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RLIP76 and Cancer

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Abstract

RLIP76 is a multifunctional membrane protein that transports glutathione conjugates of electrophilic compounds and other xenobiotics including chemotherapy agents out of cells. The protein is overexpressed in lung carcinomas, ovarian carcinomas, and melanomas. The protein also binds Ral and participates in mitotic spindle function, clathrin-dependent endocytosis, and triggers GTPase-activating protein activity. It is found throughout the cell, in membrane, cytosol, and the nucleus, and is known to shift between these compartments in response to stress. Loss of RLIP76 by antibody or antisense therapy is associated with increased sensitivity to radiation and chemotherapy. Conversely, liposomally delivered RLIP may treat poisoning and wounds.

Background

Cellular free radicals, compounds with unpaired electrons, may come from damaged mitochondria with leaky electron transport chains (1), adjacent inflammatory leukocytes with NADP oxidase complexes (2), and exposure to drugs or external ionizing radiation (3). Most biological free radicals react with oxygen or nitric oxide to yield reactive oxygen and nitrogen species such as the hydroxyl radical and peroxynitrite, respectively. These highly chemically reactive molecules modify DNA (particularly mitochondrial and telomeric DNA), proteins (including DNA repair enzymes), and lipids. One DNA product, 8-hydroxy-2'-deoxyguanosine, generates G to T transversions. The lipid oxidation product, 4-hydroxynonenal (Fig. 1A), signals critical intracellular pathways regulating proliferation and apoptosis (4). Excess cellular free radicals injure proteins and genes of the mitochondrial electron transport system amplifying the oxidative stress. Excessive oxidative stress that overcomes cellular antioxidant systems results in genotoxic stress with mutations of DNA repair and tumor suppressor genes, among others. Cells that survive these conditions may undergo malignant transformation. Evidence supporting this model includes the association of mutations in mitochondrial fumarate hydratase, succinate dehydrogenase, and cytochrome C oxidase with leiomyomas, paragangliomas, and prostate cancers, respectively (5). Loss of functional p53 secondary to G to T transversions is observed in lung and liver cancers (6). Inflammation with ulcerative colitis, Barrett's esophagus, *Helicobacter* gastritis, and asbestosis is linked to colon cancer, esophageal cancer, gastric cancer, and mesothelioma, respectively (2,6).

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Disclosure of Potential Conflicts of Interest

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The neoplastic cells avoid apoptosis by controlling intracellular free radical concentrations as well as by removing checkpoint controls such as p53. Glutathione (GSH) is the major intracellular free radical scavenger. Cancer cells overexpress the phase II enzymes, glutathione-S-transferases, in particular the isoenzyme, glutathione-S-transferases π (7). Glutathione-S-transferases catalyze the conjugation of electrophiles including xenobiotics and the endogenous products of lipid peroxidation such as 4-hydroxynonenal to GSH (Fig. 1B; ref. 8). The GSH-conjugates (GS-E) are then removed from cells by phase III detoxification enzymes. This step is vital for the cell because the conjugation reaction is reversible and also the accumulation of the conjugates leads to toxicity. The major GS-E transporters in human cells are the ATP binding cassette transporters such as those of multidrug resistance protein family, and the non-ATP binding cassette transporter RLIP76 (Ralbp1). RLIP76 accounts for up to 80% of the GS-E efflux and is the major component of phase III detoxification system (Fig. 2A). RLIP76 expression is increased in a significant fraction of human tumors. Because RLIP76 is able to export the GSH-conjugates of alkylating chemotherapy agents such as melphalan as well as nonalkylating drugs such as doxorubicin and vinorelbine, it is a major factor in the mechanisms of drug resistance. This review details our understanding of this recently discovered membrane protein and analyzes its role in the natural history of cancers.

