

# Role of RLIP76 in doxorubicin resistance in lung cancer (Review)

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**Abstract.** Lung cancer is still a major cause of cancer deaths in spite of considerable efforts in its systemic therapy. Chemotherapy, along with local irradiation is frequently employed but as a palliative therapy. Inherent and acquired resistance in NSCLC and SCLC towards chemotherapeutic agents further makes chemotherapy an incommensurable problem. The resistance mechanisms responsible for inherent DOX-resistance of NSCLC and acquired DOX-resistance in SCLC have been the subject of numerous investigations. This review will focus on the recent studies done for understanding the mechanism(s) of inherent and acquired resistance in NSCLC and SCLC and how these can be exploited for the future development of more effective novel biologic agents for the treatment of lung cancer.

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## 1. Introduction

Lung cancer is the most frequent lethal neoplasm in the world and is the leading cause of cancer deaths in the United States. It represents 12% of all cancers and 18% of cancer deaths world wide (1,2). Because of the lack of widely applicable screening methods, it is diagnosed usually in relatively advanced stages for which only palliative therapy can be attempted. The outcome of patients treated with combined chemotherapy and radiation in locally advanced disease is quite poor. In this context, inherent or acquired resistance mechanism operates to further reduce therapeutic efficacy. Better understanding of the nature of these resistance mechanisms appears to be of central importance towards the goal of developing more effective and less toxic therapy for lung cancer.

Doxorubicin (DOX) (Fig. 1A) is a highly active agent in the chemotherapeutic treatment of a number of human malignancies including breast cancer, bladder cancer, multiple myeloma, lymphoma and small cell lung cancer (SCLC) (3,4). In contrast to its high activity against SCLC, it is relatively ineffective against non-small cell lung cancer (NSCLC), which represents about 4/5 of all lung cancers. Even in SCLC, acquired resistance to DOX is common, accounting for the frequent failure to cure SCLC with DOX-based chemotherapy regimens. The resistance mechanisms responsible for inherent DOX-resistance of NSCLC and acquired DOX-resistance in SCLC have been the subject of numerous investigations (5). The inherently lower cellular accumulation of DOX in NSCLC as compared with SCLC has been frequently attributed as a major cause of DOX-resistance in NSCLC. Increased protein kinase C (PKC) particularly PKC $\alpha$  activity is closely linked with DOX-accumulation defective resistance. The mechanism through which PKC $\alpha$  can influence cellular DOX-accumulation remains unclear since regulation of ATP Binding Cassette (ABC) transporter activity by PKC $\alpha$  has not been established. PKC $\alpha$  has been shown to phosphorylate and stimulate the

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*Abbreviations:* RLIP76 (RalBP1), Ral-interacting protein; GSH, glutathione; GS-E, glutathione-electrophile conjugates; DNP-SG, dinitrophenyl S-glutathione; DOX, doxorubicin; 4HNE, 4-hydroxy-nonenal; IOVs, in-side-out vesicles; MDR, multi-drug resistance; Pgp, P-glycoprotein; MRP1, multi-drug resistance associated protein; SCLC, small cell lung cancer; NSCLC, non-small cell lung cancer; POB1, partner of RLIP76

*Key words:* RLIP76, PKC $\alpha$ , cancer, drug-resistance, glutathione-conjugate transport, doxorubicin

activity of Ral-interacting protein (RLIP76) (6-8), a novel non-ABC-transporter of DOX (9). In recent years, we have shown RLIP76, a non-ABC transporter to be involved in the inherent DOX resistance in NSCLC and acquired resistance in SCLC.

In the present review, we will discuss the studies which demonstrate that RLIP76 is a major DOX transporter and is a primary determinant of DOX resistance in lung cancer cells. We will also discuss the structural motifs of RLIP76 and some of the interacting proteins which affect its transport function and conclude with a discussion of the implication of our novel finding in understanding the mechanisms of drug resistance in lung cancer cells for the development of pharmacological methods for the treatment of lung cancer.

