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RLIP76 Targeted Therapy for Kidney Cancer

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Abstract

Despite recent improvements in chemotherapeutic approaches to treating kidney cancer, this malignancy remains deadly if not found and removed at an early stage of the disease. Kidney cancer is highly drug-resistant, which may at least partially result from high expression of transporter proteins in the cell membranes of kidney cells. Although these transporter proteins can contribute to drug-resistance, targeting proteins from the ATP-binding cassette transporter family has not been effective in reversing drug-resistance in kidney cancer. Recent studies have identified RLIP76 as a key stress-defense protein that protects normal cells from damage caused by stress conditions, including heat, ultra-violet light, X-irradiation, and oxidant/electrophilic toxic chemicals, and is crucial for protecting cancer cells from apoptosis. RLIP76 is the predominant glutathione-electrophile-conjugate (GS-E) transporter in cells, and inhibiting it with antibodies or through siRNA or antisense causes apoptosis in many cancer cell types. To date, blocking of RLIP76, either alone or in combination with chemotherapeutic drugs, as a therapeutic strategy for kidney cancer has not yet been evaluated in human clinical trials, although there is considerable potential for RLIP76 to be developed as a therapeutic agent for kidney cancer. In the present review, we discuss the mechanisms underlying apoptosis caused by RLIP76 depletion, the role of RLIP76 in clathrin-dependent endocytosis deficiency, and the feasibility of RLIP76-targeted therapy for kidney cancer.

Keywords

RLIP76; kidney cancer; drug-resistance; glutathione-conjugate transport; chemotherapeutics

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Introduction

A potential approach for improving the therapeutic efficacy of targeted therapy for kidney cancer could be to inhibit mechanisms responsible for drug-resistance or radiation-resistance. Kidney cancer cells express high levels of multiple membrane transporter proteins that can contribute to drug-resistance and may also play roles in radiation-resistance (1–5). The majority of these membrane transporter proteins belong to the ATP-binding cassette (ABC) family of proteins. Clinically, however, inhibitors of ABC-transporters have not yet been successful in improving chemotherapeutic outcomes (4–8), although alternative targeting strategies may ultimately prove clinically effective.

In general, most chemotherapeutic agents have failed to be clinically useful in the treatment of kidney cancers, although some responses to chemotherapy drugs have been seen in selected patients. Cytotoxic drugs that have been used to treat kidney cancers include platinum compounds (e.g., cisplatin, carboplatin, and oxaliplatin), anti-metabolites (e.g., 5-fluorouracil, gemcitabine, and methotrexate), taxane compounds (e.g., paclitaxel, docetaxel), anthracyclines (e.g., doxorubicin), and vinca alkaloids (e.g., vinblastine, vincristine, and vinorelbine) (1–3). However, when used as single agents, response rates with these drugs do not exceed 10%. Of these agents, the platinum compounds are known to form glutathione (GSH)-conjugates, which are subject to transport by RLIP76, a novel glutathione-electrophile conjugate (GS-E) and multidrug transporter. The anthracyclines and vinca alkaloids are direct substrates for transport by RLIP76 (9–13). In contrast, taxanes are not substrates for transport by RLIP76 (14), and there is no known direct interaction between 5-fluorouracil or gemcitabine with RLIP76. On this basis, we postulate that depletion or inhibition of RLIP76 could preferentially enhance the toxicity of those drugs that can be transported substrates (allocrites) of RLIP76. Because cytotoxic drugs are generally ineffective against kidney cancers, and so are used infrequently as compared with ‘non-cytotoxic’ targeted drugs such as sunitinib, sorafenib, and temsirolimus (15–18), it is reasonable to consider whether the efflux of these agents could be affected by RLIP76 (or whether these agents could inhibit or even activate RLIP76). Multi-drug resistance must also be considered, and can be mediated by membrane proteins, such as P-glycoprotein, multidrug resistance-associated proteins (8), and non-ATP binding cassette multi-drug transporters, such as RLIP76, which transport not only chemotherapeutic agents but also GS-Es out of the tumor cell in an ATP-dependent manner (10,11,19). Although RLIP76 is expressed in a high percentage of kidney cancer tissues and cell lines (19–29), it has not yet been explored as a targeted therapeutic strategy for kidney cancer in humans.

Although RLIP76 has not yet been targeted, multiple other chemotherapeutic strategies have been investigated for treatment of kidney cancer. For example, immunotherapy such as interferon or IL2 has been used to treat kidney cancer, but response rates are low and only selected populations have a survival benefit. Improved knowledge of cellular signaling mechanisms affected by immunotherapy and the development of targeted therapeutics has led to new, more effective therapies for kidney cancer (30–33). The introduction of the multi-specific kinase inhibitors sorafenib (BAY 43–9006; Nexavar, Bayer) and sunitinib (SU011248; Sutent, Pfizer) into the clinic has improved treatment of advanced kidney cancer (15, 16, 34). Once the optimal drug dosages and combination regimens are

developed, these agents are likely to have an even greater impact on kidney cancer survival. The recent approval of the drug temsirolimus (CCI-779; Torisel, Wyeth), the first mammalian target of rapamycin (mTOR) inhibitor approved for treating kidney cancer, offers an alternative for patients who do not respond to the kinase inhibitors, but the toxicity is greater (17,33–35). Although prolonged remissions are occasionally seen when the above agents are used, the benefit is often short-lived (36, 37), and therefore there is still a need for novel, more effective therapies that use different molecular mechanisms.

