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Minireview

The intriguing link between modulation of both multidrug resistance and ligand–toxin conjugate cytotoxicity

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Pharmacological agents which possess a chemosensitizing activity (i.e. the ability to modulate the multidrug resistance phenotype) can equally enhance ligand–toxin conjugate cytotoxicity. By confronting results obtained in both fields of research it appears that quite a number of agents, which are structurally unrelated, possess this bilateral effect. We have therefore attempted to provide a brief review of the literature and to discuss a hypothesis by which a common mechanism such as modifications in intracellular vesicle sorting and/or lipid metabolism may be implicated. We believe that these observations may provide clues for future research.

Immunotoxin; Multidrug resistance; *P*-Glycoprotein; Drug trafficking; Lipid metabolism; Chemosensitizer

1. INTRODUCTION

Metastatic cancers and malignant blood diseases are most often unresponsive to classical anticancer therapy, and several strategies aimed at developing new treatments are being pursued. Among these, two apparently distinct fields of research may be arriving at a crossroad.

The first strategy involves the utilization of pharmacological agents which are capable of increasing (as specifically as possible) the cytotoxicity of anti-cancer drugs (e.g. modulation of multidrug resistance (MDR) by chemosensitizers). The second strategy entails the development of anticancer agents with greater toxicity and specificity (i.e. ligand–toxin conjugates (LTC) such as immunotoxins (ITs)). Although, favorably modulating the sensitivity of neoplastic cells to such divers cytotoxic effectors is an attractive concept, one must recognize that, for the most part, the development of cytotoxicity-enhancers ('sensitizers') in both of these fields of research, has been based either on empirical screenings or serendipitous observations. This has mainly been due to the fact that we know little about the precise mechanisms of actions of these effectors, not to mention the eventual mechanisms of resistance. The situation would be even more extraordinary if a single sensitizer was capable of enhancing the cytotoxicity of both types of effectors, i.e. anticancer drug and LTC. Such a situation, implying that a single event induced by the sensitizer would have an impact on both types of effectors, could be of considerable interest for a better understanding of mechanisms of resistance in targeted neoplastic cells; and as a matter of fact, such a situation does exists in the uncanny observation that MDR modulators also enhance LTC activity.

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2. BACKGROUND

2.1. *Modulation of multidrug resistance*

The most widely studied form of drug resistance is the MDR phenotype which is defined as broad cross-resistance to cytotoxic drugs with no apparent structural and functional similarities [1]. Many of the drugs affected by this phenotype, such as taxol, vinca alkaloids (vinblastine, vincristine), anthracyclines (doxorubicin, daunorubicin), epipodophyllotoxins (etoposide, teniposide), actinomycin D, and mitoxantrone are important in current treatment protocols. The altered pharmacology of drugs in MDR cells (decreased accumulation and retention) appears to be mediated by a high molecular weight integral membrane protein, called *P*- glycoprotein (P-gp), that is overexpressed in MDR cells [2]. P-gp, which exhibits sequence similarities to certain bacterial transport proteins [3–5] and has ATPase activity [6] acts as a relatively unspecific energy-dependent efflux pump. Studies suggesting that the overexpression of the *MDR1* gene correlates with clinical resistance, support the hypothesis that the MDR

phenotype is involved in some aspects of resistance of cancers to chemotherapy [7–9].

Although the evidence certainly proves that P-gp can confer MDR to cancer cells [10], additional mechanisms contributing to MDR, such as modifications in intracellular drug distribution [11–17] have also largely been reported. Beck initially suggested that P-gp may somehow also be responsible for drug sequestration in acidic compartments [18], but although intracellular pH has been implicated in drug sequestration [16,17], Pastan's group has argued that the apparent increase in accumulation of drugs observed in the lysosomes and Golgi elements of MDR cells reflect more than the concentrative effect of the pH gradient in these organelles [12]. And in fact, the data presented by Hindenburg et al. implies the involvement of an energy dependent process [13]. Furthermore, Sehested et al. found increased trafficking of surface membrane material in MDR cells and proposed that this may be associated with increased drug extrusion [19]. This was consistent with the data of Warren et al. who suggested that the presence of P-gp may, in some manner, lead to the observed increased exocytosis of lysosomal enzymes [20].

The MDR phenotype can be overcome to various extents by treatment of cells with 'chemosensitizers' which increase intracellular drug accumulation specifically in MDR cells. These include calcium antagonists, calmodulin antagonists, lysosomotropic amines, cyclosporines, and other lipophilic agents [21]. Although, the mechanisms by which chemosensitizers modulate MDR is poorly understood, most appear to be either substrates for P-gp and/or inhibitors of P-gp activity [22–25]. However, modifications in intracellular drug distribution have also been reported. Both Hindenburg et al. and Schuurhuis et al. have shown that verapamil could modify the distribution of anthracyclines in drug resistant cells to that of the drug-sensitive cell phenotype [13,15], and recently our group showed that the chemosensitizer SR33557 induced a redistribution of doxorubicin in intracellular compartments of MDR cells [26]. Finally, it is also possible that some chemosensitizers may interfer with P-gp function by disrupting key regulatory sites, e.g. phosphorylation sites [27,28].

2.2. *Modulation of ligand–toxin conjugate cytotoxicity*

LTCs are conjugates of plant or bacterial toxins and a targeting vector usually composed of a monoclonal antibody but other vectors have been used, especially by Pastan's group, such as interleukines [29–31], epidermal growth factor [32], transforming growth factor [33], and insulin-like growth factor 1 [34]. Such ligand–toxin hybrids are being increasingly investigated as a means of targeting and killing tumor cells. The toxins employed in LTC construction are among the most active protein inhibitors in cell extracts [35]. These include ricin, pokeweed antiviral protein, gelonin, diphtheria toxin, and *Pseudomonas* exotoxin [36,37]. The general procedure for the construction of LTC relies on heterobifunctional reagents, such as *N*-succinimidyl 3-(2-pyridyl-dithiopropionate) which permit crosslinking between ligand and toxin [38]. However, the elimination of the cell binding component of the toxin (to prevent cross-reaction with non-targeted cells), which also plays a role in facilitating the translocation of the toxin from the endosome to the cytosol, greatly decreases the in vitro activity of LTCs [37,39]. Therefore, although these conjugates show stringent specific cytotoxicity, their clinical potential depends not only on their construction [40–42] but equally on ways of increasing their cytotoxic activity.

Among several strategies engaged in an effort to enhance LTC cytotoxicity, such as the development of more potent conjugates (e.g. using monoclonal antibody fragments [41] or chimeric fusion proteins [29–34]), potentiation of LTC cytotoxicity has been achieved in some cases with the use of sensitizers such as carboxylic ionophores [43,44], lysosomotropic amines [43–46], and calcium antagonists [47–52]. Here again, the mechanisms by which these agents enhance LTC cytotoxicity is poorly understood.

In order to be cytotoxic, LTCs need to be internalized. They bind to their membrane surface receptor, are transferred into acidic compartments (endosomes) and from there are directed either towards the Golgi apparatus or directly into the lysosome, where they are degraded [38,53,54]. Obviously, depending on the toxin moiety, limiting the access to the lysosomal route, or modifying its function, would favor both the release of toxin molecules and their translocation into the cytosol.

LTC enhancers have, in fact, been described as interfering with the intracellular routing of the ligand, most notably by interacting with the lysosome. The carboxylic ionophore monensin, for example, was first described by Jansen's group as producing a dramatic decrease in ricin A-chain-IT (RTA-IT) lysosomal sequestration and therefore increasing their number in pre- lysosomal compartments [53]. However, monensin blocks the toxicity of diphteria toxin-ITs by increasing pH and thereby preventing translocation [55]. Likewise, Pastan's group showed that verapamil inhibited the degradation of epidermal growth factor-*Pseudomonas* exotoxin (EGF-PE) conjugates most likely by interacting with lysosomes [56]. Finally, similar findings with RTA-ITs were reported by our laboratory for perhexiline and SR33557 [50–52].