## 2. RLIP76: a non-ABC transporter

RLIP76 is a stress-responsive, multi-functional membrane protein found ubiquitously in species as distant as *Drosophila* to humans (8-14). It is encoded in humans on chromosome 18p11.3 by a gene with 11 exons and 9 introns (15). RLIP76 is a 76 kDa protein product of this gene, but splice-variants include a 67 kDa peptide and longer 80 or 102 kDa peptide, (later identified as cytoctrin) (16,17). RLIP76 is frequently overexpressed in malignant cells, and plays a prominent anti-apoptotic role selectively in cancer cells through its ability to control cellular concentration of pro-apoptotic oxidized lipid by-products (18).

RLIP76 does not meet the criteria for classical ABC transporters as it does not have significant sequence homology with ABC transporters and differs from the ABC transporters in several other important aspects, including (i) lack of a classical Walker domain, (ii) lack of any close homologs in humans, (iii) integral membrane association without clearly defined transmembrane domains and (iv) its role as a direct link to Ras/Ral/Rho and EGF-R signaling through its multi-functional nature, including GAP activity, regulation of exocytosis as well as clathrin-coated pit-mediated receptor endocytosis (8,13).

Although the primary amino acid sequence of RLIP76 is known (8,10), detailed three-dimensional structure for the molecule remains to be elucidated. This knowledge will be important in understanding the transport functions as well as other activities of RLIP76. The protein lacks the well-defined trans-membrane helices of ABC transporters and there are no other RLIP76 family members with solved structure. Nevertheless, we can estimate domain behavior from sequence analysis and mutational studies. There are two ATP binding sites, one at the NH<sub>2</sub> terminus (<sup>69</sup>GKKKGK<sup>74</sup>) and the other at the COOH terminus (<sup>418</sup>GGIKDL<sup>425</sup>) (19). Both these sites are needed for substrate transport. Mutations in these sequences abrogate its ATP-binding, ATPase activity and transport function (19,20). Primary structure of RLIP76 can be divided into four regions. N terminal domain (aa 1-209), is rich in antennapedia homeodomain homologous peptides. These peptides are known to be capable of transporting wide range of substrates across membranes. The N-terminal domain also provides the membrane anchor. It has N-myristylation sites at amino acid 21-26, 40-45, and 191-196. There are vector peptide-related sequences between amino

acid 154 and 219 including the penetratin-like sequence <sup>154</sup>LTAADVVKQWKEKKKK<sup>170</sup>, a TAT-like sequence from amino acid 170-185, and a hydrophobic stretch from amino acid 203-219. Both membrane association and transport requires this domain (8,20,21). Two central domains include, one carrying a Rac/cdc42 GAP (through aa 210-357) activity, while the other binds to activated Ral (aa 391-499). A C-terminal coiled-coil domain (aa 500-655) has subsequently been shown to interact with Repl/POB1, CDK1 and Hsf-1 (Fig. 1B). These structural features link RLIP76 to a confounding array of physiological functions including clathrin-coated-pit-mediated receptor-ligand endocytosis of epidermal growth factor receptor (EGFR), insulin receptor (IR) and transforming growth factor  $\beta$  (TGF- $\beta$ ) as well as mitosis signaling (8,16,21-27). 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.

RLIP76 was cloned originally as a Ral-binding GTPase-activating protein (GAP), and predicted to be a Ral-effector, through a yeast two-hybrid screen (10). We cloned RLIP76 independently, in the search for glutathione-electrophile conjugates (GS-Es) and chemotherapy drug transporters, from a human bone-marrow cDNA library using polyclonal antibodies raised against purified human erythrocyte putative membrane transporter, DNPSG-ATPase (28-30). The identity of RLIP76 with DNPSG-ATPase was demonstrated by showing that both the human and recombinant protein could be purified by the same GS-E affinity chromatography method (9,28-30). Tissue-purified and recombinant RLIP76 have demonstrated constitutive ATPase activity that was stimulated by anionic (e.g., DNP-SG, GS-HNE,) as well as cationic (e.g., DOX) ligands (9,31). In both cells and reconstituted proteoliposomes, native and recombinant RLIP76 can transport these allocrites across membranes (9). Transport activity of purified RLIP76 fraction was only minimally affected by limited proteolytic digestion, suggesting that intact RLIP76 was not necessary for transport and that peptides originating from RLIP76 could constitute a functional transporter (9,19). This postulated hypothesis was confirmed by comparing the transport activity of intact RLIP76 with that of the N-terminal (N-RLIP76<sup>1-367</sup>) and C-terminal (C-RLIP76<sup>410-655</sup>) fragments of RLIP76 obtained from expressing the corresponding cDNA in *E. coli* and purifying these fragments using a His-tag (19). Remarkably, when both peptides purified separately were reconstituted together in artificial liposomes, transport activity was nearly equal to that of intact recombinant RLIP76 (19). These results confirmed the postulate that peptides originating from RLIP76 could reconstitute a functional transporter.

