



REVIEW

A role for intracellular immunization in chemosensitization of tumor cells?

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Acquired drug resistance represents a major cause of chemotherapy failure in patients with cancer. The characterization of the molecular pathways involved in drug resistance has provided us with new targets to overcome this problem. Many of these target proteins are often overexpressed in human cancers. A number of gene therapy strategies, including antisense oligonucleotides, ribozymes and single-chain antibodies, have been developed to achieve the selective modulation and inhibition of various cellu-

lar proteins. Thus, these approaches can be exploited to modulate the resistance phenotype of tumor cells. These gene therapy strategies represent a novel and unique way to enhance the sensitivity of tumor cells to chemotherapeutic drugs. This review will focus on the use of intracellular immunization as a means to modulate the expression of specific genetic determinants involved in the drug resistance phenotype.

Keywords: intracellular immunization; chemosensitivity; gene therapy; drug resistance; cancer

Introduction

The treatment of tumors that fail to respond to therapy or that relapse after initial response to chemotherapy has been a major clinical challenge. Acquired drug resistance and cross-resistance are major causes of chemotherapy treatment failure in a variety of cancers. Tumor cells can acquire drug resistance via several distinct mechanisms, including altered glutathione metabolism,¹ increased DNA repair mechanism,^{2,3} altered topoisomerase activity,^{4,5} and increased expression of the MDR1 gene product.^{6–14} In addition, alterations in the expression of various oncogenes, such as Bcl-2,^{15–19} p53,^{18,20–22} and cyclin D1,^{23,24} and growth factor receptors, not only can lead to the perturbation of growth regulation but may also affect the sensitivity of tumor cells to conventional chemotherapy.^{25–34} The delineation of some of the genetic determinants involved in drug resistance has enabled the development of new gene therapy-based strategies to overcome this important clinical problem.

In this context, intracellular immunization strategies have been developed to abrogate the expression of specific molecular targets. The term 'intracellular immunization' was introduced by David Baltimore in 1988 and referred to any forms of gene-transfer-based cellular resistance to viral infection.³⁵ Since then, the concept has been extended and applied to the treatment of cancer. Intracellular immunization approaches have included antisense oligonucleotides, ribozymes, transdominant negative mutants, and single-chain antibodies (sFv). Antisense oligonucleotide molecules hybridize to target

RNA to inhibit translation or induce degradation of duplex RNAs.³⁶ Ribozymes are catalytic molecules that cleave RNAs.³⁷ In contrast, sFvs and transdominant negative mutants inhibit the target molecule at the protein level.^{38,39} All these approaches are thus offering ways to modulate the expression of specific genetic determinants involved in the drug resistant phenotype. Such alterations in protein expression can be exploited to chemosensitize cancer cells. This review will focus on the potential applications of intracellular immunization to modulate the resistance phenotype of tumor cells. However, it will not cover genetic addition strategies, such as adenovirus-mediated gene transfer of p53, to enhance the sensitivity of cancer cells to chemotherapy.

Intracellular antibodies

Advances in antibody engineering techniques have made possible the isolation of the specific antigen-binding regions of immunoglobulin molecules.^{40,41} The ability to express the variable heavy and light domains of an immunoglobulin on a single molecule and the characterization of specific signals which localize proteins to precise intracellular compartments have permitted the employment of intracellular antibodies to inactivate cellular oncogenes. The two variable domains are separated by a flexible small linker peptide (Figure 1). The resultant molecule (approximately 34 kDa) has a high affinity ligand-binding capability. Intracellular expression of these antibodies has resulted in the inactivation of various target proteins in the endoplasmic reticulum (ER), the cytosol and the nucleus. The ability of these intracellular single-chain antibodies (sFvs) to inhibit specific oncogenes has been exploited to sensitize cancer cells to chemotherapeutic agents.