3. BILATERAL EFFECT OF CERTAIN PHARMACOLOGICAL AGENTS: IMPLICATION OF THE LIPID METABOLISM

3.1. *Modulation of both multidrug resistance and ligand–toxin cytotoxicity*

When one confronts the results obtained in both of

the fields of research described above, a striking observation is made: certain pharmacological agents possess both a chemosensitizing activity and an LTC enhancement activity. For example verapamil, which was the first agent reported to restore sensitivity to MDR cells by Tsuruo's laboratory [57], was later shown to be an enhancer of RTA-IT and *Pseudomonas* exotoxin-IT cytotoxicity by Pastan's group [47–49]. In both of these cases the active concentrations of verapamil were within the micromolar range. Another calcium antagonist, perhexiline, which is structurally distinct from verapamil, was reported to be a chemosensitizer by Ramu et al. [58]. Our group found that perhexiline, at the same MDR modulating concentrations, was also a strong enhancer of RTA-IT cytotoxicity [50]. Furthermore, a structurally novel calcium antagonist, SR33557, was recently described by us to be both a highly potent MDR modulator [25] and enhancer of RTA-IT cytotoxicity at micromolar concentrations [52]. And, finally, both cyclosporine A and its non-immunosuppresive analogue SDZ PSC 833 are not only potent chemosensitizers with clinical potential [59–61], but are also potent RTA-IT enhancers (Jaffrézou, J.P., Sikic, B. and Laurent, G., Cyclosporine A and cyclosporine SDZ PSC 833 enhance anti-CD5 ricin A-chain immunotoxins in human leukemic T cells, submitted for publication). Such observations (see Table I) are numerous, and evidently, this crisscrossing of reports deserves a closer look. Since the most common denominator which is potentially involved in both MDR and LTC cytotoxicity is the intracellular trafficking of the cytotoxic agents, we would like to propose the following working hypothesis: agents which possess both a chemosensitizing activity and a LTC enhancing activity act by a discrete mechanism leading to perturbations in lipid metabolism with repercussions on P-gp activity, endocytosis and intracellular vesicle sorting.

3.2. *Implication of the lipid metabolism*

Many of the agents listed in Table I which are lysosomotropic, have previously been described as interfering with lysosomal function. Lysosomes contain over 40 hydrolytic enzymes and are important sites for intracellular digestion. Intracellular membrane supply of lipids, such as cholesterol and sphingomyelin, which arrive through the process of autophagy are normally de-

Table I

Reversal of multidrug resistance (MDR) and enhancement of ligand–toxin conjugate (LTC) cytotoxicity: bilateral effect of certain pharmacological agents

Drug	MDR reversal [ref.]	Drug conc.	LTC enhancement [ref.]	Drug conc.
Calcium antagonists				
Verapamil	[23,57,62–67]	1–20 μM	[47,52]	5–200 μM
Prenylamine	[66–68]	6.6–10 μM	[51]	4 μM
Bepridil	[69]	4 μM	[51]	4–10 μM
Nifedipine	[23,64,68]	1–100 μM	[46]	6 μM
Diltiazem	[62–64]	1–100 μM	[47,51]	25–50 μM
SDB[a]	[70]	17–68 μg/ml	[71]	17–34 μg/ml
Perhexiline	[58,72]	1–10 μM	[50,51]	1–10 μM
SR33557	[25]	3–30 μM	[51,52]	5 μM
Calmodulin antagonists				
Trifluoperazine	[23,62,66,68,73,74]	1–10 μM	[48,75]	1–3 μM
Thioridazine	[73,76]	3–9 μM	[75]	3–9 μM
Chlorpromazine	[65,73,74]	4–10 μM	[48,75]	1–10 μM
Carboxilic ionophores				
Monensin	[77,78]	10–25 μM	[43,46,50,51,79]	0.05–0.1 μM
Nigericin	[77]	67 μM	[43]	0.01 μM
Lysosomotropic amines				
Chloroquine	[65,80]	3–100 μM	[43,46]	40–100 μM
Amantadine	[81]	40–66 μM	[44]	1000 μM
Other				
Cepharantine	[82]	1–2 μg/ml	[83]	1–2 μg/ml
Cyclosporine A	[59]	2–4 μM	b	2–4 μM
SDZ PSC 833	[60]	1–4 μM	b	2–4 μM

[a]Synthetic isoprenoid: *N*-solanesyl-*N*,*N*′-bis(3-4-dimethoxybenzyl)ethelenediamine.

