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Antagonist G-mediated targeting and cytotoxicity of liposomal doxorubicin in NCI-H82 variant small cell lung cancer

J.N. Moreira and R. Gaspar

Laboratory of Pharmaceutical Technology, Faculty of Pharmacy and Center for Neuroscience and Cell Biology, University of Coimbra, Coimbra, Portugal

Abstract

The aim of the present study was to characterize the interactions of antagonist G (H-Arg-D-Trp-N^{me}Phe-D-Trp-Leu-Met-NH_2)-targeted sterically stabilized liposomes with the human variant small cell lung cancer (SCLC) H82 cell line and to evaluate the antiproliferative activity of encapsulated doxorubicin against this cell line. Variant SCLC tumors are known to be more resistant to chemotherapy than classic SCLC tumors. The cellular association of antagonist G-targeted (radiolabeled) liposomes was 20-30-fold higher than that of non-targeted liposomes. Our data suggest that a maximum of 12,000 antagonist G-targeted liposomes were internalized/cell during 1-h incubation at 37ºC. Confocal microscopy experiments using pyranine-containing liposomes further confirmed that receptor-mediated endocytosis occurred, specifically in the case of targeted liposomes. In any of the previously mentioned experiments, the binding and endocytosis of non-targeted liposomes have revealed to be negligible. The improved cellular association of antagonist G-targeted liposomes, relative to non-targeted liposomes, resulted in an enhanced nuclear delivery (evaluated by fluorimetry) and cytotoxicity of encapsulated doxorubicin for incubation periods as short as 2 h. For an incubation of 2 h, we report IC_{50} values for targeted and non-targeted liposomes containing doxorubicin of 5.7 ± 3.7 and higher than 200 µM doxorubicin, respectively. Based on the present data, we may infer that receptors for antagonist G were present in H82 tumor cells and could mediate the internalization of antagonist G-targeted liposomes and the intracellular delivery of their content. Antagonist G covalently coupled to liposomal drugs may be promising for the treatment of this aggressive and highly heterogeneous disease.

Correspondence
J.N. Moreira
Laboratory of Pharmaceutical Technology, Faculty of Pharmacy
University of Coimbra
Rua do Norte, 3000-295
Coimbra
Portugal
Fax: +351-239827126
E-mail: jmoreira@ci.uc.pt

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- Doxorubicin
- Targeting
- Antagonist G
- Lung cancer

Introduction

Small cell lung cancer (SCLC) is an aggressive form of lung cancer that is highly metastatic in humans (1). SCLC accounts for 25% of all pulmonary cancers and, despite an initial responsiveness to radiotherapy and chemotherapy, the patient 5-year survival rate is only 5% (2). SCLC cell proliferation is driven by multiple autocrine and paracrine growth loops, involving multiple mitogenic neuropeptides, which play an impor-

tant role in the aggressiveness of this disease (3,4). Substances that interrupt the mitogenic signals triggered by these neuropeptides provide a new way of treating SCLC. The hexapeptide, H-Arg-D-Trp-N^{me}Phe-D-Trp-Leu-Met-NH_2, known as antagonist G, is one such substance. It works by blocking the action of multiple neuropeptides at the receptor level (5,6) and has been shown to inhibit the growth of SCLC cells both *in vitro* and *in vivo* (4,6).