RLIP76, a GTPase-activating protein, was cloned as a Ral effector protein linking Ral GTPase to Rho pathway (9–12). Even before its cloning and characterization, the transport activity associated with RLIP76 was known during the isolation of a dinitrophenyl-S-glutathione conjugate-dependent ATPase (DNP-SG ATPase) activity from RBC (13–17). Transport function was assigned to RLIP76 when its identity with DNP-SG ATPase was finally established (11,16,18,19). DNP-SG ATPase was initially characterized in human erythrocytes, and its presence in most of human tissues was shown (15,20,21). Its versatility as a transporter was established by studies showing that it catalyzed the *trans*-membrane movement of not only GS-E but also of drugs such as doxorubicin (11) and colchicine (22), which do not form GSH-conjugates. Immunoscreening of a human bone marrow cDNA library yielded the previously known protein RLIP76. Extensive structural and functional characterization of bacterially expressed purified RLIP76 established its identity with DNP-SG ATPase. RLIP76 is a 76-kDa protein with 67- and 80-kDa splice variants (the latter is cytoctrin). The gene for RLIP76 is located on human chromosome 18p11 and is identical to Ral-binding protein 1 (11). RLIP76 is expressed in most human tissues including liver, heart, lung, muscle, kidney, RBC, and WBC as well in most human tumor cell lines (15,16,20,21). Homologous proteins are found in species as distant as *Drosophila* and man (12).

RLIP76 contains ATPase activity that is stimulated by GS-E, doxorubicin, sulfates, leukotriene C₄, vinorelbine, glucorinides, colchicine, and other organic anions and cations (20–25). Both in cells and in reconstituted proteoliposomes, native and recombinant RLIP76 can transport these allocrites across membranes (11,24–34). The transport requires ATP, is saturable, and is temperature dependent (11,24). The transport activity is modulated by protein kinase C α (30,35). Phosphorylated RLIP76 shows increased transport activity for its substrates (33). Its physiologic significance in mediating transport of endogenous and pharmacologic GS-E is indicated by the fact that the V_{max} for LTC₄ efflux is ~25-fold higher for RLIP76 than multidrug resistance protein. Furthermore, anti-RLIP76 antibodies block 80% of GSHNE and doxorubicin transport in cell lines, and the inhibitors of multidrug resistance protein show much less (~30%) effect on transport activity (26). Absence of RLIP76 causes RBC lysis due to intracellular formation of mercapturic acids.

Although the primary amino acid sequence of RLIP76 is known (9,28), detailed three-dimensional structure for the molecule remains to be elucidated. This knowledge will be

important to understand RLIP76 transport function as well as other activities. The protein lacks the well-defined *trans*-membrane helices of ATP binding cassette transporters, and there are no other RLIP76 family members with solved structure. Nevertheless, we can estimate domain behavior from sequence analysis and mutational studies (Fig. 2B). There are two ATP binding sites—one at the NH₂ terminus (⁶⁹GKKKGK⁷⁴) and one at the COOH terminus (⁴¹⁸GGIKDLSK⁴²⁵; refs. 11,19). Both these sites are needed for substrate transport (19). The NH₂-terminal domain provides the membrane anchor. It has N-myristoylation sites at amino acid 21 to 26, 40 to 45, and 191 to 196. There are vector peptide-related sequences between amino acid 154 and 219 including the penetratin-like sequence ¹⁵⁴LTAADVVKQWEK¹⁷⁰, a TAT-like sequence from amino acid 170 to 185, and a hydrophobic stretch from amino acid 203 to 219. Both membrane association and transport requires this domain (36,37). The first central domain has Rac/cdc42 GAP activity, and the second central domain binds Ral. The COOH-terminal domain binds POB1, CDK1, and Hsf-1 (25). In a series of experiments over the last decade, an important role for RLIP76 in malignant cell survival and drug resistance and normal tissue response to oxidative stress has been confirmed.