Prominent among the potential other mechanisms to target are glutathione (GSH), glutathione-synthetase, glutathione-reductase, glutathione S-transferases (GSTs), cytochrome P450s, γ -glutamyl transpeptidase, and the components of mercapturic acid pathway (MAP), including the transporters for GS-E. Recent studies have established that in human and rodent cells, RLIP76 is the major GS-E transporter (19, 38). Although several ABC-transporters such as MRP1, 3 and 5, and BCRP catalyze transport of GS-E, individually, each transporter represents only a small fraction of the total GS-E efflux capacity of cells (5, 11, 39, 40). In contrast, genetic deletion of the non-ABC transporter RLIP76, led to loss of ~80% of total transport activity for GS-E and had major phenotypic effects because of sensitivity to stress or toxin-mediated apoptosis (11, 19, 41). The loss of RLIP76 transport activity for GS-E resulted in accumulation of GS-E and its electrophilic precursors (e.g., GS-HNE and 4HNE) in the tissues of these animals (41–43) and GST activity was reversibly inhibited because of accumulation of GS-E (44). The greater sensitivity of cells from RLIP76 knockout mice is apparent only when stresses such as oxidants or radiant energy are applied (42, 45). This is also evident in studies of normal unstressed mice, which exhibited no significant toxicity upon acute depletion of >50% of total tissue RLIP76 protein by antisense (46). Normal, non-malignant cell lines of endodermal, ectodermal, and mesodermal origin are generally resistant to apoptosis caused by targeting RLIP76 (47). In contrast, the greater reliance of cancer cells on the anti-apoptotic function of RLIP76 is evident in a series of studies in which RLIP76 inhibition or depletion caused apoptosis in a broad range of cancer cell types, including lung, colon, prostate, melanoma, neuroblastoma, and kidney (21, 46–49). The over-expression of components of the MAP in cancers (50), the pro-apoptotic nature of the lipid-peroxidation metabolites (51,52), the accumulation of these metabolites when RLIP76 is depleted, and the ability of chemotherapy drugs to act as competitive (substrate)-inhibitors of physiological GS-E substrates, argues strongly for a novel, integrated model of apoptosis-resistance, drug-resistance, and radiation-resistance in which RLIP76 plays a key effector role (11,13,19).

We propose that RLIP76 provides a convenient, single target that, if depleted or inhibited, will cause global inhibition of the MAP. The pro-apoptotic effect of the consequent accumulation of lipid-peroxidation metabolites itself may be sufficient to kill cancer cells, and could certainly enhance the activity of other therapeutic agents, including chemotherapy and radiation. In comparison, targeting the MAP by inhibiting GSTs (of which there are more than 20 isoenzymes) (50) or CYP-450 enzymes (more than 150 isoenzymes) (53) would be much more difficult. Indeed, targeting the GS-E efflux step by inhibiting or depleting ABC-transporters (of which there may be more than 40 members) would also be unwieldy (39, 40). In this regard, it is important to point out that deletion of *MRP1* in mice has little effect on total GS-E transport capacity in most tissues (54) while deletion of

RLIP76 results in loss of ~80% of GS-E transport (41,42,45). Furthermore, inhibition of kinases in the ERK as well as PI3K pathways by *RLIP76* depletion is more profound and consistent and is more widely apparent in a number of kidney cancer cell lines. Taken together, these studies suggest that *RLIP76* has a key role in an overarching anti-apoptosis mechanism such that inhibition of *RLIP76* could be broadly effective in the treatment of kidney cancer. This review focuses on the potential utility of *RLIP76* targeted therapy for kidney cancer, as well as the relationship among the mechanisms of cell killing induced by *RLIP76* targeting as compared with targeting of multiple kinases.

Anti-apoptotic nature of *RLIP76*

RLIP76 is a 76 kDa splice variant encoded by the human *RALBP1* gene. It is functionally and structurally homologous to a corresponding splice variant in mouse and rat, *Ralbp1*, which is encoded by the rodent *Ralbp1* gene. *RLIP76* is involved in endocytosis and tyrosine kinase receptor signaling *via* its ability to bind to AP2 and POB1 through its N-terminal and C-terminal regions, respectively (55, 56). As an ATPase which couples ATP-hydrolysis with movement of substances, *RLIP76* functions in plasma membranes as a dominant ATP-dependent transporter of several chemotherapy drugs as well as glutathionylated intermediate metabolites of the MAP. *RLIP76*-knockout (*RLIP76*^{-/-}) mouse studies have demonstrated that it is the rate-limiting step in mercapturic acid metabolism and is critically required for clathrin-dependent endocytosis (CDE) (57). Blocking the transport function of *RLIP76* causes sustained regression of human kidney cancer xenografts in nude mice without detectable toxicity (21). Apoptosis caused by *RLIP76* depletion is associated with reduced activation of survival pathways, including PI3K, ERK and Akt more markedly and more consistently sorafenib, sunitinib, or temsirolimus, three multi-kinase inhibitors used in therapy of kidney cancer (15, 16, 22). These findings suggest that *RLIP76*-targeting could be developed into a more efficacious therapy for kidney cancer.

RLIP76 over-expression is frequently associated with neoplasia, and normal cells are much less sensitive to apoptosis caused by reducing *RLIP76* function, either by depleting *RLIP76* (by antisense or siRNA) or inhibiting its transport activity at the cell surface by using specific antibodies (19, 58, 59). In several types of cancer, *RLIP76* depletion promotes apoptosis through the accumulation of pro-apoptotic lipid peroxidation products (19). Although in other cancers, *RLIP76* inhibition can disrupt receptor-ligand signaling pathways by interrupting endocytosis, this does not appear to be the case in kidney cancer, which appears inherently deficient in CDE despite expressing high levels of *RLIP76* protein. In this respect, kidney cancer appears to be unique among cancers; in most cancers CDE levels are directly correlated with expression of *RLIP76* protein. Studies involving over-expression and depletion of *RLIP76* in kidney cancer suggest that along with deficient CDE, EGF-mediated activation of Akt is also deficient in kidney cancer (19). Despite this marked deficiency in CDE, *RLIP76* depletion causes apoptosis in kidney cancer, suggesting that inhibition of CDE by *RLIP76* is not critical for the apoptosis triggered by *RLIP76* depletion.

RLIP76 as a multi-functional protein

RLIP76 appears to exert multiple functions in cells, although the relative importance of each is not known. These proposed functions based on sequence homology and experimental evidence include acting as a GTPase stimulatory protein (GAP), endocytosis scaffold or motor, chaperone, and transcription regulator (60–62). Authors have proposed that the primary function of RLIP76 is to use the ATP-dependent trans-membrane transport of anionic metabolites to drive endocytosis. This function of RLIP76 simultaneously protects cells from apoptosis and functions to determine the rate at which receptor-ligand signaling (i.e., insulin, EGF, TGF β) is terminated. This is a unique model that, for the first time, directly and mechanistically links the MAP and CDE, suggests that RLIP76 is a key regulator of tumor progression through both transport and signaling functions (11, 13).

Several lines of evidence obtained from knockout animals and cancer cells indicate that the GS-E transport function of RLIP76 is of key importance in protecting cells from apoptosis-inducing agents (12, 41, 42, 63–65). In support of this, we have demonstrated that antisense- or siRNA-mediated depletion of RLIP76 has identically potent effect on tumor regression xenograft models (21, 46, 47). Use of an antibody that bound to the cell surface domain of RLIP76 (amino acid residues 171–185) did not deplete total cellular RLIP76 (66), but still caused a similar amount regression of xenografts as did treatment with antisense, which did significantly deplete cellular RLIP76 (46). These findings strongly indicate that the critical apoptosis-related effects of RLIP76 depletion/inhibition occur because of attenuation of the membrane-associated function of RLIP76 (i.e., GS-E transport).