Entrapment of anticancer drugs in Stealth® liposomes sterically stabilized with poly(ethylene glycol) (Mr 2000) distearoylphosphatidylethanolamine conjugates, results in increased tumor accumulation and improved therapeutic efficacy. Coupling cancer cell-specific ligands like monoclonal antibodies to the surface of Stealth liposomal doxorubicin (DXR) has proved to be an efficient means of improving the cytotoxicity and therapeutic efficacy of the encapsulated drug (7,8). Small ligands like antagonist G may be more advantageous than antibodies for targeting purposes because they are chemically defined and can be manufactured in large quantities in pure form without biological contaminants. We have shown that the use of antagonist G as a targeting ligand for Stealth liposomes improved the intracellular delivery and cytotoxicity of encapsulated DXR against the classic SCLC H69 cell line compared to non-targeted liposomes (9). This interaction was shown to be peptide- and cell-specific (9). However, SCLC is believed to undergo a progression from a classical to a variant form. This transformation is associated with possible changes in surface proteins (receptors), increased cell proliferation, amplification of the c-myc proto-oncogene, and resistance to chemotherapy (10,11). Patients presenting variant SCLC are known to respond less well to chemotherapy and have shorter survival times (10).

The interaction between antagonist G and variant SCLC cell lines has not been characterized. Among the several variant SCLC cell lines available, the H82 cell line is the one that presents growing features (namely, growing as floating cellular aggregates) that are identical to the ones presented by the classic SCLC H69 cell line previously studied (9). Therefore, here we studied the interaction of antagonist G-targeted liposomes (SLG) with the human variant SCLC H82 cell line and evaluated the antiproliferative activity of encapsulated DXR.

Material and Methods

Material

Antagonist G (H-Arg-D-Trp-N^{me}Phe-D-Trp-Leu-Met-NH_2) and substance P [1-9] (H-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-NH_2) were synthesized by the Alberta Peptide Institute (Edmonton, AB, Canada). Fully hydrogenated soy phosphatidylcholine (HSPC), methoxy poly(ethylene glycol) (Mr 2000) distearoyl-phosphatidylethanolamine (mPEG-DSPE) and N-(3'-(pyridyldithio) propionoyl)amino-poly(ethylene glycol) (Mr 2000) distearoylphosphatidylethanolamine (PDP-PEG-DSPE) were generous gifts of Alza Corp. (Mountain View, CA, USA). All other chemicals were of analytical grade purity.

Cell line

The human variant SCLC cell line NCI-H82 (ATCC HTB-175) was purchased from the American Type Culture Collection and cultured in RPMI 1640 supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin (Gibco-BRL, Grand Island, NY, USA) and maintained at 37ºC in a humidified incubator containing 5% CO_2.

Preparation of liposomes

Liposomes composed of HSPC:cholesterol:mPEG-DSPE:PDP-PEG-DSPE at a

2:1:0.08:0.02 molar ratio, were prepared by lipid film hydration at 65ºC. For 8-hydroxypyrene-1,3,6-trisulfonic acid, trisodium salt (HPTS)-containing liposomes, the aqueous-space label was added during the hydration step (12). The resulting multivesicular preparations were then extruded at 65ºC sequentially through 0.2 down to 0.08 µm polycarbonate membranes (Nucleopore, Pleasanton, CA, USA) using a Lipex extruder (Lipex Biomembranes, Vancouver, BC, Canada), to provide vesicles averaging 100 nm in diameter (13), as determined by dynamic light scattering. Liposomes containing DXR (Faulding Inc., Vaudreuil, PQ, Canada) were prepared by the ammonium sulfate gradient method (14). The loading efficiency of DXR was greater than 95% and the liposomes (with a mean diameter of 100 nm) routinely contained approximately 200 µg DXR/µmol phospholipid.

Antagonist G-targeted liposomes were prepared by chemical coupling of the peptide to the end of the PEG chain of PDP-PEG-DSPE, according to a previously described method (9). The amount of coupled peptide was approximately 1 µg antagonist G/µmol phospholipid. The same procedure was used to couple a non-specific peptide, substance P [1-9].

Phospholipid concentration was determined from either the specific activity counts of the [1α,2α(n)-^{3}H] cholesteryl hexadecyl ether ([^{3}H]-CHE) tracer or by the colorimetric assay of Bartlett (15).