In addition to its transport substrates/ATP/membrane association, RLIP76 has multiple protein binding partners including Ral, Hsf-1, AP2, POB1, CDK1, protein kinase C α , and cdc42. Ral binds to RLIP76 and triggers a GAP activity on cdc42 (36,38–42). RLIP76 binds and sequesters Hsf-1 with the consequence of reduced heat shock protein expression (38). RLIP76 binds to AP2 and POB1 and inhibits clathrin-mediated endocytosis and internalization of ligand-membrane receptor complexes of epidermal growth factor receptor, insulin receptor, and tumor growth factor β (39,40). This regulates normal receptor signaling. CDK1 binds RLIP76 and translocates the protein from the surface membrane to chromosomes (39). On chromosomes, the RLIP76 ATPase activity facilitates mitosis. Protein kinase C α phosphorylates the T²⁹⁷ site on RLIP76 enhancing its transport function (30,33,35). It has been hypothesized that RLIP76 serves as a central regulator of stress response (25,28,41). During oxidative stress, increase in intracellular levels of the GS-E and protein kinase C α activation leads to RLIP76 association with the membrane and release of Hsf-1 to transcribe heat shock protein genes. Endocytosis is maximal with appropriate limited duration responses to extracellular ligands. Reduction in oxidative stress is associated with binding of RLIP76 to Hsf-1 and AP2/POB1. Heat shock protein expression is reduced and endocytosis is inhibited. Responses to extracellular signals are modified. Finally, CDK1 transfers RLIP76 to the nucleus to participate in cell division. Ral is a Ras-like guanyl nucleotide-binding protein that is carboxy terminus-geranylated and regulates cell proliferation and survival (43). Cdc42 is a member of the small Rho GTPases controlling morphogenesis and actin dynamics (44). Because RLIP76 is a central regulator in multiple pathways that respond to redox states and control cell growth, motility, division, and apoptosis, future experiments will likely help understand some of the complex mechanisms balancing metabolism, the environment, and cell proliferation or death.

RLIP76 RNA in different human tissues was determined by Northern blots (10). RLIP76 protein expression in normal and malignant cells has been measured by three assays—Western blots, tissue extract ELISA, and immunohistochemistry (27,32,45–47). RLIP76 transport activity has been quantified using cultured cell crude membrane inside-out vesicles and radio-labeled doxorubicin (16,34,46). It has been shown that normal human tissues express RLIP76 transcripts, but expression varies considerably with 10- to 12-fold higher levels in ovary and skeletal muscle, 3- to 7-fold higher in thymus, prostate, testis, small intestine, heart, brain, placenta, and kidney, and lower levels in peripheral blood leukocytes, colon, pancreas, spleen, liver, and lung (10). RLIP76 protein is expressed in all normal tissues, immortalized normal cells, and cancer cell lines examined (Table 1). Again, differences in RLIP76 content were found among normal and between different types of

neoplastic cells. Erythrocytes, breast, heart, and liver tissues had greater expression relative to colon and brain tissues as determined by immunohistochemistry. Less significant differences among normal tissues were observed by cell extract ELISA assay and Western blots analyses (15,29,32,46,47). Comparison between tumor types and normal cells was only done on cultured cell lines (46,47). Melanoma, ovary, prostate, and lung cancers had higher RLIP76 levels than breast or liver cancer or normal immortalized cultured cells (46). Functional assays of doxorubicin transport were done on erythrocytes, cultured immortalized normal cells, and cancer cell lines (Table 1). Although in most cases, doxorubicin transport rates matched RLIP76 protein content, this was not the case for lung cancers and normal erythrocytes. In the former case, increased transport rate in non-small cell lung carcinoma (NSCLC) relative to small cell lung carcinoma cell lines occurred even in the absence of different RLIP76 protein concentrations and was found to be due to T²⁹⁷ phosphorylation by protein kinase C α specifically in NSCLC (33,35). In the latter case, increased transport in erythrocytes may be due to the higher density of membrane protein on the smaller, anucleate cells relative to other normal cells. Because RLIP76 transport provides resistance to multiple chemotherapy agents and radiotherapy, examination of tumor RLIP76 content and state of T²⁹⁷ phosphorylation may be useful biomarkers for clinical outcomes.

Immunohistochemical quantification of RLIP76 in formalin-fixed, paraffin-embedded tissues has been achieved and should facilitate both retrospective and prospective studies (32,45,47). Interestingly, general associations of tumor type sensitivity with RLIP76 transport activity were found (e.g., NSCLC and melanoma low response rates, and small cell lung carcinoma and breast cancer high response rates to doxorubicin therapy). However, these limited cell line studies need to be expanded both in number and use of patient materials before conclusions are drawn. Furthermore, simple clinically applicable measurements of RLIP76 T²⁹⁷ phosphorylation are needed because transport measurements are not practical or economical on patient tumor samples.