We have proposed a model (Fig. 1) in which the ATPase and GS-E transport activities of RLIP76 are linked to clathrin-dependent endocytic vesicles that are assembled around the membrane-receptor/ligand such that signaling can be terminated through endocytosis. RLIP76 is linked to clathrin through binding with the AP2-clathrin adaptor, and links to intracellular receptor-tyrosine kinase domains through binding to POB1 (partner of Ralbp1, a homolog of Epsin) which is complexed with epsin, grb/nck, src. POB1 is the first known binding partner of RLIP76 (67,68) and has been shown to be a saturable inhibitor of the transport activity of RLIP76; as a consequence of RLIP76 binding and inhibition, augmenting cellular levels of POB1 causes apoptosis in lung cancer cells (68), and also in prostate cancer cells (67). Studies of GS-E binding mutants have shown that the endocytosis function and GS-E transport functions are inextricably linked, indicating that it is the GS-E transport activity of RLIP76 that is necessary for endocytosis (57, 68). Studies have also shown that RLIP76 binds to and is sequestered in the cytoplasm bound to the master stress/heat-response transcription factor Hsf-1, in a complex that includes HSP90 and α -tubulin (62). Furthermore, we have shown that Hsf-1 and POB1 inhibit the GS-E transport activity of RLIP76 by binding at distinct sites, and that both proteins combined cause nearly complete inhibition; the resultant increased cellular GS-E and their precursors in cells triggers massive apoptosis and necrosis in lung cancer cells (68). Whether Hsf-1, POB1, and lipid-peroxidation byproducts can play a similar role in kidney cancer is not known yet.

Molecular and cellular mechanisms of RLIP76

ATP-dependent, primary active transport of GS-E by RLIP76, arising from chemotherapy drugs or electrophilic and pro-apoptotic products of peroxidation of endogenous cellular lipids (particularly ω -6 fatty acids), is a central effector mechanism necessary for survival and motility of cancer cell. This idea has been substantiated in multiple studies of human cancer cell lines that represent the majority of all human neoplasia, and in several xenograft models (19, 20). Because RLIP76 functions to remove metabolites of mutagenic compounds, its loss should increase the levels of mutagens in cells exposed to xenobiotics, ω -6 fatty acids or chronic excessive oxidative stress, and consequently, lead to a greater incidence of cancer. We developed the RLIP76^{-/-} mouse to test this hypothesis. RLIP76^{-/-} mice are severely deficient in GS-E transport activity, CDE, PKC α -signaling, and p53-signaling, and are also cancer resistant (57). Despite having 2–3 fold higher GSH levels, the RLIP76^{-/-} mice are extremely sensitive to radiation and to chemical toxins metabolized by the MAP. The knockout mice have up to 7-fold elevated total lipid peroxidation, 4-hydroxynonenal (4HNE), its glutathione-conjugate (GS-HNE) and the aldose-reductase (AR)-mediated reduced metabolites, dihydroxynonenol (DHN) and GS-DHN, derived from peroxidation of endogenous lipids, a necessary pre-requisite for X-irradiation-induced apoptosis (11,41–43,45). The radiation-sensitivity of RLIP76^{-/-} mice is particularly remarkable considering that expression of heat-shock proteins, which play a major role in radiation-resistance, is markedly increased in these mice, but is insufficient to provide radiation protection in the absence of RLIP76. The molecular mechanisms linking RLIP76 to heat-shock proteins have been recently elucidated by studies showing that RLIP76 is the primary regulator of the activity of Hsf-1 (heat-shock factor-1), the master transcriptional regulator of the heat-shock/stress-shock response (62,68). 4HNE is a potent pro-apoptotic and genotoxic agent, whereas its metabolites are not. Higher levels of these compounds were predicted to increase cancer risk after exposure to a chemical carcinogen such as benzo[a]pyrene, which is metabolized by cytochrome p450 to electrophilic and ultimately carcinogenic diol-epoxides. These diol-epoxides are metabolized to corresponding GS-E by GST, and are also substrates for efflux by RLIP76 (38). RLIP76^{-/-} mice are highly resistant to lung carcinogenesis induced by benzo[a]pyrene, and skin-carcinogenesis induced by phorbol ester (PMA). In RLIP76^{-/-} mice, these compounds cause neither epithelial carcinogenesis nor activation of either PKC α or p53, indicate an existential requirement of RLIP76 for cancer (57). These findings and the novel relationships among glutathione-metabolism, cell survival and cell motility pathways suggested by other studies lead us to propose a novel paradigm in which GS-E transport by RLIP76 plays a central role in the malignant phenotype of kidney cancer (Fig. 2).

Studies from the our group also investigated the signaling effects of RLIP76 antisense with sorafenib, sunitinib and temsirolimus, as well as the ability of RLIP76 to transport ³H-sorafenib and ³H-sunitinib. Results of these studies revealed that sorafenib as well as sunitinib are substrates for transport by RLIP76, and thus are competitive inhibitors of GS-E transport. Furthermore, inhibition of kinase activity in the ERK and PI3K pathways as a result of RLIP76 depletion is more profound, consistent, and widely apparent in a number of kidney cancer cell lines (22).

To address whether RLIP76 plays a crucial role in defending kidney cancer cells from radiation and chemotherapeutic toxin-mediated apoptosis, authors have performed *in vivo* studies demonstrating that administration of RLIP76 antibodies, siRNA or antisense to *nu/nu* nude mice bearing Caki-2 kidney cancer cells causes significant regression of established subcutaneous xenografts (21). These studies suggest that depleting RLIP76 will cause apoptosis in kidney cancer because of RLIP76's a key survival function as a GS-E transporter that prevents accumulation of toxic and proapoptotic lipid-peroxidation products in cells. These findings are potentially of major clinical significance because they put forward targeting of RLIP76 as a unique, highly effective and functionally cancer-specific targeted therapy for kidney cancer. In addition, these studies provide a previously unknown functional link between mercapturic acid metabolism, cellular motility, endocytosis, and apoptosis.