Association of liposomes with H82 cells

[^{3}H]-CHE-liposomes were incubated with 1 x 10^6 cells/well on Falcon 48-well plates for 1 h at 37ºC, as described (7). In competition experiments, the cells were incubated with either free antagonist G (0-29 µg antagonist G/well, for 30 min at 4º or 37ºC) or antagonist G-coupled non-radiolabeled liposomes (0-0.6 µg antagonist G/well), or just liposomes for 30 min at 37ºC before the addition of [^{3}H]-CHE-SLG (0.1 mM phospholipid/well). After incubation, the cells were washed three times with cold phosphate-buffered saline (PBS), pH 7.4. The amount of liposomes associated with cells was calculated from the initial specific activity of [^{3}H]-CHE-liposomes by scintillation counting and is reported as nmol phospholipid/10^6 cells.

In some experiments, cells were plated onto 24-well plates at 2 x 10^6 cells/well. HPTS-containing liposomes, with or without coupled antagonist G, or coupled to substance P [1-9], HPTS-SLP [1-9], were added to each well (0.8 mM phospholipid/well, a total volume of 0.4 ml) and maintained at 37ºC in an atmosphere with 95% humidity and 5% CO_2 for 1 h. After washing three times with PBS, the cells were visualized and optically sectioned with an LSM-510 laser-scanning confocal microscope (Carl Zeiss, Jena, Germany) using an ultraviolet laser with emission at 364 nm for scanning.

Doxorubicin uptake

The kinetics of DXR uptake was examined for the H82 cells as a function of time both in whole cell extracts and in nuclei isolated from 50 x 10^6 cells exposed to free DXR or DXR-containing liposomes, with (DXR-SLG) or without coupled antagonist G (DXR-SL), or coupled with the non-specific peptide substance P (DXR-SLP [1-9]). The procedure for isolating nuclei and determining their DXR content has been previously described (9).

Cytotoxicity

In vitro cytotoxicity of free DXR and various DXR-containing liposome formulations was determined for H82 cells using the 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide *in vitro* proliferation assay (16). Briefly, H82 cells were seeded onto 96-well plates at 3 x 10^4 cells/well and incubated with different concentrations of free DXR,

DXR-SL, DXR-SLG, free antagonist G, empty SLG or free DXR mixed with empty SLG (at 200 µg DXR/µmol phospholipid), for 2, 24 or 48 h at 37ºC in an atmosphere with 95% humidity and 5% CO_2. At the end of incubation the cells were gently washed twice with PBS to remove the drug. The cells were then maintained in fresh medium at 37ºC in an atmosphere of 95% humidity and 5% CO_2 for up to 3 days from the beginning of the study. Cell viability was then assessed as described (17). Results are reported as IC_{50} (µM DXR, unless otherwise stated), determined from the dose-response curves.

Statistical analysis

The Student *t*-test was used to identify statistically significant differences between pairs of samples. Multiple comparisons of IC_{50} (Table 1) were performed by analysis of variance (ANOVA). The level of significance was set at $P < 0.05$ for all analyses.

Results and Discussion

Small cell lung cancer proliferation is driven by multiple autocrine and paracrine growth loops involving, among others, several neuropeptides including vasopressin, bradykinin and gastrin-releasing peptide. Binding of these neuropeptides to their receptors triggers a cascade of intracellular signals (including an increase in intracellular calcium) that culminates with DNA synthesis and cell proliferation (4). SCLC is known to undergo a transformation from a classical form that responds to chemotherapy to a variant form that grows rapidly, overexpresses the proto-oncogene c-myc and is refractory to treatment (10). The proto-oncogene c-myc is thought to play an important role in the regulation of cell division (18). Chemotherapy itself can be one of the causes for such differentiation (19). It has been previously reported that classic SCLC cell lines exhibit an increase of intracellular calcium upon the binding of several autocrine growth factors (namely, vasopressin) at the receptor level, while most variant cell lines (including the H82 cell line) show no increase in intracellular free calcium (11,20). It was hypothesized that one of the reasons for this refractoriness could be the absence in variant cells of receptors for many small mitogenic neuropeptides (20). The presence or absence of these receptors could be critical for the therapeutic success of target-based strategies against SCLC.