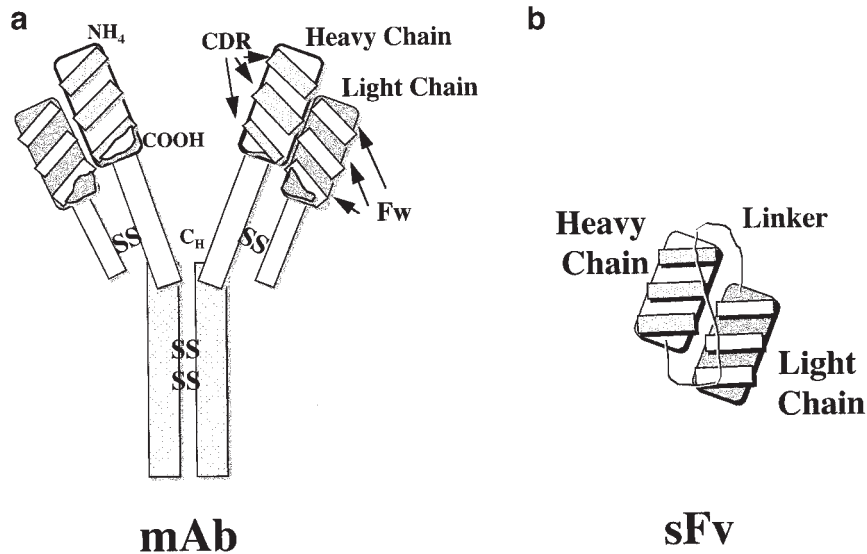


Figure 1 Structure of a monoclonal antibody and the single-chain antibody derived from it. The single-chain antibody is constructed by linking the variable heavy and light regions of the antibody using a small flexible peptide linker. The variable heavy and light regions are derived by polymerase chain reaction amplification. CDR, complementary determining region; Fw, framework; mAb, monoclonal antibody; C_H, heavy chain constant region; SS, disulfide cross-links; sFv, single-chain antibody.

erbB-2

The c-*erbB-2* proto-oncogene encodes a 185 000 kDa tyrosine kinase receptor with extensive homology to the epidermal growth factor receptor (EGFR).⁴² Clinical evidence suggest that overexpression of cell surface *erbB-2* is associated with poor prognosis in a variety of tumor types, including cancer of the ovary.⁴³ In this regard, patients with tumors demonstrating overexpression of *erbB-2* have a higher intrinsic resistance to conventional chemotherapeutic agents and a shortened relapse-free survival.^{32,43} Moreover, breast cancer cell lines selected for multidrug resistance often overexpress *erbB2* receptors.^{28,29,33,34} Recognizing that *erbB-2* plays a key role in tumor cell chemoresistance, several groups have employed strategies to modulate cell surface *erbB-2* as a means to enhance the sensitivity of tumor cells to chemotherapeutic agents.^{44,45} For example, Hancock *et al*⁴⁶ have demonstrated enhanced sensitivity to cisplatin in breast and ovarian tumor cell lines using monoclonal antibodies against *erbB-2*. Additionally, Paik and coworkers⁴⁵ have observed that breast cancer cells become sensitized to 5-fluorouracil (5-FU) when treated with monoclonal antibody against *erbB-2*.

Curiel's group has exploited a gene therapy strategy to achieve down-regulation of cell surface *erbB-2* and render tumor cells more sensitive to cisplatin. In this regard, the intracellular expression of a gene encoding an sFv directed against the *erbB-2* protein resulted in a significant reduction of cell surface *erbB-2* in ovarian cancer cells.⁴⁷⁻⁴⁹ This sFv elicited significant phenotypic changes in *erbB-2*-overexpressing tumor cells including cytotoxicity and apoptotic cell death.⁴⁷⁻⁵⁰ Furthermore, enhanced tumor cell chemosensitivity was observed *in vivo* when *erbB-2* positive tumors were treated with both the anti-*erbB-2* sFv and cisplatin in a murine orthotopic model of ovarian cancer.⁴⁴ A phase I/II clinical trial for patients with refractory ovarian cancer is currently ongoing with this sFv. In another study, NIH/3T3 cells overexpressing *erbB-2* reverted to a nontransformed phenotype after

transfection with an anti-*erbB-2* sFv.⁵¹ In all these studies, the anti-*erbB-2* sFv were directed to the lumen of the ER where the sFvs bound the extracellular domain of the *erbB-2* receptor, thereby preventing its appearance on the plasma membrane.⁵¹ Taken together, these data demonstrate the importance of *erbB-2* as a determinant of the drug resistant phenotype and the potential of sFvs to modulate this phenotype.

EGF receptor

Like the *erbB-2* receptor, EGFR is a member of the *erbB* type I family of receptors. These receptors are involved in the regulation of differentiation and cell growth.⁵² Overexpression of EGFR frequently occurs in a variety of human carcinomas including glioblastomas and cancers of lung, breast, ovary, head and neck, and bladder.^{53,54} Autocrine activation of EGFR is thought to play an important role in cancer cell growth.^{55,56} Studies with EGFR-blocking monoclonal antibodies (MAb 108, MAb 225) showed that concurrent treatment of carcinoma xenografts with cisplatin or other chemotherapeutic agents resulted in enhanced anti-tumor activity.⁵⁶⁻⁵⁹ MAb 225 is presently being used in a phase I clinical trial in combination with chemotherapy in the treatment of renal carcinoma and carcinoma of head and neck. Thus, an approach based on intracellular expression of an anti-EGFR sFv would be rational. In this regard, an ER-targeted sFv directed to the extracellular portion of EGFR was generated from the hybridoma cell line R1. Introduction of this sFv into EGFR-transformed NIH/3T3 cells via retroviral infection inhibited the transit of the receptor through the ER, leading to a reduction in the plasma membrane expression and a decrease in the EGF induced intracellular signaling.⁶⁰ A reduction in EGF-dependent colony formation was also demonstrated using this approach.⁶⁰ A second anti-EGFR sFv generated from a different hybridoma cell line (MAb 225), but also directed to the extracellular domain of EGFR, failed to cause intracellular retention of EGFR, although paracrine inhibition

of EGFR function was demonstrated.⁶¹ It is not clear why these two sFvs had such different effects, but it is likely that the structure of individual antibodies as well as their binding sites on the target proteins determine, at least in part, the final outcome.⁶⁰ Although antitumor effects were achieved *in vitro* with ER-targeted anti-EGFR sFvs, no attempts were made in these studies to determine, if any, the potential chemosensitizing effects of the anti-EGFR sFvs.

Anti-apoptotic proteins

Apoptosis plays a major role in tumor cell death induced by chemotherapeutic agents. Chemotherapy damages tumor cells, triggering a cascade of programmed molecular events that lead to activation of caspases, cytochrome C release, and cleavage of cytosolic and nuclear substrates. Any mutations or deregulation involving genes implicated in this cascade can potentially modify the response of a tumor cell to chemotherapy. The Bcl-2 family is a large family of proteins involved in the regulation of apoptosis.^{16,17} Some of these proteins function as apoptotic suppressors (Bcl-2, Bcl-X_L, Mcl-1, A1, Bcl-W) whereas other members function as promoters of cell death (Bax, Bcl-X_S, Bak, Bad, Bik).^{16,17} Because of their central role in the apoptotic cascade, these proteins have been the focus of intense research. A variety of cancers has been found to overexpress Bcl-2, including glioblastoma, and cancers of the prostate, breast, ovary and colon.⁶²⁻⁶⁶ Bcl-X_L is also overexpressed in a variety of tumors.⁶⁷⁻⁷² In addition, numerous studies have shown that overexpression of Bcl-2 and Bcl-X_L modulates the sensitivity of tumor cells to a wide spectrum of chemotherapeutic agents and gamma radiation.^{16,17} Our group has explored the utility of sFvs targeted to Bcl-2 and Bcl-X_L to enhance the sensitivity of tumor cells to chemotherapy. Bcl-2 and Bcl-X_L are mainly localized as integral membrane associated proteins, although they have also been found to be associated with other membranes, including those of the ER and the nucleus.¹⁷ We therefore hypothesized that an sFv targeted to the lumen of the ER would retain newly synthesized Bcl-2 proteins and thereby prevent their interaction with other proteins. To this end, we designed a vector that directed the sFvs to the lumen of the ER. In this vector, the sFv open reading frame has an N-terminal IgG kappa leader signal peptide which directs it to the secretory compartment of the cell. At the C-terminus, we included a KDEL peptide sequence, which provides an ER retention signal.⁷³ In a recent study with an anti-Bcl-2 sFv, we showed that concurrent treatment with cisplatin in the MCF-7 breast cancer cells resulted in enhanced antitumor activity.⁷⁴ We demonstrated that this chemosensitizing effect was correlated with an intracellular reduction of Bcl-2 protein levels. A similar sFv-mediated chemosensitizing effect was also found in ovarian cancer cell lines overexpressing Bcl-2 after treatment with CDDP or taxol.⁷⁵ The anti-Bcl-2 sFv-transduced tumor cells exhibited a five- to 10-fold reduction in the IC₅₀ compared with a control sFv. Taken together, these data suggest that sFvs targeted to anti-apoptotic proteins may have a role in cancer gene therapy in the future.

Ras proteins

Mutated active forms of Ras protein have been detected in a large number of carcinomas.^{76,77} Deregulated

expression of Ras stimulates a signaling cascade that can lead to uncontrolled cell growth and cancer.⁷⁸ In this context, