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Major Vault Protein LRP-related Multidrug Resistance

M.A. Izquierdo,[1] G.L. Scheffer, M.J. Flens, A.B. Schroeijers, P. van der Valk and R. J. Scheper

Department of Pathology, Free University Hospital, De Boelelaan 1117, 1081 HV Amsterdam, The Netherlands

INTRODUCTION

BROAD RESISTANCE to currently available chemotherapeutic drugs is a major cause of failure of cancer treatment. The identification of the mechanisms responsible for causing resistance is of considerable interest and potential clinical practice. The problem of drug resistance has been investigated in the laboratory by using drug-selected cancer cell lines. Biochemical studies have described distinct differences between parent cells and the corresponding resistant sublines [1]. Frequently, upon exposure to natural product drugs, mammalian cells acquire resistance to many structurally and functionally unrelated compounds, including anthracyclines, epipodophyllotoxins, vinca alkaloids and taxanes. Such cells are referred to as displaying a multidrug resistance phenotype (MDR) [2–4].

To date, in experimental systems, MDR is known to be conferred by two proteins, the *MDR1* gene product, P-glycoprotein (Pgp), and the *MRP* gene product, multidrug resistance protein (MRP) [5, 6]. These two proteins belong to the ATP-binding cassette (ABC) superfamily of transporter proteins [7]. The mechanism(s) by which Pgp mediates drug resistance, as well as its possible physiological function and clinical relevance, have been reviewed extensively by others [2–4]. The mechanism by which MRP mediates drug resistance is beginning to be elucidated, but the clinical relevance of MRP is still unknown. Besides ABC transporters, other mechanisms may exist that contribute to diverting cytostatic drugs from their intracellular targets, thereby conferring MDR.

We have identified a protein overexpressed in a non-Pgp MDR lung cancer cell line, and termed it lung resistance-related protein (LRP) [8]. The subsequent identification of LRP as the human major vault protein [9] led to the convergence of our research with that regarding recently described cellular organelles named vaults [10, 11]. In this article we review the data that led us to believe that vault-related MDR, as reflected by LRP-overexpression, represents a marker of drug resistance and may constitute a novel mechanism of MDR.

ISOLATION OF LRP AND ASSOCIATION WITH NON-PGP-MEDIATED MDR

Experimental evidence for other causes of MDR is largely based on MDR cell lines that do not overexpress Pgp [12]. One of these Pgp-negative MDR cell lines is 2R120, a non-small cell lung cancer cell line derived by exposure of its parental SW1573 to increasing concentrations of doxorubicin [13]. The 2R120 subline shows moderate levels of resistance to doxorubicin, vincristine, and etoposide (4- to 45- fold) [13]. The 2R120 cell line was chosen by us to investigate further non-Pgp-mediated MDR. BALB/c mice were immunised with 2R120 cells and the monoclonal antibody LRP-56 was selected for strong immunoreactivity with 2R120 cells compared to SW-1573 cells [8]. LRP-56 specifically reacted with a 110 Kd protein, LRP, which was overexpressed in 2R120 cells, but not in parental SW-1573 or revertant 2R120 cells. Interestingly, the Pgp-overexpressing 2R160 cell line, a SW-1573 subline selected at a higher concentration of doxorubicin, had a very low LRP-expression even compared to parental SW-1573 cells [8]. Since its identification, LRP has been found to be overexpressed in a large number of Pgp-negative drug-selected MDR cell lines, indicating that LRP-overexpression is a frequent feature in non-Pgp-mediated MDR [8, 14]. LRP-overexpressing MDR cell lines include small cell lung cancer (GLC4/ADR), fibrosarcoma (HT1080/DR4), breast cancer (MCF7/Mitox and MCF7/MR), and myeloma (8226/MR40) [8]. Thus, LRP may be overexpressed in cell lines from a variety of tumour types which have been selected with drugs from different chemical classes. Upregulation of LRP can be observed early during the process of drug selection in various series of MDR sublines, such as those derived from the SW-1573 and GLC4 cell lines [8, 15]. This observation suggests that the LRP-associated mechanism is already involved in low or moderate levels of drug resistance.

The concomitant overexpression of LRP and MRP appears to be a frequent event in non-Pgp MDR cell lines. Most of the MDR cell lines listed above also show increased levels of MRP, often associated with amplification of the *MRP* gene [16, 17]. Intriguingly, the *LRP* gene has been localised to the short arm of chromosome 16 (16p11.2), which is 27 cM proximal to the *MRP* gene (16p13.1) [18]. This raised the possibility that the *LRP* gene was simply co-amplified with the *MRP* gene. However, this possibility was eliminated when amplification of the *MRP* gene, but not the *LRP* gene, was found in SW1573/2R120 and GLC4/ADR cells [18]. Furthermore, in HT1080/DR4 cells, both genes were amplified in the homogeneously stained region hsr(7)(p12p15), but only *MRP* gene sequences were contained within hsr(18q) [18]. These data indicate that, although both the *MRP* and *LRP* genes

Correspondence to R.J. Scheper.
[1]Present address: Hospital Duran i Reynalds, Av. Casteldefels Km 2.7, 08907 Hospitalet de Ll., Barcelona, Spain.

map to the short arm of chromosome 16, they are rarely co-amplified and are not normally located within the same amplicon.

LRP and Pgp are rarely simultaneously overexpressed in drug-selected MDR cells. Increased levels of these proteins have been reported in MCF7/D40 breast cancer cells and certain 8226 myeloma sublines [8, 19]. Remarkably, in independently isolated 8226 MDR sublines with similar Pgp content, the resistance levels were substantially higher in a LRP-overexpressing subline than in an LRP negative subline, suggesting that the LRP-associated mechanisms of MDR may further contribute to the MDR phenotype of certain Pgp-overexpressing cells [19]. However, usually Pgp-positive MDR cell lines do not overexpress LRP, for example, the non-small lung cancer SW-1573/2R160, the ovarian carcinoma A2780AD, and the myeloma 8226/Dox6 and 8226/Dox40 cell lines [8].

LRP overexpression has also been reported in Pgp/MRP negative MDR cell lines, such as the mitoxantrone-selected MCF7/MR cell line [14]. In these cells, the LRP-associated mechanism of MDR may play a prominent role.

LRP IS THE HUMAN MAJOR VAULT PROTEIN

A cDNA library was made from the MDR human fibrosarcoma cell line HT1080/DR4 and a cDNA coding for the *LRP* gene was isolated [9]. Sequencing of the cloned cDNA demonstrated a single open reading frame of 2688 base pairs coding for an 896 amino acid protein with a calculated Mr of 100 kD. Confirmation of the isolation of full-length cDNA was obtained by ^{35}S-immunoprecipitation of the predicted 110 kD protein from both control LRP-overexpressing MDR tumour cells and LRP-transfected MOP8 cells, but not from MOP8 cells transfected with an irrelevant plasmid [9]. The deduced LRP amino acid sequence gave no indication of transmembrane fragments or the ATP binding "active transport" signature that is characteristic for ABC-transporter proteins such as Pgp and MRP. Comparative sequence analysis indicated that LRP shares 57 and 87.7% amino acid identity with the major vault protein α from *Dictyostelium discoideum* [20] and the major vault protein of *Rattus norvigecus* [21], respectively. Thus, LRP was identified as the human major vault protein [9], which is the most abundant component of recently described cellular organelles termed vaults [10, 11].

VAULTS: CELLULAR ORGANELLES IN SEARCH OF A FUNCTION

Vaults were first identified by negative staining and transmission electron microscopy in 1986, as contaminant particles of clathrin-coated vesicle preparations derived from rat liver [10, 11]. The term "vaults" was chosen by Rome and associates to describe the morphology of the particles, which consist of multiple arches reminiscent of those that form cathedral vaults [11]. Vaults are complex ribonucleoprotein particles that, in the rat, are composed of a major vault protein of 104 kD (accounting for >70% of the mass of the particle), three minor proteins of 210, 192, and 54 kD, and a small RNA molecule [10, 11, 22]. The vault components are assembled in a barrel-like structure of approximately 57 × 32 nm with a molecular mass of approximately 13 MDa, composing the largest ribonucleoprotein body reported to date (three times the size of a ribosome). The vault particle has 2-fold symmetry, and each half can be opened into a flower-like structure that contains eight petals surrounding a central ring [23] (Figure 1). These dynamic structural variations are likely to play a role in vault function. Vaults have been isolated from various species including the lower eukaryote *D. discoideum*, amphibians (frog), birds (chicken), and mammals (rat, rabbit, cow). Despite the complex composition and structure of vaults, they are highly conserved among phylogenetically dissimilar species, supporting the notion that their function is essential to eukaryotic cells [10, 22]. To date, the function of vaults is unknown. Most vaults are present in the cytoplasm but a small fraction of vaults are localised to the nuclear membrane and nuclear pore complexes (NPC) [24]. Here, structural similarities support the hypothesis that vaults constitute the central plugs of the nuclear pore complex. This raises the possibility that vaults mediate the bidirectional transport of a variety of substrates between the nucleus and the cytoplasm [24].

Initial immunocytochemical and immunohistochemical studies using the monoclonal antibody LRP-56 have constituted the first step to elucidate the significance of vaults in normal and malignant human tissues, as well as their relationship to drug resistance. Antibodies raised against the entire vaults were reported to be specific for the major vault protein, the immunodominant component of vaults, and their immunoreactivity related to the total amount of vaults [10, 22]. Thus, LRP-56 immunoreactivity probably represents vault distribution as well. LRP-56 displays a characteristic cytoplasmic punctate-staining pattern [8], in agreement with the predominant localisation of vaults (Figure 2).

DISTRIBUTION OF THE MAJOR VAULT PROTEIN LRP IN HUMAN NORMAL TISSUES

Proteins related to drug resistance *in vitro* have been demonstrated in normal human tissues, where they appear to play a protective role against toxic compounds. The malignant counterparts seem to retain this function and thus, neutralise the effects of cytotoxic drugs. By immunohistochemistry, LRP has been found to be widely distributed in normal human tissues [25]. The specificity of LRP-56 for the major vault protein LRP was demonstrated by the precipitation of the predicted approximately 110 kD protein from several normal

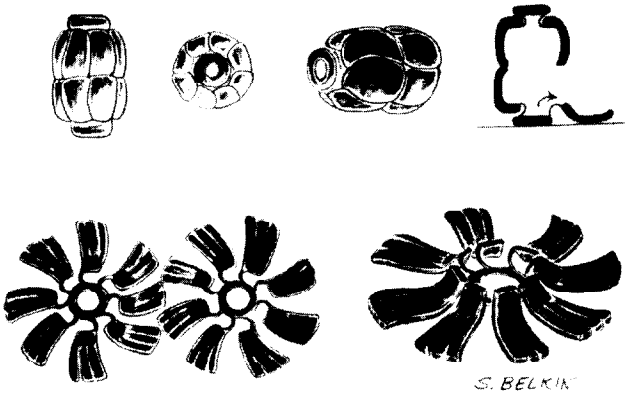

Figure 1. Model showing the folding of vault flowers into vaults. Top row: side, end, and angled views of an intact vault, with a cross-sectional view showing one of two possible pathways leading to unfolding (see text). Bottom row: paired vault flowers, derived from a single vault; (right) three-dimensional rendering of a single vault flower viewed at an angle. Reproduced from *The Journal of Cell Biology*, 1991, Vol. 112, pp. 225–235 by copyright permission of The Rockefeller University Press.

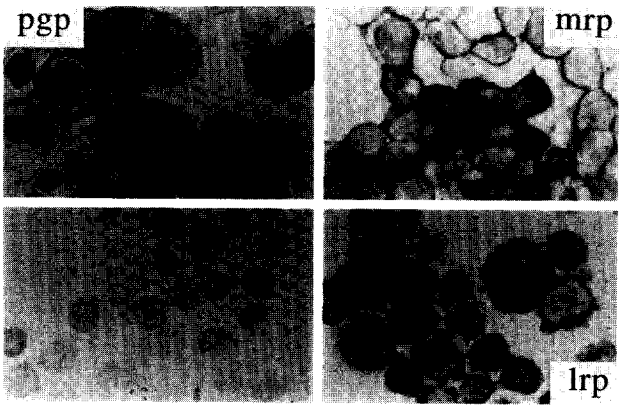

Figure 2. LRP-56 displays a characteristic cytoplasmic punctate staining pattern, in MDR tumour cells, as compared to the predominantly membranous staining of Pgp and MRP. (a) Pgp-staining of 2R160 cells; (b) MRP-staining of GLC4/ADR cells; (c) 2R120 staining with irrelevant control antibody; (d) LRP-staining of 2R120 cells.

tissues, peripheral blood, bone marrow, and tumours. In some tissues, such as normal colon, an additional band of approximately 105 kD was detected [25]. A similar doublet has been reported in *Dictyostelium discoideum* and *Xenopus oocytes* supporting the view that different isoforms of the major vault protein may exist [22].