In the present study we have shown that the covalent attachment of antagonist G at the end of PEG-grafted (Stealth) liposomes gave results that were consistent with receptor-mediated internalization of liposomes by the human variant SCLC H82 cell line. This resulted in improved intracellular delivery and cytotoxic activity of encapsulated DXR relative to non-targeted liposomes.

The differences in cellular association in the experiments carried out at 4º and 37ºC

Table 1. Cytotoxicity of various doxorubicin formulations against H82 cells.

Incubation time (h)	DXR-SL	DXR-SLG	DXR	DXR + empty SLG	Empty SLG (mM phospholipid)	Free antagonist G (µM antagonist G)
2	>200	5.7 ± 3.7	3.4 ± 1.2	2.2 ± 0.2	>1	148 ± 4.4
24	60.9 ± 22.9	5.5 ± 4.9	1.2 ± 0.2	1.1 ± 0.2	>1	57.8 ± 7.2
48	21.3 ± 7.7	6.4 ± 2.8	0.6 ± 0.5	0.8 ± 0.6	>1	39.3 ± 19.5

Data are reported as the mean ± SD of the IC_{50} values (µM DXR) for 3-7 independent experiments, unless otherwise stated, and were extrapolated from the dose-response curves. DXR = doxorubicin; DXR-SL = non-targeted liposomes containing doxorubicin; DXR-SLG = antagonist G-targeted liposomes containing doxorubicin; SLG = antagonist G-targeted liposomes.

with [^{3}H]-CHE-SLG (Figure 1) suggests that antagonist G-targeted liposomes were being internalized by H82 cells. At 37ºC, where both binding and internalization take place, SLG associated with the cells approximately 20-30-fold more than SL, SLP [1-9] or SL in the presence of free antagonist G. At 4ºC, a 2.2- to 2.7-fold decrease in the cellular association of SLG was observed, which is to be expected if the component of cell-liposome association due to liposome internalization by the tumor cells was inhibited at this low temperature. Based on the assumption that there are 7.7 x 10^{12} liposomes/µmol phospholipid (7), we estimate that, after a 1-h incubation, within the phospholipid concentration range tested, there were 3,600-12,000 SLG liposomes internalized/cell.

Images of optically sectioned cells obtained by confocal microscopy showed that after 1 h at 37ºC SLG liposomes containing the fluorescent dye HPTS (HPTS-SLG) were distributed both on the cell surface and intracellularly (Figure 2A). Under the same conditions, no staining was detectable when the cells were treated with either HPTS-SL (Figure 2B) or HPTS-SLP [1-9] (Figure 2C).

Association of [^{3}H]-CHE-SLG with H82 cells was competitively inhibited only when cells were pre-incubated with non-radiolabeled SLG (Figure 3B) at a concentration that was 48-fold lower than the highest amount of free antagonist G that showed no inhibition of binding either at 4º or at 37ºC (Figure 3A). This result is also consistent with the explanation that the association of SLG with H82 cells was taking place mainly through a receptor-mediated process, possibly involving multivalent binding sites. The amount of coupled antagonist G needed to reach 50% of cell-liposome association inhibition was 0.03 µg (Figure 3B). In a control experiment, liposomes pre-incubated with the tumor cells did not interfere with the cell-liposome association of [^{3}H]-CHE-SLG (data not shown). The covalent linkage of antagonist G to the PEG-grafted liposomes appears to have increased the avidity of the peptide for its receptors due to the multivalent presentation of the peptide, that is, the presence of several attached-peptide molecules allows one liposome to bind more than one receptor at once. DeFrees et al. (21) have shown that the use of sialyl Lewis glycolipid coupled to liposomes was a stronger inhibitor of E-selectin-dependent cell adhesion than free glycolipid. Overall, these studies reinforce

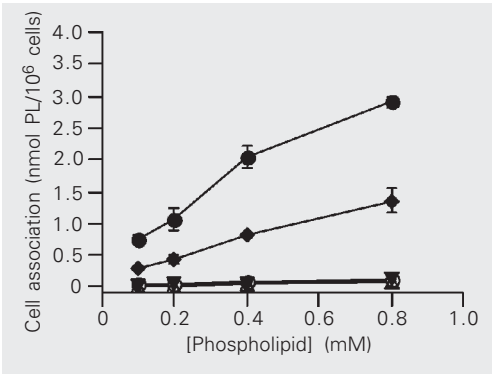

Figure 1. Association of [^{3}H]-CHE-labeled liposome formulations with SCLC H82 cells. H82 cells (1 x 10^6 cells) were incubated with different liposomal formulations (0.1-0.8 mM phospholipid (PL)/well) containing SL (open circles) or SLP [1-9] (inverted triangles) at 37ºC or SLG at 4ºC (lozenges) or 37ºC for 1 h (filled circles) or SL in the presence of free antagonist G at an antagonist G/phospholipid molar ratio of 1:200 (open triangles). Each point is the mean ± SD for 3-4 samples. SCLC = small cell lung cancer; SL = non-targeted liposomes; SLG = antagonist G-targeted liposomes; SLP = substance P-coupled liposomes.

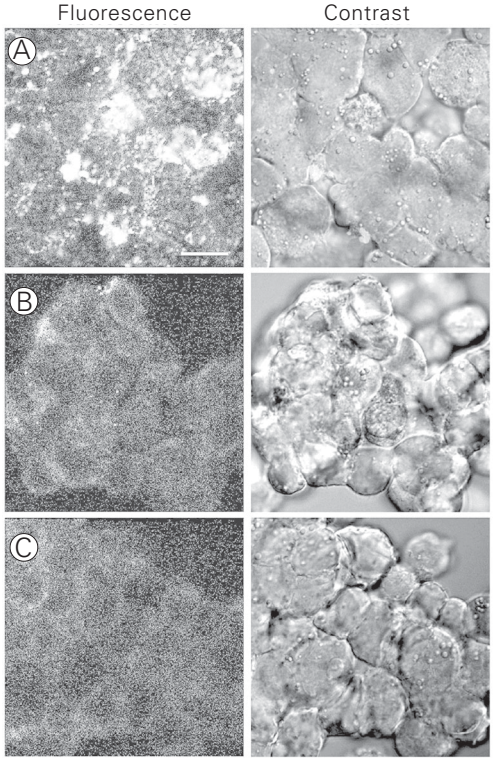

Figure 2. Association of HPTS-containing liposomes with SCLC H82 cells. H82 cells (2 x 10^6 cells) were incubated with liposomes (0.8 mM phospholipid/well) at 37ºC for 1 h. H82 cells were incubated with SLG (A), SL (B) or SLP [1-9] (C). After washing with cold PBS, the cells were visualized with an LSM-510 laser-scanning confocal microscope. HPTS = 8-hydroxypyrene-1,3,6-trisulfonic acid, trisodium salt; SCLC = small cell lung cancer; SLG = antagonist G-targeted liposomes; SL = non-targeted liposomes; SLP = substance P-coupled liposomes. Scale bar for all panels = 10 µm.