## 3. Physiological significance of RLIP76

One of the physiologic functions of RLIP76 is regulation of intracellular concentration of the electrophilic intermediates of oxidative lipid metabolism by mediating efflux of GS-E formed from oxidative degradation of arachidonic acid, including leukotrienes and the 4HNE-GSH conjugates. RLIP76 mediated transport of amphiphilic chemotherapeutic agents such as anthracyclines and vinca alkaloids as well as

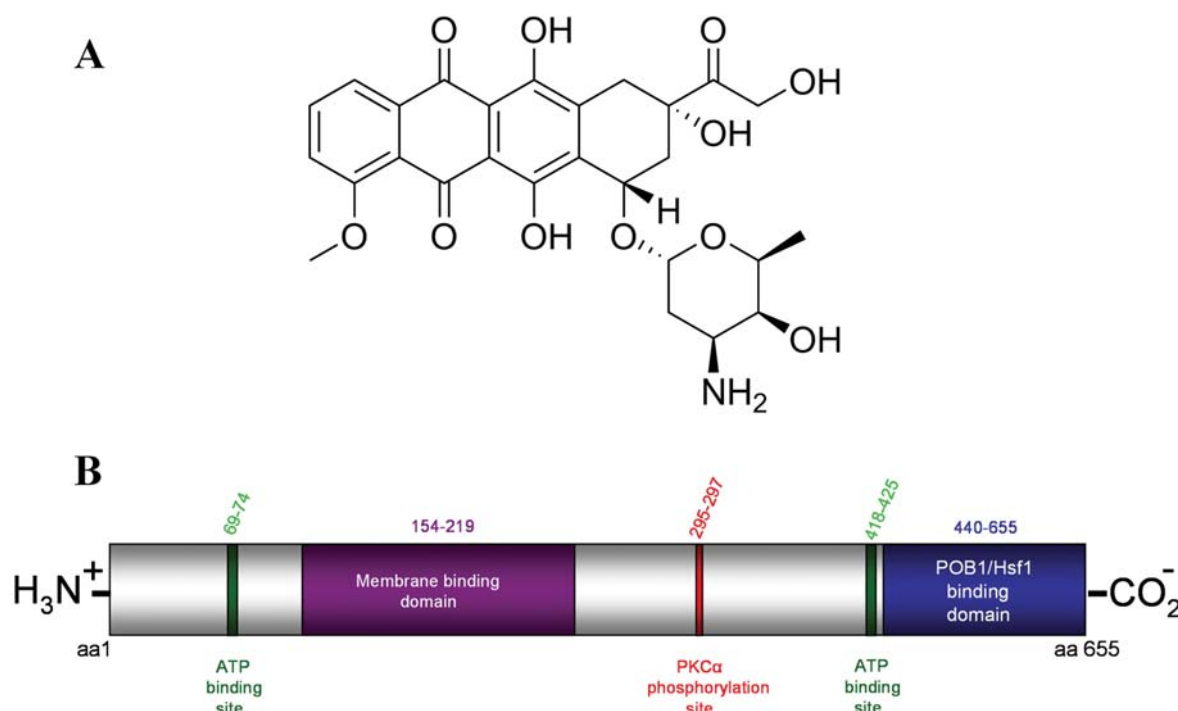


Figure 1. Structure of doxorubicin (panel A). Schematic representation of RLIP76 domains (panel B).