[b]Jaffrézou J.P., Sikic B.I., and Laurent, G. (1993) Cyclosporine A and cyclosporine SDZ PSC 833 enhance anti-CD5 ricin A-chain immunotoxins in human leukemic T-cells (submitted for publication).

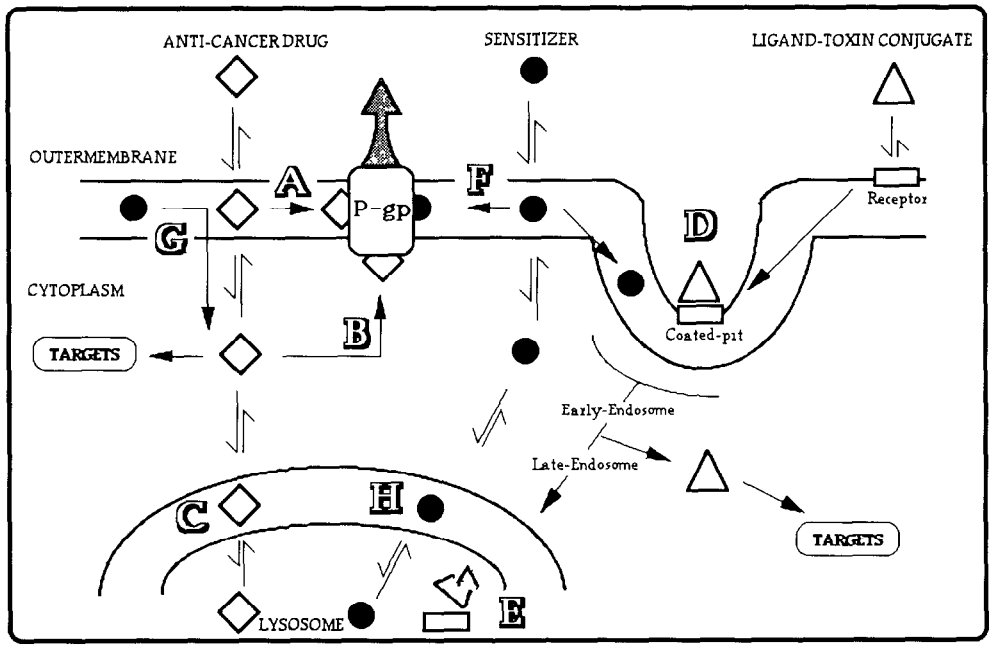

Fig. 1. Scheme of how certain pharmacological agents, can both modulate multidrug resistance (MDR) and enhance ligand–toxin conjugate (LTC) cytotoxicity. (1) Cytotoxic drugs (open diamonds) interact with membrane lipids and can be captured, before they reach there target, by P-gp either directly from the lipid bilayer (A) and/or from the cytoplasm after their diffusion (B). These drugs may also interact with intracellular membranes and acidic vesicles such as lysosomes (C). (2) LTCs (open triangles) are generally internalized through coated pits (D) and traffic towards lysosomes where they are degraded (E). A portion of the internalized LTCs escape this fate and reach cytoplasmic targets. (3) Sensitizers (closed circles) may modulate MDR either by directly inhibiting P-gp (F) and/or by displacing cytotoxic drugs from the lipid bilayers thereby preventing their expulsion by P-gp (G). Sensitizers may also interact with intracellular vesicles (such as lysosomes, H), by either disrupting lipid metabolism and/or modifying pH, leading to the observed redistribution of cytotoxic drugs. Sensitizers may increase LTC cytotoxicity by re-routing LTC through membrane channels, by-passing degradation and releasing active toxin into the cytoplasm, or by interferring with lysosomal or vesicular processing by changing the pH or lipid metabolism

graded in lysosomes [84,85]. In this perspective, cationic amphiphilic drugs (CADs) have been described as concentrating in the lysosomes and inhibiting the intralysosomal breakdown of lipids. This leads to the induction of lipidosis, which is characterised by an increase in cellular phospholipids and the appearance of lamellated inclusion bodies (for review see [86]). The cationic amphipatic nature of most sensitizers suggests that a strong interaction can and will occur with certain polar lipids resulting in complexes formed by hydrophobic and electrostatic forces [87]. The ability of these putative lysosomotropic agents to induce lysosomal storage of polar lipids (these agents seemed to be also accumulated in lysosomes together with lipids) and perturbing membrane function has been described by Lüllmann et al. [88,89]. The presence of such, morphological alterations by sensitizers were first described by Pastan's group with the abnormal appearance of lysosomes in verapamil treated cells [56]. Since then, Akiyama's group and our laboratory have shown that cells treated with thioridazine [90], cepharantine [83], SDB [71], perhexiline [50], and SR33557 [51,52] all show the presence of many autophagosomes and electron-dense lysosome-like bodies.