Clinical-Translational Advances

Inhibition of RLIP76 function or expression by monoclonal antibody or antisense therapy will expose cells and tissues to sequelae of oxidative stress. Based on the findings of increased electrophiles in cancer cells as well as increased RLIP76 protein and transport, cancers may be more dependent on RLIP76 than most normal tissues. To test this hypothesis, polyclonal rabbit anti-RLIP76 antibody and RLIP76 siRNA from Dharmacon Research Laboratories were administered to normal and tumor cells in culture and to murine tumor models (46). Selective antitumor efficacy was observed both *in vitro* and *in vivo*. Antibody at 40 $\mu\text{g}/\text{mL}$ and siRNA at 20 $\mu\text{g}/\text{mL}$ produced 60% and 80% inhibition of cell survival for melanomas and lung cancer cell lines, but <5% and 20% inhibition of cell survival for breast and liver cancer and normal immortalized cells *in vitro* (46). When combined with chemotherapy (cisplatin, vinorelbine, doxorubicin, daunorubicin, vincristine, vinblastine, mitomycin C, and melphalan), antibody or siRNA produced synergistic tumor cell kill with a combination index of 0.001 to 0.3 (47–49). These observations support the additional role of RLIP76 in tumor cell drug resistance. In contrast to the modest effects of anti-RLIP76 therapy alone in tissue culture, complete regressions were observed in murine-established tumor models of B16 melanomas, H358 and H520 NSCLC, and SW480 colon carcinomas with single 200- μg doses of antibody or siRNA (46,47). When antibody was given weekly with cisplatin (1 mg/kg) and vinorelbine (0.2 mg/kg), complete remissions occurred more rapidly. Although animals treated chemotherapy alone died from tumors with 4 months, most animals receiving antibody either alone or in combination with chemotherapy survived disease-free for >1 year. Progress on anti-RLIP76 therapy will require clinical development of a reagent. Humanization of a monoclonal antibody seems

the most direct route. Prospective tumor biomarkers of oxidative stress, RLIP76 phosphorylation, or RLIP76 expression may help select patients and predict clinical response.

Another application of the RLIP76 technology is to increase its levels in cells by delivery of RLIP76-loaded proteoliposomes. This has permitted survival of normal tissues after the stress of external radiation (32). I.p. administration of RLIP76 proteoliposomes to mice produced increased tissue levels of RLIP76 in liver, kidney, heart, and brain. Four hundred micrograms of RLIP76 proteoliposomes given up to 3 days after 1,000 cGy irradiation of mice improved survival (32). There are likely many uses for an effective systemic antifree radical agent. Hand-foot syndrome secondary to cancer chemotherapies may be alleviated by topical RLIP76 (50). Similarly, esophageal injury from chest radiotherapy may be palliated with oral RLIP76 (51). RLIP therapy may reduce side effects of cancer therapy. Development of these treatment modalities will require adequate production of RLIP76 and formulation for both regional and systemic delivery.

Conclusions

RLIP76 is a fundamental link between biochemical pathways of GSH-linked metabolism of xenobiotics and stress-defense signaling pathways. It represents a stress-resistance effector that plays a pivotal role in defending normal cells from poisons, and cancer cells from apoptosis. Thus, augmentation of RLIP76 in normal cells that are stressed or injured may be a pharmacologic method for treatment of poisoning and wounds, and controlled depletion or inhibition of RLIP76 should naturally target malignant cell that rely on the mercapturic acid pathway to protect themselves from apoptosis. The rate regulatory role of RLIP76 in endocytosis has many unexplored implications with respect to this mechanism that functions to terminate signaling initiated by ligand-receptor binding.