GS-E produced during oxidative metabolism places this multi-functional protein in a central role as a resistance mechanism for preventing apoptosis caused by chemotherapeutic agents. RLIP76 is also involved in variety of external/internal stressors, including oxidative stress, heat shock and radiation (25,32-34). Though RLIP76 is involved in many activities, in the present section we will discuss three major functions of RLIP76:

**Protection against heat-shock and oxidative stress.** Induction of RLIP76 was an early response to heat shock or oxidants, prior to the appearance of previously known heat shock proteins or antioxidant enzymes (32). When K562 cells were exposed to mild heat shock (42°C, 30 min) or oxidative stress (50  $\mu$ M H<sub>2</sub>O<sub>2</sub>, 20 min) and allowed to recover for 2 h, there was an enhanced level of 4HNE, a several fold induction of hGST 5.8, which catalyzes the formation of GS-HNE, and induction of RLIP76, which mediates the transport of GS-HNE from cells. Cells preconditioned with stress shock, transported GS-HNE at a 3-fold higher rate, and increased efflux of GS-HNE was observed. The increased efflux of GS-HNE was blocked by coating the cells with antibodies against RLIP76, confirming that GS-HNE is transported by RLIP76. The stress-preconditioned cells with induced hGST 5.8 and RLIP76 acquired resistance to 4HNE and H<sub>2</sub>O<sub>2</sub> mediated apoptosis by suppressing sustained activation of JNK and caspase-3. The protective effect of stress preconditioning against H<sub>2</sub>O<sub>2</sub> or 4HNE induced apoptosis was abrogated by coating the cells with anti-RLIP76 IgG, which inhibited the efflux of GS-HNE. These results showed that the cells acquired resistance to apoptosis by metabolizing and excluding 4HNE at a higher rate and suggested that the intracellular concentrations of GS-HNE and endogenous GS-E modulated stress-mediated signaling (32).

**Transport mediated drug-resistance.** RLIP76 serves an anti-apoptotic function by preventing accumulation of endogenously derived GS-E. Endogenous GS-E, which forms as a result of oxidant or radiant stress, compete for efflux with a number of chemotherapy drugs including DOX, daunorubicin, colchicine, vincristine, vinblastine and vinorelbine, GS-E derived from alkylating agents or platinum coordinates which are known substrates for transport by RLIP76 (8,9,28,31,35-37).

The role of RLIP76 in mediating DOX resistance was subsequently tested in K562 human leukemia cells by comparing the effect of transfection of empty vector vs. vector-containing full-length RLIP76 cDNA. RLIP76 was overexpressed ~4-fold in Western blots as well as by RT-PCR analysis. The DOX and DNP-SG transport activity of RLIP76-transfected cells increased ~5-fold and IC<sub>50</sub> of DOX increased approximately 5-fold. As would be expected for a GS-E transporter, the IC<sub>50</sub> for 4HNE, an endogenous alkylating agent, also increased approximately 3-fold. The effect of increased RLIP76 expression was also evaluated by increasing RLIP76 in cells by delivering it using proteoliposomes containing RLIP76. H358 lung cancer cells showed avid uptake of RLIP76, with cellular RLIP76 protein increasing in a linear manner with respect to the amount of RLIP76 proteoliposomes used. Resistance to DOX increased in a linear fashion with respect to the amount of RLIP76 in proteoliposomes (9,38).

**RLIP76 protects against radiation toxicity.** Since generation of reactive-oxygen species (ROS) is a common pathway for high-energy radiation as well as heat shock and oxidant exposure, we summarized that RLIP76 would also afford protection against radiation. We tested this hypothesis by determining the effect of increased RLIP76 on radiation-sensitivity. Because



we had previously demonstrated that RLIP76 containing proteoliposomes were avidly taken up by cultured cells and would result in an increased cellular RLIP76 level as well as DOX resistance, we treated SCLC cells (H182 and H1618) in culture with liposome reconstituted in the presence of purified RLIP76 prior to the exposure to a single dose of radiation at 500 cGy. After radiation, cells were serially cultured by reinoculation into medium each day at a density of  $5 \times 10^5$  cells/ml, and cell density was determined at 24 h intervals. Cells enriched with RLIP76 were significantly more resistant to radiation compared to control cells. These results suggested that accumulation of GS-E formed from reactive intermediates of lipid peroxidation contributes significantly to radiation toxicity and that increased efflux of these by-products rendered cells resistant to radiation (8,22).