Because kidney cancers contain significantly more RLIP76 as compared with normal tissues, it is possible that the cytoprotective function of RLIP76 could play an important role in kidney cancer. This reasoning was bolstered by recent observations that targeting the mTOR pathway provides a valuable and effective therapy for kidney cancer; recent studies by others have shown that RLIP76 functions through an mTOR independent protective mechanism (33, 35). Results of our studies demonstrate that targeting of RLIP76 for depletion by antisense DNA or siRNA, or inhibition by an antibody caused marked regression of established Caki-2 xenografts after a single dose (21). Weight gain of mice was not affected by depletion or inhibition of RLIP76, and no signs of toxicity as assessed histologically were seen even after >50% depletion of RLIP76 in normal tissues such as heart, lung, liver, and kidney (46). This degree of efficacy and concomitant lack of toxicity compares favorably with other biologically targeted pre-clinical approaches (Table I).

Studies using RLIP76^{-/-} mice have shown that RLIP76 is the primary rate determinant of CDE (41, 45, 57), an important component of responses to hormonal signaling and regulator of cellular growth, differentiation, architecture, mobility, and death. RLIP76 is the first known effector of Ral, which regulates cell structure and motility (11,19,56,60–62,69,70). In addition, studies in a broad spectrum of cancers have shown that RLIP76 content correlates directly and closely with CDE as well as with resistance to chemotherapy drugs. Kidney cancer is a clear exception, in which CDE is nearly absent, but the transport activity and drug-resistance phenotype typically associated with over-expression of CDE remains intact. The lack of CDE in kidney cancer appears to be due to over-expression of truncated inhibitory splice-variants of RLIP76, and can be reversed by over-expression of wild-type RLIP76. This suggests the possibility that loss of normal RLIP76 function is the cause of loss of CDE, and perhaps underlies the unique signaling pathway characteristics in kidney cancer. Despite the lack of CDE, kidney cancer cells still undergo apoptosis upon RLIP76 depletion; this indicates that the anti-apoptotic function of RLIP76 is mediated primarily through its effects on preventing accumulation of pro-apoptotic lipid-peroxidation products through its GS-E transport activity. Most interestingly, RLIP76 inhibition causes very marked and consistent changes in ERK and PI3K in several kidney cancer cell lines, suggesting that targeting RLIP76 could be more effective than targeting ERK or PI3K agents clinically. Present review demonstrate whether the anticancer mechanisms triggered by RLIP76 depletion are related to its transport function, to determine the relationship between

these mechanisms and the known mechanisms proposed for the action of existing clinical therapies such as sunitinib, sorafenib, or temsirolimus, and to determine whether kidney cancers can be radio-sensitized by depleting RLIP76.

RLIP76 depletion affects heat-shock responses in kidney cancer cells

Studies by Hu and Mivechi (62) have shown that RLIP76 is a primary regulator of Hsf-1, the transcription factor considered the master controller of the heat-shock response. Numerous heat-shock proteins and other chaperones are transcriptionally up-regulated by Hsf-1 in response to chemical oxidant stressors as well as radiant stressors that augment cellular lipid peroxidation levels. In RLIP76^{-/-} mice, expression of heat-shock proteins such as Hsp1 α , Hsp40, Hsp105, mammalian stress protein 1, stress-induced phosphor-protein 1 and insulin-like growth factor binding protein, have all been shown to be increased 2–5 fold by real-time qPCR (71). Whether modulation of expression of heat-shock protein by depleting or inhibiting RLIP76 happens in kidney cancer cells is not known yet. However, if it does occur, it is possible that cells with increased Hsf-1 levels would have a relatively lower RLIP76 transport activity due to inhibition, such that the effects of RLIP76 depletion could be exacerbated. In RLIP76^{-/-} MEFs, transfection with wild-type RLIP76 functions to suppress Hsf-1 activity to the base line level seen in RLIP76^{+/+} MEFs, whereas Hsf-1-binding-deficient mutants of RLIP76 should not be able to suppress Hsf-1 (68).

RLIP76 in kidney cancer

GS-E transport by RLIP76 plays a central and essential role in protecting cancer cells from apoptosis caused by endogenous and exogenous electrophilic compounds (11, 13, 19). The existential need of RLIP76 for cancer is highlighted by our studies showing that mice deficient in RLIP76 are highly resistant to cancer caused by one of the most potent known ultimate (initiator and promoter) carcinogens, benzo[a]pyrene (57). Other investigators have identified RLIP76 as the first known Ral-effector, and predicted that it is a link between the Ral pathway that controls cell motility and membrane plasticity, and the Ras pathway that serves an important survival function (56, 60, 61, 69, 70). The molecular mechanisms for these functions were unknown, and have been elucidated by our group in multiple models, including the knockout mouse (19, 41). We have established that RLIP76 is a substrate-stimulated ATPase that couples ATP-hydrolysis with movement of substances (substrates transported across membranes are referred to as allocrites). Purified RLIP76 is necessary and sufficient to cause the trans-membrane movement of mercapturic acid-precursors and glutathione-drug/metabolite-conjugates when reconstituted in completely artificial liposomes (10, 12, 38, 65). Blocking the transport function sensitizes cancer cells to chemotherapy drugs. Even in the absence of chemotherapy drugs, inhibition of the trans-membrane transport activity of RLIP76 causes apoptosis selectively in cancer cells, which appear to rely more heavily than normal cells on the ability of RLIP76 to minimize cellular concentration of highly pro-apoptotic endogenous metabolites originating from oxidation of ω -6-fatty acids (linoleic and arachidonic acids) in cellular membranes. Our observations that GS-E transport and CDE are deficient in RLIP76^{-/-} mice, and that RLIP76 mutants lacking GS-E transport activity also do not support endocytosis, serve to reconcile the two views of RLIP76 (as a Ral-effector and as a GS-E transporter) (57).

