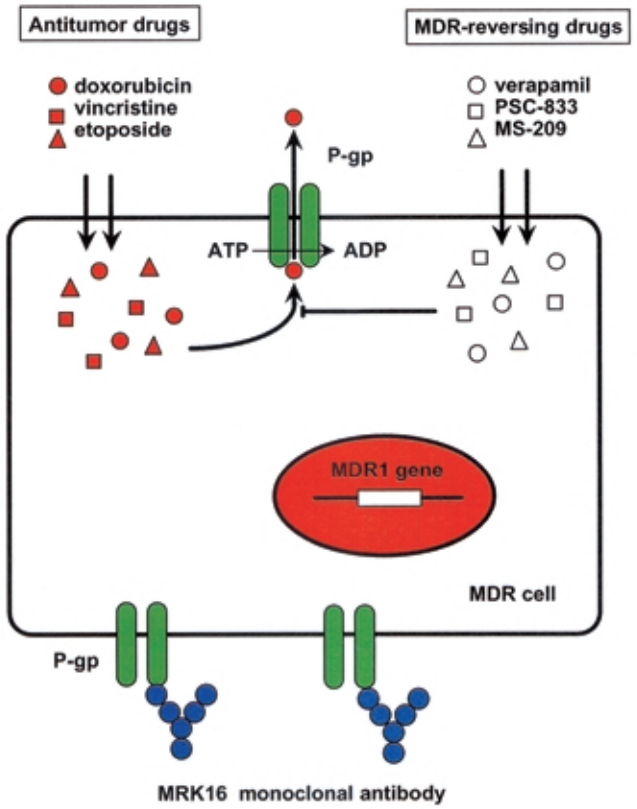

Fig. 1. P-Glycoprotein-mediated multidrug resistance and its reversal. P-Glycoprotein, encoded by the *MDR1* gene, transports various anticancer drugs from MDR cells in an energy-dependent manner. MDR-reversing agents, such as verapamil, PSC-833 and MS-209, inhibit P-glycoprotein-mediated drug transport. MRK16, a monoclonal antibody against P-glycoprotein is also useful to reverse MDR.

In addition to small-molecular P-gp inhibitors, monoclonal antibodies to P-gp are useful to target this protein in drug-resistant cancers. Our research group has developed immunotherapeutic approaches for overcoming MDR using anti-P-gp monoclonal antibodies, MRK-16 and MRK-17.[3, 14] The combination of anti-P-gp monoclonal antibodies with P-gp inhibitors may also be useful in reversing P-gp-mediated MDR. The combination of MRK-16 and PSC-833 synergistically reversed MDR *in vitro* and *in vivo*.[15] Mechanistic analysis showed that PSC-833 increased MRK-16 binding to P-gp.

It may be possible to protect hematopoietic cells from toxic side effects of cancer chemotherapy by transducing the *MDR1* gene. Although this gene is expressed in various normal tissues as mentioned above, it is not widely expressed in bone marrow cells. Lack of protection by the drug transporter may be one of the reasons for severe bone marrow cell suppression by many chemotherapeutic agents. Therefore, successful introduction of the *MDR1* gene would allow hematopoietic cells to survive increased doses of chemotherapy. For this purpose, retrovirus-mediated gene transfer can be used, because of the potential for stable vector integration and expression.[16] This therapeutic approach is interesting because it could be applied to a wide range of currently resistant solid tumors.

2. Proteasome as a target against drug resistance

2-1. Proteasome inhibition for reversal of stress-mediated resistance of solid tumors

Resistance to chemotherapy is a principal problem in treating the most commonly seen solid tumors. Tumor cells, *in vivo*, are often exposed to such conditions as glucose deprivation, hypoxia, low pH, and other nutrient deprivation.[17, 18] These microenvironmental conditions are based upon inadequate vascularization in solid tumors, regardless of their origin or location. The microenvironment itself has been thought to be a major mechanism of drug resistance because it hinders drug access to tumor cells and reduces the oxygen radicals generated by antitumor drugs. The drug resistance can also be associated with decreased cell proliferation, cell-cell contact and adhesion of cancer cells to extracellular matrix. In addition, the microenvironmental stress conditions may select tumor cells that have decreased apoptotic potential through genetic alterations, thereby leading to resistance to apoptosis induction by antitumor drugs. Of further importance is that the stress conditions also induce drug resistance without such genetic alterations in tumor cells.