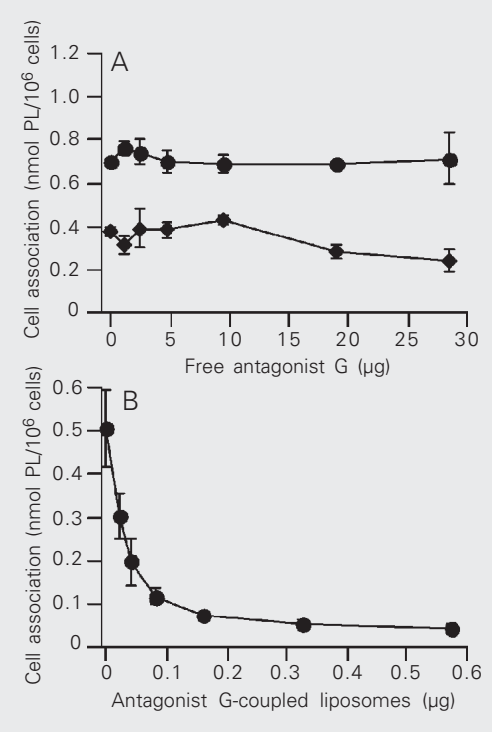

Figure 3. Competitive inhibition of the association of [^{3}H]-CHE-SLG with SCLC H82 cells. H82 cells (1 x 10^6 cells) were pre-incubated for 30 min with: *A*, 0-29 µg of free antagonist G, either at 4ºC (lozenges) or 37ºC (circles); *B*, 0-0.6 µg of antagonist G covalently linked to non-radiolabeled SLG at 37ºC (circles). Competitive inhibition was determined by adding [^{3}H]-CHE-SLG (0.1 mM phospholipid (PL)/well), either at 4º or 37ºC and incubating for 1 h. Each point is the mean ± SD for 3 samples. [^{3}H]-CHE-SLG = [1α, 2α(n)-^{3}H] cholesteryl hexadecyl ether-antagonist G-targeted liposomes; SCLC = small cell lung cancer; SLG = antagonist G-targeted liposomes.

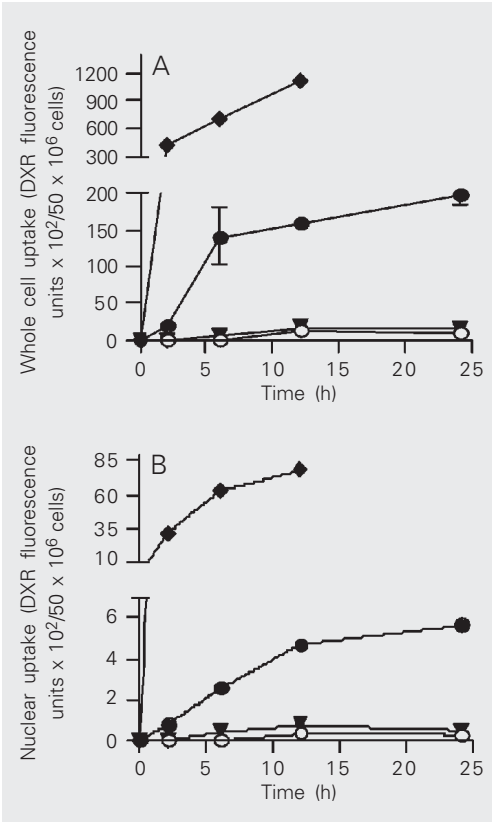

Figure 4. Kinetics of DXR or DXR-containing liposome uptake by SCLC H82 cells. Twenty µM of free DXR (lozenges) or DXR-containing liposomes (DXR-SL, open circles; DXR-SLG, filled circles, and DXR-SLP [1-9], triangles), were incubated with 50 x 10^6 H82 cells at 37ºC for 24 h. DXR was measured in whole cell extracts (A) or in isolated nuclei (B). The background was subtracted from the values at each time point. Each point is the mean ± SD of 3 samples. DXR = doxorubicin; SL = liposomes; SLG = antagonist G-targeted liposomes; SLP = substance P-coupled liposomes.

the idea that liposomes might be a powerful tool for the efficient presentation of ligands to cell surface receptors that require multivalent contact.