Kidney cancer cells frequently have mutations in the *VHL* tumor suppressor gene, which result in inappropriate accumulation of HIF α , thereby driving the transcription and secretion of growth factors, including VEGF, PDGF, TGF α , and EGF (type 1 ligand), and erythropoietin (3,30,72–79). These in turn bind their cognate receptors and activate signaling in the Ras and PI3K pathways. The Ras pathway is activated in more than half of all kidney cancers (17, 18, 80). Interruption of Ras signaling is known to induce apoptosis in kidney cancer cells, and this formed the rationale for sorafenib, sunitinib, and other receptor tyrosine kinase inhibitors (RTKIs), which can terminate this signaling (2,81). We have found that phosphorylation of ERK and PI3K is dramatically and consistently decreased in human kidney cancer cell lines upon RLIP76 depletion, to a greater extent than that observed with RTKIs such as sunitinib, sorafenib, or temsirolimus (22). Furthermore, our studies using radio-labeled ³H-sorafenib and ³H-sunitinib provided the initial demonstration that these two drugs are substrates for transport by RLIP76, and thus are competitive inhibitors of GS-E transport (22). These findings raise that possibility that a significant mechanism of action of these drugs in kidney cancer could also be through apoptosis related to increased accumulation of HNE and GS-HNE. In addition, RLIP76 may have a role in mediating radiation-resistance in kidney cancer cells. Because a primary mechanism of cell killing after X-irradiation is through the genotoxic effects of lipid-hydroperoxides produced as a consequence of OH radicals produced as a result of radiation, these findings offer additional evidence for the overall model in which RLIP76 protects cells by removing toxins generated during oxidative stress.

Collectively, studies of RLIP76 in kidney cancer indicate that it serves as a key effector function in cancer cell survival and is a valid target for cancer therapy. They also confirm that inhibitory modulation of RLIP76 transport activity at the cell surface is sufficient for antitumor effects, indicating that the GS-E transport function of RLIP76 is a central apoptosis preventing and invasion promoting mechanism in kidney cancer.

RLIP76 inhibition

Because there are no known specific small molecule inhibitors of RLIP76, and because anti-RLIP76 IgG appears to be satisfactory pharmacological inhibitor, yielded significant remissions of cancer in xenograft models, our laboratory have pursued antibody approaches to inhibiting RLIP76. We have developed rabbit-anti-human anti-RLIP76 antibodies to full length RLIP76 as well as to the cell surface epitopes of RLIP76 (comprised of aa^{171–185}) and shown that the purified IgG fraction from these antibodies are effective at triggering apoptosis in cancer cells and at inducing regression in established xenografts of kidney cancer (21). As controls, we developed corresponding antibodies to intracellular epitopes of RLIP76, which do not inhibit the transport activity of RLIP76 in intact cells and do not cause apoptosis (66).

RLIP76 depletion

Our laboratory have studied a number of siRNA and phosphorothioate antisense DNA molecules targeted to different regions of RLIP76, and concluded that the most specific and effective depletion occurs when targeting the nucleotide region 510–555 (encoding

aa^{171–185}). The methods used to select and design the particular siRNA and antisense DNA constructs are published, and the resulting targeting agents have been shown to be effective and selective in depleting RLIP76 (47,63).

Striking anti-neoplastic effects that are not associated with evident toxicity (in terms of either weight loss or metabolic effects) have been seen for the antibody, antisense and siRNA in a kidney cancer xenograft model of Caki-2 cells (21). Hsd: Athymic *nu/nu* nude mice were injected subcutaneously in the flanks with 2×10^6 Caki-2 cell suspensions in 100 μ l PBS. When tumors reached a cross-sectional area of $\sim 40 \text{ mm}^2$, animals were randomized for treatment with pre-immune serum, scrambled siRNA, scrambled antisense DNA, RLIP76 antibodies, RLIP76 siRNA and RLIP76 antisense. Treatment consisted of 200 μ g of RLIP76 antibodies, siRNA or antisense in 100 μ l PBS, intraperitoneally. Control groups were treated with 200 μ g/100 μ l pre-immune serum, scrambled siRNA or scrambled antisense DNA. Tumors were measured in two dimensions using calipers. Treated mice had rapid and dramatic reductions in tumor sizes, whereas uncontrolled tumor growth was observed in the control groups. Xenograft-bearing mice treated with RLIP76 antibody, RLIP76 siRNA or RLIP76 antisense were alive at 177 days without evidence of recurrence, whereas control mice were censored by day 42. Weight gain was comparable to non-tumor-bearing controls, and no overt toxicity was evident.

Histopathological examination of paraffin-embedded tumor xenograft sections as observed by hematoxylin and eosin staining revealed that RLIP76 depletion reduced the number of tumor blood vessels and restored normal morphology, when compared to controls. Collectively, *in vitro* and *in vivo* studies revealed that depletion of RLIP76 had potent antiproliferative, antiangiogenic and prodifferentiation properties in kidney cancer, yet spared normal cells.

The effectiveness of the RLIP76 antisense to deplete RLIP76 in mouse tissues was also demonstrated in separate studies of non-tumor-bearing C57B mice by sacrificing animals for analyses at 24 h after a single *i.p.* dose of RLIP76 antisense (200 μ g/100 μ l PBS/mice). Western blot analyses of tissues confirmed detectable level of RLIP76 in tissues from scrambled antisense treated animal and undetectable RLIP76 in RLIP76 antisense treated animals (41, 46). Overall, all three agents exhibited marked anti-neoplastic effects. These findings indicate that RLIP76 is a key survival protein for kidney cancer cell, and that its depletion/inhibition results in regression of human kidney cancer xenografts without any apparent toxicity to animals.

The marked effectiveness of RLIP76-targeted therapy compares favorably with other promising targeted agents in kidney cancer. ZD1839 (gefitinib, Iressa), a tyrosine-kinase inhibitor, reduces the rate of growth of SKRC-49 renal cell xenografts in nude mice when administered by gavage daily for 15 days. The combination of paclitaxel and ZD1839 was more effective in slowing growth, but regression was not observed (82). Ly294002, a PI3K inhibitor, did cause delayed and incomplete regression by 30 days in xenografts of 786-0 renal cell carcinoma (*VHL*-deficient), when treatment was started before established visible tumor was present (83). Verotoxin, a Gb3 (globotriaosylceramide) targeting agent, caused regression of very small tumors, but if treatment was started when ACHN renal cell tumors

were established (at least 6–7 mm in each dimension), relatively little growth inhibition was seen (84). An anti-CD26 monoclonal antibody was effective in xenografts of Caki-2 (*VHL*-expressing), but growth inhibition occurred rather than regression (85). The NFκB-targeted drug BAY-11–7085 has recently been shown to nearly completely inhibit the growth of established Caki-1 or 786-0 cell xenografts, but also did not cause regression (86). Incomplete regression was seen by day 30 in xenografts of SKRC-52 (*VHL*-deficient) renal cell carcinoma treated with DAPT (γ-secretase inhibitor) targeted at interrupting Notch signaling (87). Thus, targeted therapeutics for kidney cancer cell aimed at RTK, PI3K/Akt, NFκB, or Notch do not appear to be as effective as RLIP76 inhibition or depletion in comparable animal xenograft models. It should also be noted that xenograft studies of sorafenib, sunitinib, temsirolimus, or other agents in development have not demonstrated this dramatic an effect. The greater effectiveness of RLIP76 targeted therapy can be understood in terms of a chemical as well as biochemical signaling model (Figs. 1 & 2).