Pathophysiological stress conditions in culture commonly cause the glucose-regulated response of cancer cells, which is characterized by induction of endoplasmic reticulum (ER)-resident stress proteins, GRP78 and GRP94.[19] The stress response can be mediated by the unfolded protein response or ER stress response pathway that is induced following the accumulation of unfolded proteins in the ER. It is important to recall that overexpression of GRP78 is, indeed, observed in clinical settings. A possible link between glucose-regulated stress response and drug resistance was first reported by Shen *et al*.[20] They showed that GRP-inducing conditions caused significant resistance induction to doxorubicin in Chinese hamster ovary (CHO) cells. Subsequently, the stressed CHO cells were shown to become resistant to etoposide as well as to doxorubicin, both of which are antitumor topoisomerase (topo) II poisons. We also showed that such resistance induction occurred in human cancer cell lines.[21] Thus, glucose-regulated stress response is associated with development of cellular resistance to topo II poisons.

Topo II poisons stabilize the cleavable complex, an intermediate product of the topo II-catalyzed reaction.[22] Accumulation

of cleavable complexes is thought to lead to eventual cell death, and a decrease in the number of cleavable complexes could confer drug resistance. In agreement with this, a decreased expression of topo IIα occurs under glucose-regulated stress conditions.[21] We found that proteasome inhibition attenuated the inducible resistance by inhibiting the topo IIα depletion induced by glucose starvation and hypoxia as well as by the chemical stressor A23187.[23, 24] The topo IIα restoration was seen only at the protein level, indicating that topo IIα protein depletion occurred through a proteasome-mediated degradation mechanism. In agreement with this restoration, the stress-induced etoposide resistance was effectively prevented *in vitro* by the proteasome inhibitor lactacystin.[24] Furthermore, lactacystin significantly enhanced the antitumor activity of etoposide in the refractory HT-29 xenograft. In this regard, a recently developed proteasome inhibitor, PS-341, which is now in phase I and II clinical trials,[25] may also have potential for overcoming stress-induced resistance.

2-2. Nuclear proteasome accumulation as a novel mechanism of drug resistance

During the course of the above-described studies, we found that stress conditions stimulated nuclear accumulation of proteasome in HT-29 human colon cancer cells.[23, 26] Accumulation under these conditions was also observed in A2780 ovarian cancer cells, suggesting that this response occurred regardless of the cell's origin. No changes were detected in the total expression levels of proteasome. Recently, we found that nuclear proteasome accumulation during glucose starvation was attenuated by stable expression of a mutant type of proteasome subunit, XAPC7, that lacked the nuclear localization signal (NLS).[27] It is important that the expression of NLS-defective XAPC7 also diminished the induction of resistance to the topo II-directed drugs etoposide and doxorubicin. Under normal conditions, however, the NLS-defective XAPC7 had little effect on either nuclear proteasome distribution or etoposide sensitivity. These results demonstrate that nuclear proteasome localization, possibly through up-regulation of the NLS-dependent transport, is involved in the development of drug resistance under stress conditions (Fig. 2).

We also demonstrated that XAPC7/dNLS-transfected cells showed slightly higher expression levels of topo IIα under glucose-starvation conditions than did XAPC7/WT-transfected cells.[27] This observation raises the possibility that degradation of nuclear proteins, including topo IIα, is facilitated by nuclear proteasome accumulation under stress conditions. Although the exact mechanisms of drug resistance mediated by nuclear proteasome accumulation remain to be determined, our observations suggest that inhibiting nuclear distribution of proteasomes can be a selective approach to preventing stress-inducible drug resistance of solid tumors. Indeed, microenvironmental stresses are not observed in normal tissues, and the NLS-dependent pathway appears important particularly for stress-induced nuclear transport of proteasomes in cancer cells. Thus, further studies into the mechanisms of nuclear proteasome transport would provide tumor-selective strategies to circumvent the inducible resistance to topo II-directed drugs.