Distinct patterns of LRP expression in normal tissues have been noticed [25]. High LRP-expressions have been observed in epithelia of the bronchus, digestive tract, as well as in keratinocytes, adrenal cortex, and macrophages. In other species, vaults are also abundant in certain epithelial cells (i.e. rat intestine) and macrophages (i.e. rabbit alveolar macrophages). Relatively high expression has also been found in proximal tubules of the kidney, transitional urothelium, ductal pancreatic cells, and germ cells. Varying levels have been observed in other organs including trophoblast, Purkinje cells and endothelial cells at different locations, such as brain and testes blood-barriers, as well as in fibroblasts. The ubiquitous presence of LRP in distinct cell types suggests that vaults fulfil a common basic function(s) in all cells, although cell type-specific roles cannot be excluded.

Many tissues with high LRP expression also display increased levels of other drug resistance associated proteins such as Pgp and MRP. They include epithelia of the bronchus and digestive tract, and macrophages [26–28]. Based on these localisations, a detoxifying role has been proposed for Pgp. These tissues are exposed to an array of toxic metabolites and xenobiotics and it would not be surprising that other molecules or cellular organelles contribute to cellular protection. It is tempting to speculate that vaults may protect the nucleus from nuclear toxins.

EXPRESSION OF THE MAJOR VAULT PROTEIN LRP IN HUMAN MALIGNANCIES AND ITS RELATIONSHIP WITH DRUG RESISTANCE

LRP in drug-unselected cancer cell lines

We first studied the expression of LRP in a panel of 61 human cancer cell lines used at the National Cancer Institute (U.S.A.) for screening of new anticancer drugs [29]. The panel includes cell lines derived from cancers of the colon, kidney, lung, breast, ovary and brain, as well as from melanoma and leukaemia [30]. The expression of Pgp and MRP in this panel was also investigated [29, 30].

LRP and MRP were found to be expressed at varying levels in 78 and 87% of the lines, respectively, whereas Pgp was detected at relatively low levels in only 24% of the lines [29]. These findings suggest that the vault and MRP-associated mechanisms of resistance are widespread in human malignancies. Furthermore, because these cell lines are not drug-selected, these mechanisms may play a role in inherent drug resistance. Cell lines derived from cancers that are generally considered to be poorly responsive to chemotherapy, such as colon or kidney cancer, displayed high levels of LRP-expression. In contrast, leukaemia cell lines showed low levels of LRP [29].

The NCI panel of cell lines had been previously characterised for *in vitro* response to a variety anticancer agents including seven classical MDR-related drugs and fifteen classical MDR-unrelated drugs [30]. Among the three MDR-associated proteins, LRP showed the greatest individual value as a marker of *in vitro* resistance to both MDR-related drugs (doxorubicin, vincristine) and, unexpectedly, also to some non-classical MDR drugs (cisplatin, carboplatin and melphalan) [29]. The LRP-associated phenotype of drug resistance seems to be broad, including drugs which are not substrates for Pgp or MRP. Thirty-seven cell lines (61%) co-expressed LRP and MRP, and eleven of these lines also showed Pgp expression. High levels of two or three MDR-related proteins were in general associated with high levels of drug resistance [29]. These findings suggest that multiple mechanisms, in particular those associated with LRP- and MRP-expression, may be already involved in drug-unselected cell lines.

LRP in clinical specimens

The presence of vaults in human tumours, as revealed by LRP-expression, and its correlation with clinical parameters are in the early stages of investigation. The data obtained so far and summarised below, suggest a potential role for vaults in clinical drug resistance.