Studies from the Liu laboratory (88) demonstrated that RLIP76 is over-expressed in U937 cells and the knockdown of RLIP76 expression by shRNA inhibited proliferation, induced apoptosis, blocked cell cycle progression, and increased chemo-sensitivity to daunorubicin in these cells by inducing caspase-mediated apoptosis. This effect occurred through upregulation of the proapoptotic protein Bax and downregulation of cyclin D1, cyclin E, and Bcl-2, in a dose-and time-dependent manner. Expression of cleaved PARP was also increased after RLIP76 depletion as a function of dose and time. Collectively, these results indicate that induction of apoptosis in U937 leukemia cells by RLIP76 shRNA probably occurs through a caspase-dependent pathway and suggesting RLIP76 as regulator of expression of apoptosis-related molecules. Interestingly, normal cells were unaffected by RLIP76 shRNA treatment. RLIP76 knockdown also significantly enhanced the cytotoxicity of daunorubicin, and the combination of daunorubicin and RLIP76-targeted shRNA exerted a greater anti-proliferative effect than daunorubicin treatment alone, confirming the role of RLIP76 in chemoresistance in U937 cells (88).

Wang et al. and Wu et al. reported that over-expression of RLIP76 is associated with neoplasia and is necessary for tumor growth and bone metastasis. Depletion of RLIP76 diminished orthotopic tumor growth of PC3 cells and inhibited spontaneous metastasis (23,27). RLIP76-targeted therapy using antibodies, siRNA, or antisense led to durable and complete remission in xenograft models of human cancers (13). These studies support the *in vivo* anticancer efficacy of RLIP76 depletion and the lack of toxicity to non-cancerous tissues; suggest that therapeutic strategies targeting RLIP76 may provide a broad-spectrum anti-neoplastic approach. p53 is a well-characterized transcription factor that functions as a tumor suppressor, inhibiting cell growth and inducing apoptosis in response to DNA damage, primarily through induction of p21 and Bax. Many human cancers contain a p53 mutation, which can mediate resistance to chemotherapy-induced apoptosis. Thus, the studies from the Chen laboratory concluded that RLIP76 may trigger apoptosis of tumor cells, irrespective of p53 status (27).

In another study, Wang et al. also reported that inhibition of RLIP76 expression in U87 and U251 glioma cell lines by RLIP76 siRNA significantly suppressed tumorigenicity and induced apoptosis in an endotopic xenograft mouse model independent of p53 status.

Survival probability was higher in glioma patients exhibiting lower RLIP76 expression. These studies indicate that RLIP76 over-expression is associated with higher tumor grade and shorter survival, and that inhibition of RLIP76 signaling may be a potential treatment for malignant glioma. These results also suggest that RLIP76 expression may be a key prognostic index for cancer patient survival (27, 28). The potential use of targeting RLIP76 in combination with other chemotherapy agents is another area of active research. Wang et al. (28) evaluated the combined effects of RLIP76 shRNA plus the antitumor drug temozolomide, on the U87 and U251 glioma lines *in vitro*. Combined RLIP76 knockdown and temozolomide treatment inhibited cell proliferation *in vitro* more effectively than either treatment alone. Furthermore, RLIP76 downregulation enhanced chemosensitivity to temozolomide. These cell culture studies have led to important insights but do not prove the *in vivo* efficacy of the combinatorial effect of RLIP76 and chemotherapy drug(s).

A previous study by Drake et al. has demonstrated that RLIP76 over-expression confers to broad resistance of multiple chemotherapy including cisplatin, melphalan, doxorubicin, and mitomycin-C, and inhibition of RLIP76 using antibodies results in increased cytotoxicity, indicating the potential role of RLIP76 as a target in the treatment of leukemia (89).

Radiation sensitivity

We have shown that RLIP76 confers radiation-resistance in normal and in cancerous cells, and that the overall effect of RLIP76 depletion is greater than the effects exerted by signaling proteins including Akt, JNK and MEK (42, 45). Based on studies of RLIP76^{-/-} mice and other histologies of cancer cells, we reasoned that RLIP76 modulation would directly affect radiation-sensitivity and resistance of kidney cancer cells. To test this postulate, we determined the sensitivity of Caki-2 human kidney cancer cells to X-irradiation in dose-response studies using 100–1000 cGY single dose X-irradiation, followed by colony-forming assays. Cells pretreated with RLIP76-liposomes were least sensitive to radiation, and delivery of recombinant RLIP76 to cells *via* a liposomal delivery system completely reversed radiation-sensitivity. At each radiation dose, survival was significantly greater when cells were pretreated with RLIP76-liposomes before radiation exposure. These findings confirmed that RLIP76-liposomes are radio-protective agents (22, 42) and suggest that depleting RLIP76 may be effective in improving the efficacy of radiation therapy in kidney cancer.

The results of these studies showed that both, 4HNE as well as GS-HNE were increased by about three-fold in the RLIP76^{-/-} mouse liver tissue (42,43). These studies demonstrated for the first time that loss of RLIP76 results in the accumulation of endogenously generated electrophiles and their GSH-conjugates *in-vivo*. A key element of this review, that RLIP76 depletion will uniformly result in increased 4HNE and GS-HNE levels in kidney cancer, can be directly assessed through a novel and highly sensitive and specific LC-MS assay for measurement of GS-HNE, 4HNE, as well as other ω -6 fatty acid metabolites (42, 43).

Discussion

RLIP76 was discovered simultaneously by three different groups 20 years ago (60, 69, 70) as a downstream effector for the Ral GTPases. It originally had three names: RLIP76 (in human), RalBP1 (in rat) and RIP1 (in mouse), although the former two are now most commonly used. The proposed mechanism of action for RLIP76-targeted therapy is radically different from most current approaches, which are largely focused on chemicals that modify kinases or phosphatases. The rationale of this review is based on an older observation, that cancer cells have increased activity of the MAP, typified by increased levels of the previously considered rate limiting step of mercapturic acid production, GSTs (50). Because a large number of carcinogens and alkylating or platinum containing antineoplastic agents are metabolized to mercapturic acids, and because genotoxic and apoptotic toxins generated from oxidative stress caused by high energy radiation must also be detoxified by metabolism to mercapturic acids, the MAP remains of critical important not only in carcinogenesis, but also in drug-resistance.