2-3. Other mechanisms of proteasome-mediated drug resistance

The proteasome is a major site for degradation of abnormal or irrelevant and regulatory proteins, such as transcription factors, oncoproteins and cyclins. Consequently, proteasome can be involved in various types of drug resistance.[25] For example, NF-κB signaling is dependent on proteasome activity, and this pathway plays a role in protecting against drug-induced apoptosis in certain cell types. Indeed, PS-341 has been shown to block the protective response and to sensitize cancer cells to topo I inhibitors. The proteasome is also involved in a possible repair mechanism of camptothecin (CPT)-induced cleavable complexes through degrading topo I. Thus, proteasome inhibition may be useful for overcoming various types of drug resistance.

However, proteasomes are ubiquitously expressed in normal tissues and play essential roles in various cellular functions. Therefore, total inhibition of proteasome activities might have adverse effects. In this context, regulatory events upstream of the proteasome-mediated degradation, such as phosphorylation and ubiquitinylation of substrates, will be interesting to explore as potential targets. With respect to topoisomerase-targeted drug resistance, it will be especially important to elucidate the regulatory mechanisms of the stress-induced topo IIα and the CPT-induced topo I degradation (Fig. 2). Modification of topoisomerases with SUMO-1, a ubiquitin-like protein, may also be interesting. We recently identified the major sumoylation site of topo I and found that sumoylation enhances the cleavable complex formation and apoptosis induced by CPT.[28] Thus, additional research on regulatory events upstream of the proteasome-mediated degradation could provide novel and specific strategies for overcoming drug resistance.

3. Glyoxalase I as a target in apoptosis resistance

3-1. Apoptosis-resistant tumor cells

Apoptosis is an active cell death mechanism that plays a role in several biological processes. Various antitumor agents have been reported to elicit apoptosis in tumor cells.[29] This implies that blocking the apoptosis signaling could be another mechanism for multidrug resistance to chemotherapy. Since tumor development is caused by an upset of balance between cell growth and death, abnormality in the machinery of apoptosis often occurs in tumors, causing a chemotherapy-resistant phenotype of the tumor cells. To determine the molecular basis of resistance to antitumor agent-induced apoptosis, we isolated and characterized several drug-resistant mutants that were also resistant to apoptosis induced by antitumor agents.[30] To identify the factors responsible for the apoptosis resistance, we performed a cDNA subtractive hybridization with mRNA from human monocytic leukemia U937 and its variant UK711, which is resistant to apoptosis induced by antitumor agents.[31, 32] We found that glyoxalase I was selectively overexpressed in the apoptosis-resistant UK711 cells.

3-2. Glyoxalase I as an apoptosis resistance factor

We observed an elevated expression of glyoxalase I in several apoptosis-resistant tumor cells. Glyoxalase I is an essential component in pathways leading to the detoxification of methylglyoxal, a side product of glycolysis.[33] Methylglyoxal is a reactive dicarbonyl compound and is involved in a variety of detrimental processes, including advanced glycation end product (AGE) formation with protein and DNA modification.[34] Meth-

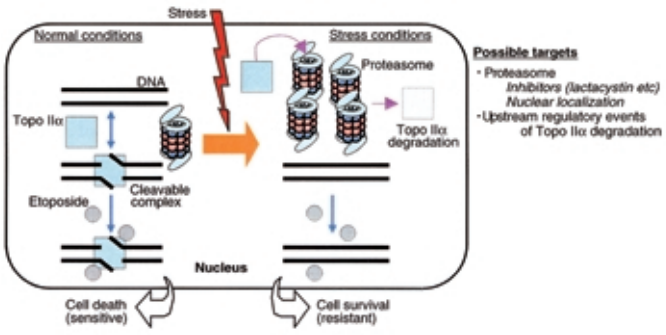

Fig. 2. Possible mechanisms of stress-induced topo II-directed drugs.

ylglyoxal is hypothesized to be cytotoxic, owing to its protein- or DNA-modifying properties. In tumor cells, the high glycolytic activity caused by rapid deregulated growth increases the intracellular levels of methylglyoxal. To eliminate cytotoxicity, detoxification systems are required. The principal route for methylglyoxal catabolism is the glyoxalase pathway, which consists of two enzymes, glyoxalase I and glyoxalase II.[33] The regulation of these enzymes in tumor cells and their involvement in drug resistance, however, have not been fully elucidated.