#### 4. Doxorubicin resistance in lung cancer

DOX, an anthracycline antibiotic, is one of the most widely used anticancer drugs, effective in a wide variety of human cancers (39-41). Its efficacy, like that of other anticancer drugs, is hampered by innate and acquired drug resistance in cancer cells. Multiple mechanisms of DOX resistance have been elucidated. Although alterations in cellular antioxidant defenses (42), topoisomerases (43), and stress and apoptosis signaling pathway proteins (44) are found in many DOX-resistant cells, drug-efflux mechanisms which create a DOX-accumulation defect appears to be the most common alteration (44,45). The ABC-family of multi-specific transporters including P-glycoprotein (Pgp, ABCB1), multidrug-resistance protein (MRP1, ABCC1), and multiple other MRP-related transporters have been shown to mediate DOX-transport and resistance (46-48). Pgp was the first to be identified among these transporters (49), and differed from MRP and related transporters in that the later transporters use not only the weakly cationic anthracycline and vinca alkaloids as substrates, but can also transport GS-E of endogenous and exogenous alkylating agents (including a number of chemotherapy drugs). Though the ABC-transporters play a significant role in protecting many cancer cell types from DOX, *in vitro* and clinical studies indicate the involvement of other DOX-transporters as well (49,50). Multiple potential mechanisms for DOX-efflux have been identified in lung cancer cells, primarily belonging to the ABC-transporter super-family, though rigorous evidence for DOX-transport activity in isolated systems is lacking for most. P-glycoprotein (mdr-1 gene product, Pgp) is the prototypical and best characterized membrane protein which has been shown to mediate the ATP-dependent transmembrane transport of DOX, as is a major cause of a DOX-accumulation defective phenotype in many types of human cancers, but its role in inherent resistance to DOX in lung cancer is likely limited because of its relatively low expression in lung cancer cells (51). Other ABC-super family transporters such as the MRP1 (ABCC1, multidrug-resistance associated protein) and BCRP (ABCG2) are more significant determinants of DOX transport in lung cancer cells (51,52). However, our recent studies in panels of SCLC and NSCLC have shown that RLIP76 contributes approximately 2/3 of total DOX-transport activity with the remainder being contributed to by ABC-transporter (51).

#### 5. RLIP76 is a major DOX transporter

Our laboratory showed for the first time, that RLIP76 can mediate ATP-dependent transmembrane movement of not only anionic GS-E (DNP-SG), but also weakly cationic chemotherapeutic drug (DOX) in several independent systems (8,9). This was proved by experimental evidence such as i) transport studies on proteoliposomes (reconstituted with either asolectin or defined phospholipids) containing purified recombinant RLIP76; ii) acquired resistance to DOX and 4HNE in the cells transfected with cDNA encoding RLIP76; iii) acquired resistance to DOX in the cells upon direct incorporation of RLIP76 by fusion of the cells with proteoliposomes. These results confirmed and indicated that RLIP76, in addition to its role in signal transduction, can catalyze transport of GS-E and xenobiotics, and may contribute to the multidrug resistance phenomenon. In a variety of transport studies, we have proved that DOX is an excellent substrate for ATP-dependent efflux by RLIP76. Because drug-efflux mechanisms mediate drug accumulation defects as well as resistance to the cytotoxic effects of drugs, the function of RLIP76 as a drug transporter would predict that overexpression of this protein should render resistance to the drugs which are its allocrites. We initially addressed this hypothesis by comparing DOX sensitivity with the expression of RLIP76 as well as transport activity between wild-type and DOX exposure derived, resistant H69 SCLC cell lines. The 3- and 30-fold DOX-resistant variants tested had similar levels of MRP and undetectable levels of Pgp. Reconstituted RLIP76 catalyzes ATP-dependent uptake of  $^{14}\text{C}$ -DOX into vesicles. The activation energy for transport of the DOX was 96 kJ/mol, and the temperature optimum was at 37°C with inactivation at 45°C. The calculated kinetic parameters for DOX transport in reconstituted RLIP76 are as follows:  $K_m$  for ATP, 4.6 mM;  $K_m$  for DOX, 3.1  $\mu\text{M}$ ;  $k_{cat}$  for DOX (assuming half of the protein was reconstituted in transport-competent orientation), 1.9  $\text{s}^{-1}$  (9).