RLIP76 is upregulated in lung (26), bladder (90, 91) and ovarian (92) cancers, and intriguingly, has been shown to act as a membrane transporter that pumps GS-Es, including chemotherapeutics, out of cells (20). Blockade of RLIP76 by various approaches has been shown to increase the sensitivity of cancer cells to radiation and to synergize with chemotherapeutic drugs, such as anthracyclines and vinca alkaloids (vinorelbine) to further enhance apoptosis in cancer cells. These *in vitro* effects have translated to pronounced *in vivo* effects in tumor xenografts (26, 46). Another early study found that blockade of RLIP76 with specific antibodies synergized with doxorubicin to cause apoptosis in NSCLC (59). Conversely, ectopic administration of RLIP76, such as by transfection in cancer cell lines or with proteoliposomes *in vivo*, can restore transport and the efflux of xenobiotics, and enhance drug-resistance in the tumors, reversing the regressive effects of RLIP76 blockade (19, 63).

RLIP76 appears dispensable in un-stressed non-malignant cells, but is essential for cancer cells. The complete lack of CDE in the absence of RLIP76 (i.e., in RLIP76 knockout mice), and the necessity of the GS-E transport function of RLIP76 for CDE to occur, itself represents a novel paradigm. These studies offer strong support that RLIP76 is an overarching anti-apoptosis mechanism that, if inhibited, can be more broadly effective in the treatment of various carcinomas.

RLIP76 blockade is more effective than the currently available multi-kinase inhibitors, sunitinib, sorafenib, and temsirolimus at inducing regression of in kidney cancer growth in pre-clinical xenograft studies. RLIP76 depletion also reduced expression of p-ERK and p-PI3K in various kidney cancer cells, and to a significantly greater extent than any of the three clinically available kidney cancer drugs such as sunitinib, sorafenib, and temsirolimus known to target these pathways (22). As expected, sorafenib and sunitinib affected the ERK pathway more than the PI3K pathway, which was preferentially affected by temsirolimus; however, these effects were inconsistent among different cell lines as compared with the effect of RLIP76 antisense. Blockade of RLIP76 may enhance the activity of sunitinib and sorafenib because these drugs are transported substrates of RLIP76 (22). These studies show

that kidney cancer cell lines studied were very sensitive to apoptosis by targeted depletion of RLIP76, and those important survival kinases PI3K and ERK activation was inhibited by RLIP76 depletion.

RLIP76 depletion or inhibition will increase the cytotoxicity of chemotherapy drugs that are subject to transport by increasing the accumulation of these drugs in cells upon inhibition or depletion of RLIP76. Targeting RLIP76 for depletion or inhibition led to growth regression of xenografts of kidney cancer cell lines independent of *VHL* mutation or TRAIL sensitivity, indicating that targeting RLIP76 represents an exceptionally broad-spectrum effective anti-neoplastic strategy. Based on these observations, we conclude that cell proliferation and apoptosis are valid biomarkers to assess RLIP76 response in future clinical trials. Herein, we have discussed recent work supporting the validity of RLIP76 as a target in kidney cancer, and the functional model in which RLIP76 provides protection from chemical and radiant stress through its transport activity. These studies also show that anti-RLIP76 IgG, RLIP76 siRNA and RLIP76 antisense alone exerts antineoplastic activity in xenografts model of human kidney cancer, and that RLIP76 depletion increases radiation-sensitivity of kidney cancer cells. We thus conclude that function of RLIP76 as a multi-specific stress-protective GS-E / drug-transporter is necessary for the characteristic apoptosis-resistant phenotype of kidney cancer.

Conclusions

Because cancer is a multi-stage process, it requires multi-targeted approaches in order to develop an effective treatment regimen. Studies summarized in this review article provide strong evidence that RLIP76 has therapeutic properties in kidney cancer yet may not cause side effects that plague so many current cancer therapies. RLIP76 depletion causes significant cytotoxicity to cancer cells and also inhibits angiogenesis, which is the key survival mechanism for kidney cancer growth and metastasis. In addition, identification of additional targets of RLIP76 will help us understand the molecular mechanisms underlying its anticancer properties. Preventing and treating advanced kidney cancer involves choosing drugs that not only efficiently kill tumor cells but also spare normal cells to avoid potential loss of kidney function. Therefore, the multi-target-mediated tumor inhibition caused by targeting RLIP76, which does not appear to be cytotoxic to surrounding normal mesangial cells, represents a novel and effective strategy for the treatment of kidney cancer in the clinical setting. Therefore, the impact could be potentially very significant in decreasing both the incidence and prevalence of kidney cancer.

Depleting RLIP76 by antisense or siRNA, or inhibiting it by antibody, causes sustained regression of human kidney cancer xenografts in nude mice without detectable toxicity (21). Apoptosis caused by RLIP76 depletion is associated with reduced activation of survival pathways including PI3K, ERK and Akt. These reductions are more marked and more consistent than observed with sorafenib, sunitinib, or temsirolimus, three multi-kinase inhibitors used in therapy of kidney cancer.

The marked, broad-spectrum effectiveness of RLIP76 targeting suggests that RLIP76 could play roles in fundamental mechanisms of carcinogenesis and drug-resistance common to

most cancers. Because RLIP76 depletion is effective in a number of cell types, including p53 null, Ras- or *VHL*-mutated, or TRAIL resistant, it is certainly possible that neither these, nor PI3K or ERK are necessary for its apoptotic effects.

The cancer-specific nature of RLIP76-targeting seems apparent from studies in mice, which have shown no deaths due to treatment, and no overt effects on blood counts or histology. Indeed, the most remarkable effects seen with RLIP76 antisense administration are a 30–40% decrease in blood glucose, cholesterol and fatty acids, all of which are apparently beneficial. We do not know whether a similar non-toxic and beneficial profile will be observed in higher animals; primate studies are thus mandated.

These findings also suggest that RLIP76-targeting could be developed into a more effective therapy for kidney cancer, because cells would undergo apoptosis rather than transformation due to the lack of RLIP76, indicates that RLIP76 represents a fundamental mechanism for protection of cancer cells against apoptosis. Most likely, drugs targeting RLIP76 will be most useful in combinatorial therapies with classical chemotherapeutic agents. Therefore, this review focuses on identifying and characterizing membrane transport mechanisms which mediate drug-resistance in malignancies.