The mRNA expression of glyoxalase I as well as its enzyme activity was significantly elevated in several drug-resistant cells, including UK711, UK110, and K562/ADM, as compared with their parental cells. Since these mutant cell lines were populations that survived after treatment with etoposide or adriamycin, it is possible that the development of drug resistance is accompanied by this overexpression of the enzyme. When overexpressed in human leukemia cells, glyoxalase I inhibited etoposide- and adriamycin-induced apoptosis, indicating the direct involvement of the enzyme in apoptosis suppression caused by these drugs. The expression of glyoxalase I inhibited upstream signaling of apoptosis leading to caspase protease activation. Abnormalities in the glyoxalase system are also linked with human disease states including diabetes, malaria and muscular dystrophy, and several inhibitors of glyoxalase I have been designed from analogues of the hemithioacetal substrate based on glutathione derivatives.[35] We tested the effect of the glyoxalase I inhibitors on the apoptosis resistance of human leukemia cells. We found that cotreatment with *S-p*-bromobenzylglutathione cyclopentyl diester (BBGC), a cell-permeable inhibitor of glyoxalase I, selectively enhanced etoposide-induced apoptosis in resistant UK711 cells, but not in parental U937 cells.[31] These results indicate that glyoxalase I inhibitors are effective drug resistance-reversing agents in human leukemia cells.

3-3. Glyoxalase I as a target in cancer chemotherapy

We quantitatively measured glyoxalase I enzyme activity in 38 human solid tumor cell lines used in our anticancer drug-screening system.[32] We found that glyoxalase I was not only overexpressed in prostate and colon cancers, but also its activity was higher in all of the cancer cell lines than in the normal tissue samples that we examined (Fig. 3). These observations suggest that the increase of glycolytic activity could be associated with tumorigenicity as well as the drug-resistant phenotype. Among several cancer cells, we found that glyoxalase I was frequently elevated in human lung carcinoma cells. We next examined the antiproliferative effect of the glyoxalase I inhibitor. A positive correlation between cellular glyoxalase I activity and BBGC sensitivity was observed in the lung cancer cell lines.[32] Human lung cancer cells expressing higher glyoxalase I activity underwent apoptosis when treated with BBGC, but cells expressing lower activity did not. BBGC induced stress-activated protein kinase, c-Jun N-terminal kinase 1 (JNK1) and p38 mitogen-activated protein kinase (MAP kinase) activation, leading to caspase activation in glyoxalase I-overexpressing tumor cells. Moreover, the glyoxalase I inhibitor significantly inhibited xenografted human lung cancer and human prostate cancer growth (Fig. 4). We observed no decrease in the body weight of glyoxalase I inhibitor-treated mice throughout the experiments. Our results indicate that glyoxalase I is a tumor-specific target enzyme especially in human lung cancer cells, and glyoxalase I inhibitors are potent chemotherapeutic agents with minimal cytotoxicity. When we compared the glyoxalase I inhibitor with standard anticancer drugs, the pattern of drug sensitivity to BBGC in several human solid tumor cell lines differed from those of other antitumor drugs, such as DNA topoisomerase inhibitors, alkylating agents and antimitotic agents. These observations suggest that glyoxalase I inhibitors could be anticancer drugs with a novel mode of action.

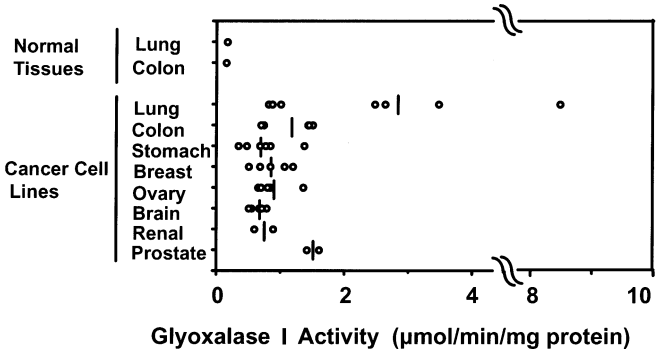

Fig. 3. Glyoxalase I activity in human solid tumor cell lines and human normal tissue samples. Cytosolic fractions were isolated, and glyoxalase 1 assays were performed. Bars, means for each cell line.