To prove further, we generated RLIP76<sup>+/+</sup> and RLIP76<sup>-/-</sup> mice colonies, based on genotyping (33). Replacement of RLIP76 in RLIP76<sup>-/-</sup> mouse tissues were examined in animals given 200  $\mu\text{g}/0.2\text{ml}$  purified RLIP76 liposomes, i.p. RLIP76 was clearly detected in mouse tissues at 24 h after an RLIP76-liposomes dose (33). Consistent with the observed function of RLIP76 as a transporter of DOX in cell culture studies (51), DOX transport in membrane vesicles was found to be decreased in RLIP76<sup>-/-</sup> mice tissues. A greater than 80% loss of GS-E and DOX transport activity was seen in the RLIP76<sup>-/-</sup> mouse tissues (8,33). Delivery of RLIP76 to RLIP76<sup>-/-</sup> animals via liposomal delivery system completely reversed transport mechanisms (33,53). Greater than 80% loss in liver and heart tissue of total DOX and GS-E transport activity in RLIP76 knockout mice strongly supports the hypothesis that RLIP76 can contribute substantially to total DOX transport capacity in cancer cells. Sequence analysis of RLIP76 indicates the presence of four potential PKC $\alpha$  phosphorylation sites (8). Since increased PKC $\alpha$  activity is correlated with increased DOX-resistance and decreased cellular DOX-accumulation, and because RLIP76 can be activated through phosphorylation by PKC $\alpha$  (7), mutational studies in RLIP76 at PKC $\alpha$  phosphorylation sites proved that the regulation of

RLIP76 by PKC $\alpha$  is a significant determinant of inherent and acquired DOX-resistance in lung cancer (7,54).

## 6. Impairment of transport of RLIP76 sensitizes lung cancer cells to doxorubicin

Our studies proved that RLIP76 is a major transporter of DOX. If this assertion were true, inhibition of the transport function of RLIP76 after treatment with RLIP76 antibody should significantly enhance the cytotoxicity of DOX in these cells. Using highly specific anti-RLIP76 IgG at the concentration of 50  $\mu$ g/ml, we treated both NSCLC and SCLC for 16 h followed by addition of DOX in these cells. Our studies confirmed that anti-RLIP76 antibody markedly enhanced the cytotoxicity of DOX in NSCLC and SCLC. Further increase in IgG concentration showed saturation, which is consistent with a specific antigen and antibody interaction. The interaction of anti-RLIP76 IgG and DOX was markedly synergistic (CI 0.36 $\pm$ 0.17). The degree of synergy between anti-RLIP76 IgG and DOX was found to be significantly greater (CI near 0.001) than that between DOX and Herceptin (an anti-Her-2/neu humanized monoclonal antibody used in therapy of human breast cancer). Overall, the sensitizing effect of anti-RLIP76 IgG to DOX was somewhat more prominent for SCLC as compared to NSCLC. Mechanism on anti-RLIP76 IgG mediated cytotoxicity was explained by a model built on our previous studies in which a physiologic function (i.e. GS-E efflux) of RLIP76 exerts an anti-apoptotic effect, and DOX inhibits this physiologic function. Our studies showed that specific inhibition of the transport function of RLIP76 by RLIP76 IgG can trigger apoptosis and can increase DOX cytotoxicity synergistically (55).

## 7. Role of PKC $\alpha$ and RLIP76 in transport-mediated DOX-resistance in lung cancer

In order to answer the question whether PKC $\alpha$  or RLIP76 or both are the cause for differential drug-resistance observed in NSCLC and SCLC, we determined the expression of PKC $\alpha$  and RLIP76 in several NSCLC and SCLC cell lines. Western blot analysis using antibodies against PKC $\alpha$ , with equal amount of crude protein from either cell line indicated a higher expression of PKC $\alpha$  in all NSCLC cell lines as compared to SCLC cell lines. Depletion of PKC $\alpha$  by siRNA, in several NSCLC and SCLC cell lines, showed a decrease in the drug resistance (IC<sub>50</sub> for DOX) of NSCLC cell lines to the level observed with SCLC cell lines. Same phenomenon was observed in DOX-transport studies using proteoliposomes reconstituted with purified RLIP76 from PKC $\alpha$ -depleted NSCLC and SCLC. These results directly indicate that PKC $\alpha$  plays a major role towards differential DOX-resistance of NSCLC and SCLC (7,54,56).