Relevance to Human Health

RLIP76 is a multifunctional transporter and signaling protein functioning as a xenobiotic/oxidative-stress/radiation defense mechanism. Despite improvement in clinical response rates with newly developed targeted multi-kinase inhibitors, death due to resistance, metastases, or toxicity remain major impediments to the cure of kidney cancer. Recent publications have shown that RLIP76 blockade is highly effective in causing regression of tumor growth in kidney cancer xenografts and that blocking RLIP76 also inhibits invasion. These findings are potentially of major clinical significance because they promise a unique, highly effective and functionally cancer-specific targeted therapy for human kidney cancer. These findings are also of pivotal basic significance because they define a novel paradigm that provides a previously unknown functional link between mercapturic acid metabolism, cellular motility, endocytosis, and apoptosis. Given the specific effects of RLIP76 depletion that regulate both the incidence and progression of kidney cancer, we strongly believe that evaluation of RLIP76 efficacy in pre-clinical models will have a significant impact on the rational use of RLIP76-blocade in human kidney cancer control.

In summary, RLIP76 expression is positively correlated with the degree of malignancy, and RLIP76 over-expression predicts decreased survival of cancer patients. Over-expression of RLIP76 enhanced tumorigenicity and suppressed apoptosis, whereas downregulation of RLIP76 expression significantly suppressed tumorigenicity and promotes apoptosis. Furthermore, RLIP76 and its downstream effectors Rac1 and JNK are crucial signaling molecules regulating the tumorigenicity and apoptosis of cancer cells, identifying RLIP76 as an ideal prognostic marker and an attractive therapeutic target for the treatment of cancer. Collectively, the current review shows that RLIP76 is a novel therapeutic effect, which is relevant for controlling and treating kidney cancer.

Future Perspectives

We predict that the depletion or inhibition of RLIP76 will cause apoptosis and necrosis in kidney cancer cells by increasing accumulation of proapoptotic electrophilic lipids, particularly 4HNE and GS-HNE. Accumulation of these metabolites can trigger proliferation, differentiation, and cytokine responses at low concentrations, and apoptosis or necrosis at high concentrations. Depletion or inhibition of RLIP76 will increase the sensitivity of kidney cancer cells to chemotherapeutic agents because of increased accumulation of the drugs in cells. RLIP76 suppression should increase the radio-sensitivity of kidney cancer because of the increased accumulation of proapoptotic electrophilic lipids and their glutathionylated metabolites, which need RLIP76 for efflux from cells. The ability of RLIP76 to suppress angiogenesis and VEGF expression in kidney cancer, without affecting normal cells, represents a much-desired ability of an ideal therapeutic drug for kidney cancer. Thus, RLIP76 therapy could represent a reasonably safe, effective and clinically relevant choice for kidney cancer in humans. The current body of research warrants further studies to standardize the doses, routes of administration, organ specificity and bioavailability in humans.

As a Ral effector, RhoGAP, and adapter protein, RLIP76 has been shown to play important roles in endocytosis, mitochondrial fission, cell spreading and migration, actin dynamics during gastrulation, and Ras-induced tumorigenesis. Additionally, RLIP76 is also important for stromal cell function in tumors, as it was recently shown to be required for efficient endothelial cell function and angiogenesis in solid tumors. However, RLIP76 knockout mice are viable, and blockade effects appear to be selective for implanted tumors in mice, suggesting the possibility that RLIP76-targeting drugs may be successful in clinical trials. In this review, we outline many cellular and physiological functions of RLIP76 in normal and cancer cells, and discuss the potential for RLIP76-based therapeutics in kidney cancer treatment.

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The abbreviations used are

RLIP76	<u>R</u> al- <u>i</u> nteracting protein
CDE	clathrin-dependent endocytosis
GSH	glutathione
GS-E	glutathione electrophile conjugates
4HNE	4-hydroxy nonenal
PI3K	phosphatidylinositol 3-kinase
RCC	renal cell carcinoma

VEGF	vascular endothelial growth factor
VHL	von Hippel-Lindau

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Figure 1. Schematic representation of RLIP76 domains

RLIP76 is a 655 amino acid protein with ATP binding sites at residues 69 to 74 and 418 to 425. The membrane-binding domain is from residues 154 to 219. The NH₂ terminus has an AP2 binding domain. The middle domain has the Rho/Rac GAP function. RLIP76 plays a role in the linking of Ras and Ral. RLIP76 can also regulate signaling downstream of Ras to cJun through its GAP activity toward the Rac/Rho family GTPase. Termination of signaling is mediated by endocytosis of the receptor-ligand complex. We propose that the ATPase activity of RLIP76 plays a significant role in this process, possibly as an energy transducer, and that this ATPase activity is coupled with efflux of endogenous substrate allocrites, particularly GS-E. The Ral-binding domain and POB1/cdc2 binding domain are the first half and second half of the COOH terminus region, respectively. RLIP76 plays a key effector role in different signaling pathways through its GS-E transport activity.

Figure 2. Proposed model for RLIP76 function

A model for control of signaling by RLIP76 through regulation of cellular levels of HNE and its metabolites (GS-HNE and GS-DHN). RLIP76 is a transporter of glutathione-conjugates of carcinogenic electrophiles (GS-E) arising from xenobiotic compounds as well as endogenously generated electrophilic compounds, particularly metabolites of lipid-peroxidation of ω -6 fatty acids (linoleic, γ -linolenic, arachidonic). Excessive consumption of ω -6 fatty acids is known to be associated with an increased risk for cancer.

Table 1

Relative efficacy of targeted therapies in kidney cancer xenograft models

Cell Line	Target	Agent	Dose	Response	Ref
Caki-1	CD26	anti-CD26	10 mg <i>i.p.</i> d 4–60	Inhibited progression	85
786-0	PIK3	LY-294002	75 mg/kg/wk <i>i.p.</i> × 4 wk	~50% regression	83
SKRC-52	γ -secretase	DAPT	10 mg/kg <i>s.c.</i> d 1–3 q wk × 5	Inhibited progression	87
SKRC-49	EGF-RTK	ZD-1839	100 mg/kg <i>p.o.</i> (with paclitaxel 2 mg/kg) d 1, 8, 15	Inhibited progression	82
786-0 & Caki-1	NF κ B	BAY 11-7085	5 mg/kg <i>i.p.</i> × 35 d	Inhibited progression	86
ACHN	Gb3	Verotoxin	0.1 μ g <i>i.p.</i> × 1	Regression and rapid regrowth	84