3-4. Further insights in the mode of action of glyoxalase I-related factors in cells

Presently, the precise mechanism of apoptosis induced by glyoxalase I inhibitors is still unknown. Inhibition of glyoxalase I leads to the accumulation of methylglyoxal in cells. Since methylglyoxal is capable of inducing DNA modification and protein cross-links, DNA damage or inactivation of some anti-apoptotic proteins by methylglyoxal could be an initial activator of apoptosis. Recently, several researchers have outlined the specific roles of intracellular methylglyoxal in apoptosis. Godbout *et al.* reported that methylglyoxal enhances cisplatin-induced apoptosis by activating protein kinase Cδ.[36] It was also reported that tumor necrosis factor (TNF) induces a substantial increase in intracellular levels of methylglyoxal that leads to the formation of a specific methylglyoxal-derived advanced glycation end product.[37] Methylglyoxal modification of proteins has also been detected in several human cancer cell lines. We recently identified heat-shock protein 27 (Hsp27) as a major methylglyoxal-modified protein in cancer cells.[38] We showed that methylglyoxal modification was involved in Hsp27 oligomerization to prevent cytochrome *c*-mediated caspase activation during apoptosis. These data suggest that methylglyoxal modification of proteins could be a targeted process and that methylglyoxal may function as a signal molecule during the regulation of cell death. Further studies to clarify the role of in-

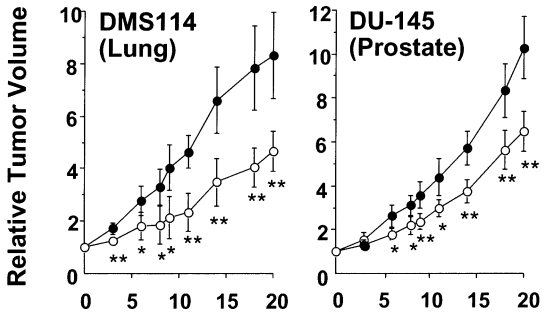

Fig. 4. Effect of BBGC on tumor growth in nude mice bearing human cancer xenografts. Tumor-bearing mice were treated with BBGC daily from days 0 to 8 at doses of 0 (●, control) and 100 mg/kg (○). The data shown are means±SD of five mice. Statistical evaluations were performed using Student's *t* test, comparing the group receiving BBGC treatment with the group receiving control treatment. * $P<0.05$, ** $P<0.01$.

tracellular methylglyoxal will give novel insights into the regulation of cell growth and provide rationale for glyoxalase-targeted chemotherapy.

4. Akt-mediated survival-signaling pathway

4-1. Akt as a promising target for cancer chemotherapy

The susceptibility of cells to apoptosis appears to be dependent on the balance between pro-apoptotic and survival (anti-apoptotic) signals. The fact that diverse chemotherapeutic drugs induce apoptosis, while engaging different intracellular targets, raises the possibility that anticancer drugs may induce apoptosis by decreasing survival signals, such as the Akt-mediated survival-signaling pathway.

The serine/threonine kinase Akt (also known as PKB or RAC-PK) is the cellular homologue of the retroviral oncogene product v-Akt.[39] A number of reports indicate that growth factors and cytokines stimulate cell survival. After stimulation with growth factors and cytokines, phosphatidylinositide-3-OH kinase (PI3K) is activated and phosphorylates phosphoinositides. The interaction of the generated phospholipid second messenger molecule, phosphatidylinositol 3,4,5-trisphosphate (PIP_3), with the pleckstrin homology domain of Akt recruits Akt to the plasma membrane, where it is phosphorylated at two key regulatory sites, Thr^{308} (by 3-phosphoinositide-dependent protein kinase-1 (PDK1)) and Ser^{473} (by an as-yet-unidentified kinase PDK2) residues. Phosphorylation at both residues is necessary for full activation of Akt and the subsequent control of biological responses, including apoptosis inhibition (by phosphorylating the pro-apoptotic Bcl-2 family member Bad, the caspase family member caspase-9, the Forkhead transcription factor protein family, and IκB kinases)[39] and cell cycle progression (by phosphorylating $p21^{Waf1/Cip1}$, $p27^{Kip1}$, and MDM2).[39, 40] In this section, we document the regulation of the Akt signaling pathway by Hsp90 and the role of Akt-mediated survival-signaling pathway during chemotherapeutic drug-induced apoptosis.