The difference in RLIP76 activity between NSCLC and SCLC is not related to the amount of RLIP76 protein in these cells rather to the specific activity of RLIP76. Our studies showed that specific activity of highly purified RLIP76 from six SCLC cell lines were approximately half that observed for seven NSCLC. SCLC and NSCLC have inherently different sensitivity to DOX, SCLC being significantly more

sensitive than NSCLC, our findings for the first time suggested that differences in RLIP76 between NSCLC and SCLC plays a significant role in inherent difference in DOX sensitivity in these two distinct types of lung malignancy (17,51,55).

Further, we investigated the mechanisms how RLIP76 activity is higher in NSCLC as compared with SCLC by site-directed mutagenesis studies of RLIP76 gene. Sequence analysis of RLIP76 indicated the presence of four (S<sup>118</sup>, T<sup>297</sup>, S<sup>353</sup> and S<sup>509</sup>) potential PKC $\alpha$  phosphorylation sites (7,54). In deletion mutant analyses of potential phosphorylation sites in RLIP76, we identified T<sup>297</sup> and S<sup>509</sup> as targets for phosphorylation by PKC $\alpha$ . Phosphorylation at T<sup>297</sup> increased DOX-transport activity by 2-fold for RLIP76 purified from recombinant source, or from three SCLC (H69, H1417 and H1618) and three NSCLC, one each derived from H226 (squamous), H358 (bronchio alveolar) and H1395 (adenocarcinoma). ATP-dependent transport of DOX for each of these mutants indicated that PKC $\alpha$  can activate RLIP76, and that the majority of this effect is abrogated by deletion of T<sup>297</sup>.

In our initial studies (17), we have found that both SCLC and NSCLC cell lines contain similar amounts of RLIP76 protein (0.53 $\pm$ 0.04% of total membrane protein, n=12), but the specific activity of RLIP76 from NSCLC was ~2-fold greater than from SCLC, both in ATPase activity and transport activity. Post-translational modifications as well as differences in susceptibility to limited tryptic digestion were suggested to be the reason for this difference. Our studies showed the presence of a 44 kDa peptide resulting from limited proteolytic digestion of RLIP76 from NSCLC, which was absent from SCLC. The N-terminal sequence of this 44 kDa peptide revealed the sequence TTETE, beginning at T<sup>294</sup>. Pre-treatment of the protein from SCLC with PKC $\alpha$  caused the appearance of this band. The 44 kDa band was not seen upon PKC $\alpha$  pre-treatment of rec-RLIP76 del<sup>297-299</sup>. These findings indicate that in NSCLC, RLIP76 exists predominantly as a phosphorylated form at T<sup>297</sup>, and its characteristic susceptibility to serine protease at T<sup>294</sup> confers a significantly greater DOX-transport activity (7).

## 8. RLIP76 is essential for PKC $\alpha$ mediated DOX-resistance

The role of RLIP76 in PKC $\alpha$ -mediated resistance signaling was analyzed by determination of IC<sub>50</sub> for DOX in RLIP76<sup>+/+</sup> and RLIP76<sup>-/-</sup> mouse embryonic fibroblasts (MEFs) in the presence and absence of PKC $\alpha$ . Although RLIP76<sup>-/-</sup> MEFs were significantly more sensitive to DOX as compared to RLIP76<sup>+/+</sup> MEFs (IC<sub>50</sub> 25 vs. 125 nM), depletion of PKC $\alpha$  only affected the DOX-sensitivity of RLIP76<sup>+/+</sup> MEFs by 2.2-fold. These results demonstrate that the effect of PKC $\alpha$  mediated drug resistance requires the presence of functional RLIP76; that is, the primary function of PKC $\alpha$  with respect to DOX-resistance in NSCLC is to keep RLIP76 in its active phosphorylated state. The complete reversion of the resistance phenotype from NSCLC to that of SCLC upon depletion of PKC $\alpha$  and the complete inability of PKC $\alpha$  to affect resistance in RLIP76<sup>-/-</sup> MEFs strongly supports this conclusion.