The increased levels of cell-liposome association of SLG relative to SL or SLP [1-9] resulted in a more efficient intracellular delivery of DXR when it was encapsulated in antagonist G-targeted formulations, both to the whole cells and to the nuclei (Figure 4A,B). When delivered by SLG, accumulation of DXR, both into whole cells and nuclei, was faster and higher (10- to 20-fold) than when the drug was delivered either by SL or SLP [1-9]. Interestingly, in whole cell extracts DXR from SLG accumulated more rapidly and plateaued at 6 h compared to drug accumulation in the nucleus, which plateaued at around 12 h. This may be related to either uptake of some free DXR released from the liposomes prior to internalization or to the release of the drug from endosomes (22). The accumulation of free DXR both in the whole cell extracts and in the nuclear fractions, was more rapid and occurred to a greater extent than that of the liposomal samples. This does not necessarily lead to improved tumor accumulation *in vivo* due to the higher volume of distribution of free DXR relative to that of liposomal DXR (23).

We then determined whether the improved intracellular drug delivery by the targeted formulation would translate into an improved antiproliferative activity against the H82 cell line (Table 1). Interestingly, after an incubation of only 2 h, DXR-SLG was as toxic as free DXR ($P > 0.05$) and approximately 35 times more toxic than DXR-SL ($P < 0.001$). After 24- and 48-h incubations, the differences in cytotoxicity between DXR-SLG and free DXR were not significant ($P > 0.05$), and while the differences between DXR-SLG and DXR-SL decreased, they were still statistically significant ($P < 0.001$). The absence of any differences between free DXR and free DXR in the presence of empty SLG at 200 µg DXR/µmol phospholipid ($P >$

0.05), independent of incubation time, suggested that there was no contribution of antagonist G to the observed cytotoxicities. The IC_{50} values for empty SLG and free antagonist G were much higher than the concentrations of phospholipid and antagonist G at the IC_{50} for DXR-SLG at all incubation times, confirming that the cytotoxic activity of this sample was exclusively due to the more efficient delivery of the encapsulated drug by SLG.

The cytotoxicity results demonstrate that binding and internalization of DXR-SLG contribute to an increased level of cytotoxicity against the H82 cell line compared to DXR-SL. The results for DXR uptake into whole cells plateaued before that in isolated nuclei, suggesting that the release of DXR from the endosomes may be delayed. How this delay in DXR release from endosomes, which will affect the rate at which the drug reaches intracellular sites of action, impinges on the overall cytotoxicity of targeted formulations remains to be assessed. In spite of a possible delay in the release of DXR from the endosomes, however, we observed that the targeted formulations had a cytotoxicity that was similar to that of the free drug *in vitro*.

Overall, based on the data presented here, it may be inferred that receptors for antagonist G in H82 cells were present and functionally active, and were not turned off by the differentiation process that took place. These results, along with the high affinity of antagonist G for the vasopressin receptor (6), which is expressed on the surface of variant SCLC cells (11,24), suggest that this could be the main receptor involved in the internalization of SLG. Moreover, the small differences between the amounts of coupled antagonist G necessary to inhibit 50% of cellular association of SLG, both in the H82 variant and the classical H69 SCLC cell lines (9), suggested a similar density of receptors for antagonist G between these two subtypes of SCLC cells. This was confirmed by the similar cellular association patterns of either fluorescent radiolabeled liposomes or DXR-containing liposomes (9).

The similar cytotoxic activity of DXR-SLG against the H82 variant and the H69 SCLC cell lines (9), where c-myc proto-oncogene is either not detectable or present at only trace levels (18), suggests that the delivery of liposomal DXR through receptor-mediated endocytosis could be an efficient way to overcome the problems of c-myc overexpression by variant cells. Moreover, the long circulation half-lives of SLG (9), a crucial feature for *in vivo* applications, give antagonist G-targeted liposomes containing DXR, or antisense oligonucleotides against c-myc, potential for the treatment of variant small cell lung cancer.

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