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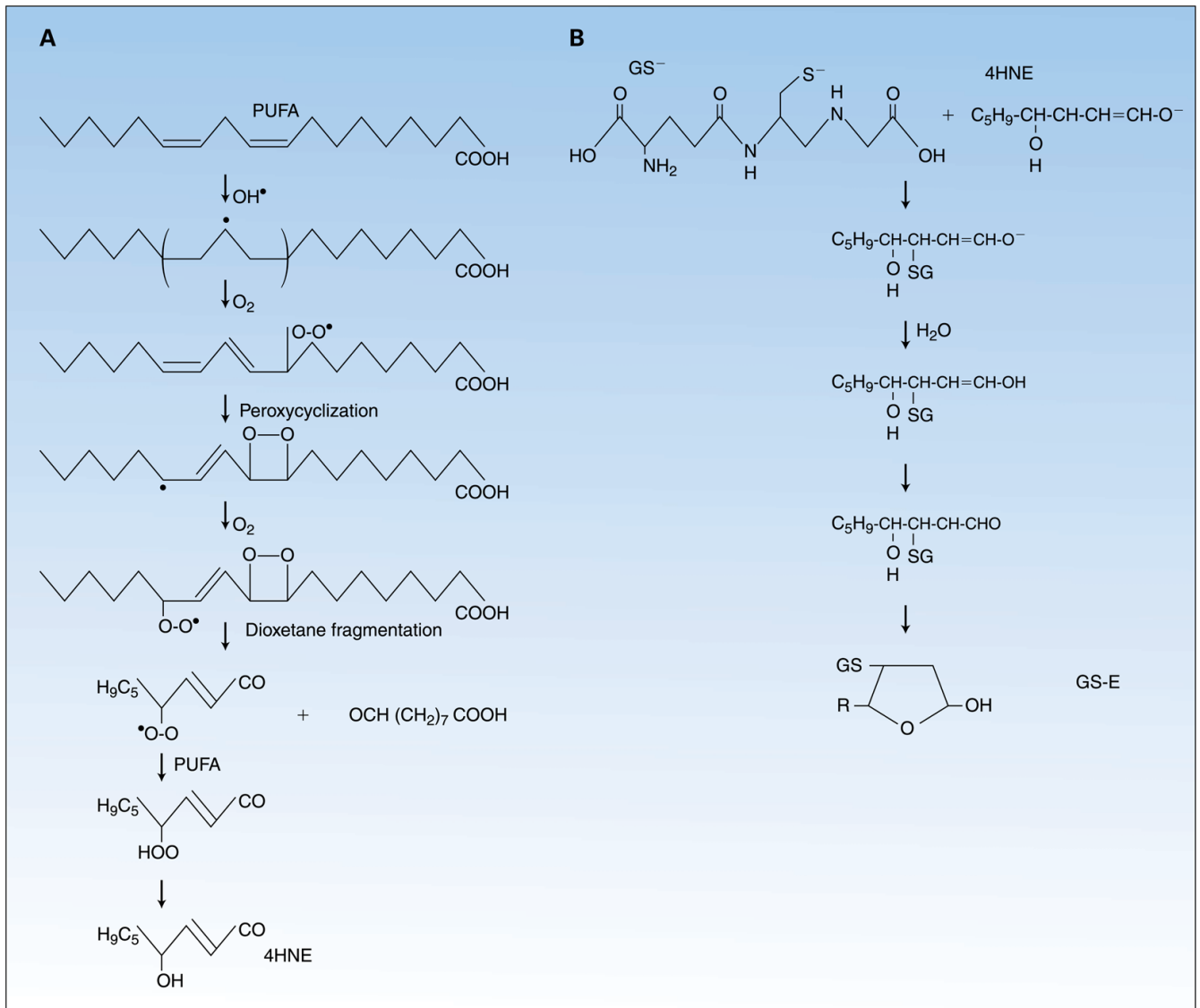
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**Fig. 1.**

A, generation of 4-hydroxynonenal from linoleic acid, a polyunsaturated fatty acid. A free hydroxyl radical abstracts H and makes a lipid radical. Oxygen addition produces a peroxy radical. Peroxycyclization follows, and then dioxetane fragmentation occurs. H extraction from PUFA then generates 4-hydroxynonenal. **B**, GSH is composed of glutamic acid, cysteine, and glycine with a γ -glutamyl linkage. Bound to glutathione-S-transferases, the thiol group is deprotonated by an adjacent tyrosine. GS-E reacts with 4-hydroxynonenal to form an intermediate. This intermediate reacts with water to seize a proton and release a negatively charged hydroxyl ion. The enol then rearranges through the ketoenol tautomerism to the aldehyde. The aldehyde undergoes oxo-cyclo tautomerism and forms the cyclic 5-membered hemiacetal form. This GS-E is a substrate for RLIP76.