To find a solution to the important question whether RLIP76 is upstream or downstream of PKC $\alpha$ -mediated pathway, we depleted the PKC $\alpha$  in MEFs. Depletion of PKC $\alpha$

causes 30% decrease in rate of proliferation of MEFs. This decrease is absent in RLIP76 gene knockout (RLIP76<sup>-/-</sup>) MEFs. PKC $\alpha$  stimulate DOX-resistance by increased efflux, in RLIP76<sup>+/+</sup> MEFs while RLIP76<sup>-/-</sup> MEFs lack this phenotype completely. These results lead to the definitive conclusion that RLIP76 is a downstream regulator of PKC $\alpha$  and is required for PKC $\alpha$ -mediated signal proliferation (54,56).

### 9. RLIP76 inhibition by POB1 and Hsf-1 induces DOX sensitivity

POB1 (partner of RLIP76), also known as REPS2, was discovered in screening for the RLIP76-binding proteins using the yeast two-hybrid system along with REPS1 (24,25,57). Hsf-1 (heat shock factor-1) is a transcription factor that is known to regulate cellular heat shock response through its binding with the multi-specific transporter protein, RLIP76 (23,25). In the unstressed state, Hsf-1 is sequestered in a complex with tubulin, HSP90 and RLIP76 (23). Stress or constitutively active Ral-GTP binding to RLIP76 triggers the release of Hsf-1 and its migration to the nucleus, where its transcription factor activity is important for the expression of heat shock proteins (25).

Since inhibition or depletion of RLIP76 has been shown previously to selectively target lung cancer cells for apoptosis and sensitize them to DOX toxicity (58), we reasoned that augmenting cellular POB1 or Hsf-1 should inhibit efflux of DOX from NSCLC, resulting in increased accumulation and thus increased cytotoxicity of DOX. POB1 caused a saturable inhibition of <sup>14</sup>C-DOX transport with maximal inhibition of about 50%. The addition of Hsf-1 also caused saturable inhibition of transport, with a maximum of 40%. In the presence of both proteins, 90% inhibition of <sup>14</sup>C-DOX transport was observed. The additive nature of transport inhibition is consistent with distinct but perhaps overlapping binding sites for POB1 (aa<sup>499-655</sup>) and Hsf-1 (aa<sup>440-655</sup>), both being present in the C-terminal region (25). These striking findings predict that simultaneous augmentation of POB1 and Hsf-1 would very effectively inhibit RLIP76 transport activity.

### 10. Conclusions

Studies summarized here demonstrate that RLIP76 is a primary determinant of DOX-resistance, and that PKC $\alpha$  mediated accumulation defect and DOX-resistance in NSCLC is primarily due to differential phosphorylation of RLIP76 in SCLC and NSCLC. Depletion of RLIP76 in these cells not only triggers apoptosis but also synergistically increases DOX efficacy. These studies are of great importance in deciding the treatment for lung cancers as well as future drug development. RLIP76 phosphorylation or RLIP76 expression can be used as a marker which could help in selecting patients and predicting clinical response. Humanization of a monoclonal antibody or antisense seems the most direct route. Another application of the RLIP76 technology is to increase its levels in cells by delivery of RLIP76-loaded proteoliposomes which may reduce side effects of cancer therapy. Hsf-1 and POB1 augmentation in lung cancer cells inhibit RLIP76 transport activity; their liposomally encapsulated form could

also be used clinically as a therapeutic agent. Development of these treatment modalities will require adequate production of RLIP76 and formulation for both regional and systemic delivery. As discussed above, RLIP76 is a major drug transporter in conferring drug resistance in lung cancer and inherent resistance in NSCLC, future drugs with poor transport through RLIP76 will be more efficient in skirmishing against lung cancer.

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