Morphological alterations, such as those described above, have been linked to the interaction of the sensitizers with certain lipids such as phosphatidylserine [91], and sphingomyelin [25,50–52,54] as well as certain lysosomal enzymes such as acid sphingomyelinase [25,50–52], camthepsin D and acid phosphatase [92]. Moreover, chlorpromazine and chloroquine have been shown to directly affect phospholipid synthesis [93–95]. Chlorpromazine, for example is a potent inhibitor of phospholipase A1 [94,96], and of protein kinase C [97]. Finally, although sensitizers such as cyclosporine A and monensin may not induce phospholipidosis, the former has been described as possibly binding to lipid domains and thereby provoking changes in both membrane potential and lipid order [98], and the latter has recently been shown to directly inhibit sphingomyelin synthesis [99].

3.2.1. Enhancement of ligand–toxin cytotoxicity

Several reports have shown that interference with lysosomal function in particular with lipid metabolism by lysosomotropic agents may not only lead to delayed digestion of cellular lipids, but also of cellular and endocytosed proteins. Monensin, for example, has been shown to disrupt membrane trafficking and block the function of the trans Golgi apparatus [91] and to delay

RTA-IT degradation [52]. Other studies have shown that chemosensitizers such as propanolol (a lysosomotropic amine) [100], trifluoperazine, chlorpromazine, and thioridazine (calmodulin antagonists) also inhibit lysosomal degradation of low density lipoproteins and/or EGF-PE conjugates [75]. In the calcium antagonists family, which has been described as inhibiting the degradation of low-density lipoproteins [90], inhibition of intracellular EGF-PE conjugate degradation has been shown by Pastan's group for verapamil [56] and its analog SDB [71], and by our group for RTA-ITs by perhexiline and SR33557 [50–52].

3.2.2. Reversal of multidrug resistance

From the observations described above, it is relatively easy to imagine how interference with the lipid metabolism could lead to increased LTC cytotoxicity by modifying the intracellular trafficking leading to a more favorable cytotoxic pathway. It is also conceivable that these interferences with lipid metabolism would have an impact on MDR. Indeed, Higgins and Gottesman proposed that P-gp may interact with drugs which are intercalated within the lipid bilayers [101]. These authors also proposed that differences in membrane lipid composition could effect drug partitioning and therefore explain the variable resistance patterns seen for P-gp in different tumor cell lines. Presumably, chemosensitizer-induced modifications in membrane lipid composition would have similar repercussions. Moreover, two recent studies have clearly shown that P-gp mediated ATP hydrolysis is dependent on the lipid environment [102,103]. Finally, it is also conceivable that alterations in lipid metabolism induced by CADs may also bring about changes in receptor-mediated events [104,105] thereby perturbing pathways such as the phospholipid-signaling pathway which in turn can effect protein phosphorylation [106,107], and perhaps P-gp.

4. CONCLUSION

By confronting results obtained both in the fields of MDR modulation and LTC enhancement, it appears that quite a number of agents, which are structurally unrelated, possess a bilateral effect. Several lines of evidence suggest that alterations in lipid metabolism may be a factor. Of course caution should always be exercised in extrapolating some sort of definite link. Nevertheless, these observations, which suggest that their may indeed exist a link between the mechanism(s) of action involved, do lead some investigators, as we have seen, to simultaneously explore both fields of research.

Although our hypothesis is certainly provocative, and their is still little data with which to elucidate a common mechanism, the concept that sensitizers, most of which have been described as interacting with either endocytosis, intracellular vesicle sorting, and/or lipid metabolism, play a role in both MDR modulation and LTC enhancement may provide clues for future research (see Fig. 1).

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