An initial screening of 174 tumour specimens comprising 27 tumour types demonstrated LRP-expression in 63% of the cases [25]. Very often tumours were either mostly LRP negative or showed LRP immunoreactivity in the majority of the cells. The lowest frequency of LRP-expression was noted in germ cell tumours, Wilms' tumours, rhabdomyosarcoma, Ewing's sarcoma, and acute myeloid leukaemia. The more undifferentiated areas of neuroblastomas were mostly LRP negative, whereas scattered mature ganglion cells consistently showed high LRP levels, an observation confirmed by others [31]. Three of four small cell lung cancer specimens and a significant proportion of ovarian carcinomas (approximately 25%) were LRP negative. The vast majority of other solid tumours were predominantly LRP positive. For instance, colon, renal, pancreatic and endometrial cancers expressed LRP in all the specimens studied [25]. The distribution of LRP in clinical specimens was consistent with the *in vitro* data indicating the widespread distribution of the LRP-associated mechanism of drug resistance in human malignancies [29] and fairly reflected the responsiveness of different tumour types to chemotherapy [25]. Schadendorf and colleagues studied the immunohistochemical expression of Pgp, MRP and LRP in 21 primary and 37 metastatic malignant melanomas [32]. Pgp was detected in only one case. MRP and LRP were

expressed in 50 and 62% of the tumours, respectively, with no difference between primary and metastatic tumours. Among the metastatic specimens, no difference in MRP-expression was found between those taken prior to or after chemotherapy. In contrast, a significant correlation was found between prior chemotherapy treatment and increased LRP levels of expression. MRP and, in particular LRP, may be expected to provide insight into the drug resistance phenotype in malignant melanoma.

A preliminary report on the expression of LRP in 42 lung cancer specimens showed LRP positivity in 83% of squamous cell carcinomas, in 59% of adenocarcinomas, and in 36% of large cell undifferentiated carcinomas [33]. In contrast, only 5% of small cell lung cancers, the most chemosensitive subtype of lung cancer, expressed LRP. No correlation between LRP-expression and clinicopathological parameters was found in this heterogeneous and relatively small group of tumours.

LRP as predictor of response to chemotherapy and prognoses

Studies in different cancers have been initiated to investigate whether the expression of LRP in cancer specimens is predictive of response to chemotherapy and prognosis.

Childhood acute lymphoblastic leukaemia (ALL). In one study including 30 patients with relapsed childhood ALL, the expression of LRP, but not of Pgp, was significantly associated with an increased *in vitro* resistance of fresh leukaemic cells to daunorubicin (DNR) [34]. Next, M.L. den Boer (Free University Hospital, Amsterdam; unpublished data) studied the expression of Pgp, MRP and LRP in initial and relapsed childhood ALL. In addition, the accumulation of and the *in vitro* cytotoxicity to DNR were determined in leukaemic cells. Pgp- and MRP-expression were not correlated with resistance to DNR, and there was no difference in the levels of these proteins between initial and relapsed leukaemias. In contrast, the expression of LRP in 33 patients was weakly correlated ($P = 0.06$) to DNR resistance. Moreover, relapsed patients showed a significantly higher percentage of LRP positive cells than initial patients. These studies concluded that LRP, but not Pgp or MRP, may be a clinically relevant resistant protein in childhood ALL.

Acute myeloid leukaemia (AML). List and colleagues studied the prognostic significance of Pgp- and LRP-expression in adult AML [35]. Among 69 evaluable patients studied prospectively, both Pgp- and LRP-expression were associated with poor response to induction chemotherapy that included different combinations of DNR, arabinoside cytosine, mitoxantrone and etoposide. However, only LRP had independent prognostic significance in a logistic regression model. Only 32% of LRP positive patients achieved remission compared to 68% of LRP negative patients. In addition LRP, but not Pgp, approached significance as predictor for overall survival. Hart and coworkers studied samples from 60 patients with AML [36]. *LRP* gene expression was measured by RT–PCR and found to be significantly increased only in relapsed/refractory disease. No correlation was found between LRP transcript levels and *MDR1* or *MRP* gene expression.

Ovarian carcinoma. We investigated the expression of Pgp, MRP, and LRP in the tumours of 57 women with FIGO stage III/IV ovarian cancer [37]. These patients were treated with platinum and alkylating agent based chemotherapy. Sixteen and sixty-eight per cent of cases expressed Pgp and MRP, respectively. The detection of Pgp and MRP had no prognostic value in this group of patients. Seventy-seven per cent of the tumours expressed LRP. LRP positive tumours had a significantly inferior response to chemotherapy (8% complete, 36% partial, and 56% no response) as compared to LRP negative tumours (50% complete, 30% partial, and 20% no response) [37]. Furthermore, the expression of LRP was significantly associated with a shorter interval until tumour progression and shorter overall survival [37]. The predictive value of LRP is remarkable because most LRP-overexpressing MDR cell lines do not show crossresistance to platinum or alkylating agents. However, this finding agrees with the correlation between LRP-expression and sensitivity to cisplatin and melphalan in drug-unselected cancer cell lines [29]. This might indicate that LRP is frequently co-expressed with other resistance mechanisms. A direct role for the LRP-associated mechanism of resistance to some non-classical MDR, including platinum-based, drugs deserves further investigation. Alternatively, LRP may be associated with a more aggressive biological behaviour.