4-2. Identification of Akt and PDK1 as Hsp90 client proteins

To find the molecules that interact with Akt, we performed immunoblot analysis after immunoprecipitation of Akt with an anti-Akt pAb. We could determine that Hsp90 was an Akt-binding protein.[41] Hsp90 is an abundant and highly conserved protein involved in a diverse array of cellular processes. In contrast to other heat-shock proteins, Hsp90 is not required for maturation or maintenance of most proteins *in vivo*. Most of the identified cellular targets were signaling proteins and bound to Hsp90 via the NH_2-terminal ATP- and Hsp90-inhibitor geldanamycin-binding domain or the COOH-terminal oligomerization domain. Interestingly, Akt binds to Hsp90 through the middle domain of Hsp90 (amino acids 327–340 of Hsp90β). This result was supported by the fact that geldanamycin could not inhibit Akt binding to Hsp90. Hsp90 acts as a chaperone for unstable signal transducers and keeps them poised for activation until they are stabilized by conformational changes associated with signal transduction. Examination of the role of Hsp90 in Akt signaling by inhibiting Akt-Hsp90 binding with binding domain-containing Akt deletion mutants revealed that the blockade of Akt-Hsp90 binding inactivated Akt and increased the sensitivity of the cells to apoptosis-inducing stimuli, such as growth-factor withdrawal and chemotherapeutic drugs. We confirmed that Hsp90 binding to Akt was responsible for protection of Akt from protein phosphatase 2A (PP2A)-mediated dephosphorylation.[41] Therefore, Akt-Hsp90 binding might play an important role in the stabilization of Akt kinase activity. Recently, we also reported that endothelial nitric oxide synthase (eNOS) formed a complex with Hsp90 through binding to Hsp90's middle domain.[42] Because Akt-dependent phosphorylation of eNOS at Ser^{1179} was promoted by Hsp90, Hsp90 may function as a scaffold for Akt and its substrates.

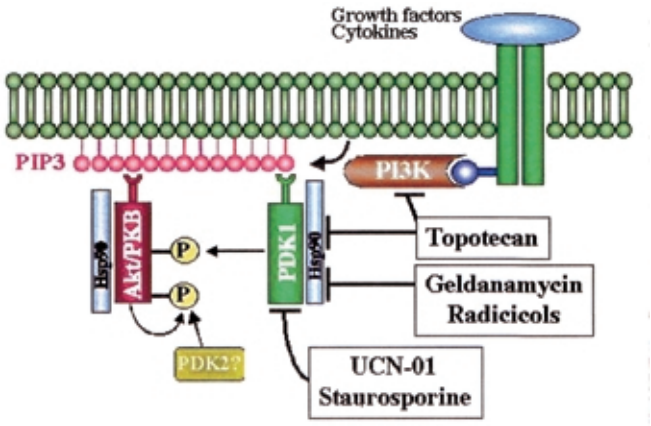

Fig. 5. Schematic representation of molecular targets of topotecan, UCN-01, and Hsp90 inhibitors (geldanamycin and radicicol and their analogues).