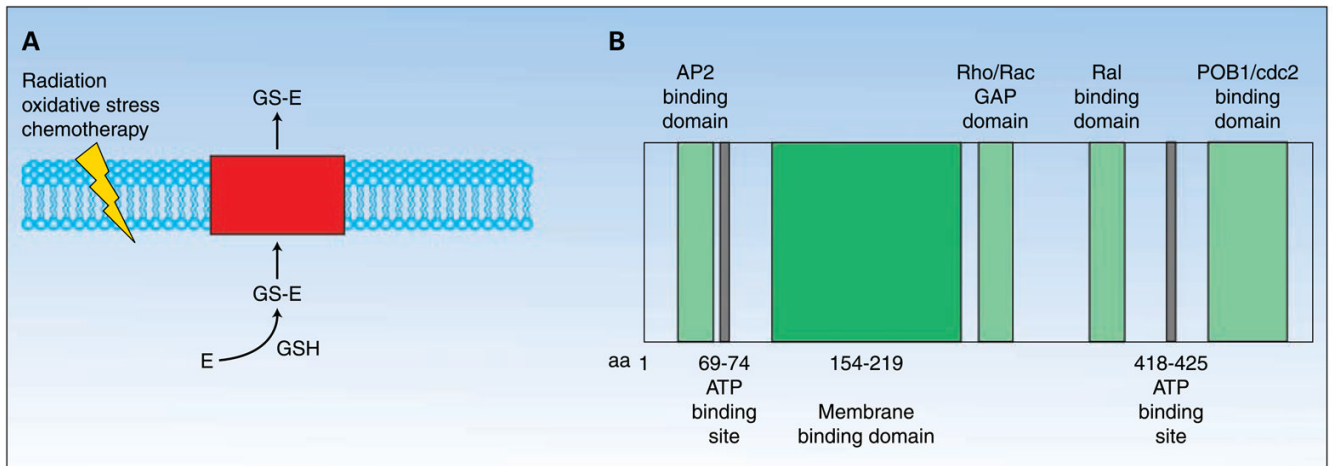


Fig. 2.

A, schema of RLIP76 ATPase – dependent efflux of GSH conjugates with electrophiles. *E*, electrophile. B, 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. The Ral binding domain and POB1/cdc2 binding domain are the first half and second half of the COOH terminus region, respectively (25).

Table 1

Cell line and tissue RLIP76 protein expression and doxorubicin transport

Cell type	Cell	RLIP76 protein ($\mu\text{g}/10^8$ cells)	^{14}C -doxorubicin transport activity in IOVs (pmol/min/mg)
Melanoma cell line	DG-1	63	384
Ovarian Ca cell line	OVCAR-3	53	298
Prostate Ca cell line	PC-3	46	211
NSCLC cell line	H358	37	184
NSCLC cell line	H520	38	—
NSCLC cell line	H226	36	—
NSCLC cell line	H2347	40	—
SCLC cell line	H1618	32	96
SCLC cell line	H1417	28	—
SCLC cell line	H182	35	—
Breast Ca cell line	MCF7	13	38
Breast Ca cell line	MDA-MB231	13	40
Breast Ca cell line	T-47D	12	37
Liver Ca cell line	HepG2	17	55
Nonmalignant			
Lung epithelium	HLBEC	22	46
Lung endothelium	HLMVEC	19	40
Aorta smooth muscle	HAVSM	15	40
Umbilical endothelium	Human umbilical vascular endothelial cell	14	36

Tissue type	Tissue	RLIP76 protein ($\mu\text{g}/\text{g}$ tissue)	^{14}C -doxorubicin transport activity in IOVs (pmol/min/mg)
Peripheral blood	Erythrocytes	25	250
Heart tissue*	Heart	34	145
Liver tissue	Liver	39	—
Muscle tissue	Muscle	22	—
Lung tissue	Lung	21	235
Placenta tissue	Placenta	8	—

Abbreviations: SCLC, small cell lung carcinoma; IOV, inside-out vesicles.

* Mouse tissue. All others human. Protein content was based on erythrocyte standard and scanned immunoblots (15,44). Cell line data were directly from references or scanned (44,45). Transport data from references (16,28,32,34).