In conclusion, the studies performed to date agree in designating LRP as a potentially useful marker of clinical drug resistance. The fact that LRP appears to show prognostic value in different cancers treated with dissimilar chemotherapeutic regimens is certainly remarkable. These results agree with the capacity of LRP to predict a broad resistance phenotype in the drug-unselected cancer cell lines [29].

POSSIBLE MECHANISM OF VAULT-RELATED MDR

The data reviewed in the present article support a correlation between the amount of vaults, as reflected by LRP immunoreactivity, and resistance to anticancer drugs. Whether increased expression of vaults is sufficient or necessary to confer drug resistance requires further study. Transfection of only the *LRP* gene, coding for the human major vault protein, has failed to confer MDR [9]. This finding does not exclude a role of vaults in MDR considering that the complete vault particle will be required for functional activity.

Based on current knowledge regarding MDR and vaults, the following functional model is proposed. MDR cancer cells often display an altered intracellular distribution of drugs as compared to parental cells [38,39]. MDR cells distribute daunorubicin into the perinuclear region and, subsequently, redistribute the drug away from the nucleus into a punctate cytoplasmic pattern, whereas parental cells localise daunorubicin in a diffuse nuclear and cytoplasmic pattern [38, 39]. Reduced nuclear accumulation of daunorubicin has been reported in the LRP-overexpressing MDR cell line 2R120 [40]. The perinuclear and cytoplasmic structures mediating daunorubicin redistribution within MDR cells are unknown. Vaults appear to be good candidates. Considering the large size and abundance of vaults in the cytoplasm, the punctate staining pattern of LRP-56, as well as the evidence supporting a role of vaults as transporter units of the nuclear pore complexes, it is tempting to hypothesise that vaults can mediate drug resistance by regulating both the cytoplasmic redistribution and the nucleocytoplasmic transport of drugs (Figure 3). To test this hypothesis, the role of vaults in mediating drug resistance must be supported by gene knockout/antisense experiments in which vault structure is

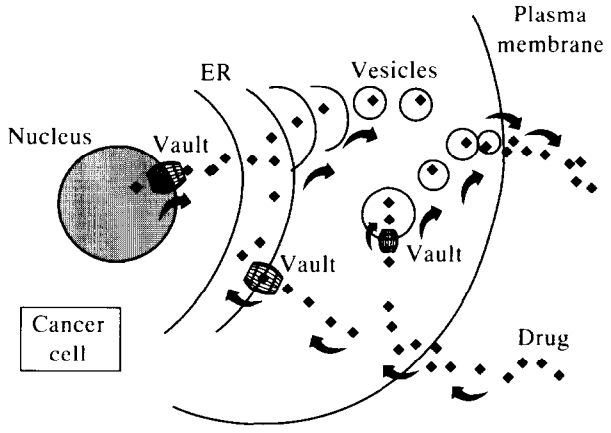

Figure 3. Hypothetical role of LRP in drug-resistance. LRP/vaults may mediate nucleocytoplasmic and vesicular transport of drugs. Through exocytotic vesicles the drugs would be extruded from the cell.

disrupted and their function is suppressed, this resulting in reversal of resistance.

CONCLUSION

The understanding of mechanisms mediating MDR is fundamental for the development of strategies aimed at preventing or circumventing cytostatic drug resistance, thus improving results of chemotherapy. It remains possible that, besides ABC-transporters, alternative mechanisms may mediate transport of drugs. The molecular, genetic and clinical data reviewed here indicate that the vault-related mechanism may be involved in such transport. Further studies into the role of vaults in drug resistance are warranted. Ultimately, the results of these studies may lead to the identification of molecules able to prevent or antagonise vault-related drug resistance.

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