Although Hsp90 inhibitors (e.g., geldanamycin, radicicol, and radicicol analogues) could not inhibit Akt-Hsp90 binding, treatment of the cells with Hsp90 inhibitors downregulated the phospho-Akt (Thr^{308}) level. Moreover, Hsp90 inhibitors did not directly affect Akt kinase activity *in vitro*. Therefore, Hsp90 inhibitors might suppress Akt signaling mainly by inhibiting upstream Akt kinases (e.g., PDK1, PI3K, PDK2). We recently showed that upstream Akt kinase PDK1 was a Hsp90 client protein.[43] Treatment of the cells with Hsp90 inhibitors suppressed the PDK1-Hsp90 binding *in vivo* and decreased the amount of PDK1 without directly inhibiting PDK1 kinase activity. The proteasome was involved in the PDK1 degradation after inhibiting PDK1-Hsp90 binding. Treatment of proteasome inhibitors increased the amount of detergent-insoluble PDK1 in Hsp90 inhibitor-treated cells. Therefore, the association of PDK1 with Hsp90 regulates its stability, solubility, and signaling. These results indicate that Hsp90 plays an important role in the Akt signaling pathway by binding to both Akt and PDK1. Recently, 17-allylaminogeldanamycin (17-AAG) was reported to suppress Akt kinase activity by inhibiting PI3K activity in HER2-overexpressing breast cancer cells.[44] Moreover, Akt was degraded by proteasome after 17-AAG treatment[44] in addition to the previously reported caspase-mediated cleavage.[45, 46] These results indicate that Hsp90 might be a promising target for developing new chemotherapeutic drugs to suppress the Akt-mediated survival-signaling pathway.

4-3. Upstream Akt kinase PDK1 is a molecular target of some chemotherapeutic drugs

Screening of chemotherapeutic drugs that possess direct effects on the Akt pathway revealed that staurosporine and a water-soluble CPT analogue, topotecan (10-hydroxy-9-dimethylaminomethyl-(*S*)-camptothecin), in addition to the above-mentioned Hsp90 inhibitors, possessed the ability to decrease the phospho-Akt (Thr^{308}) level in cells (Fig. 5).

Because the staurosporine analogue UCN-01 (7-hydroxystaurosporine) exhibited potent anti-tumor activity in *in vivo* and *in vitro* tumor models, its clinical usefulness has been investigated in the United States. UCN-01 was originally isolated as a selective protein kinase C (PKC) inhibitor from the culture broth of *Streptomyces* sp. The PKC family was thought to be a major target of UCN-01; however, it is still unclear whether PKC is the sole target of UCN-01 and how PKC inhibition is involved in its anti-tumor activity. Examining the effect of UCN-01 on

the Akt signaling pathway revealed that UCN-01 directly suppressed upstream Akt kinase PDK1 with an IC_{50} value lower than 33 n*M* in *in vitro* and *in vivo* assays.[47] UCN-01's inhibitory effect on PDK1 was also observed in xenografted murine NL-17 and human PC-3 tumors. By contrast, UCN-01 had marginal effects on Akt and PI3K activities. Overexpression of the active form of Akt diminished the cytotoxic effects of UCN-01, indicating that UCN-01 may exert its cytotoxicity in part by inhibiting the PDK1-Akt survival pathway. Because UCN-01 has already been proved to have potent anti-tumor activity *in vivo* and *in vitro*, the Akt-PDK1 pathway may be a promising target for tumor treatment.

We also tried to identify the molecular target(s) of topotecan on the Akt signaling pathway because topotecan decreased the Akt kinase activity in cells.[48] Topotecan is a novel topoisomerase I inhibitor that has shown activity against numerous human tumor cell lines and xenografts. Topotecan has also shown clinical activity in small cell and non-small cell bronchogenic carcinoma, ovarian carcinoma and myeloid leukemia, and has been approved for the treatment of relapsed ovarian cancer and small cell lung cancer. Interestingly, Akt dephosphorylation could not be seen in topotecan-resistant A549/CPT cell line. This fact suggests that Akt inactivation plays an important role in the exhibition of topotecan-mediated cytotoxic effects. Topotecan was revealed to exert its cytotoxic effects in part by suppressing the kinase activities of upstream Akt kinases PI3K and PDK1. The PI3K-Akt signaling pathway appears to play an important role in angiogenesis. Examination of topotecan's effect on endothelial cells revealed that topotecan downregulated the *in vitro* migration of endothelial cells.[49] Transfection of constitutively active *akt* cDNA restored topotecan's inhibitory effect on endothelial migration, indicating that topotecan might exert its antiangiogenic activity by downregulating the PI3K-Akt signaling pathway.

Because the Akt signaling pathway is also associated with metastasis formation of some tumor cells,[50] the pathway (particularly upstream Akt kinase PDK1) is a promising new target for developing chemotherapeutic drugs to treat tumors (Fig. 5).

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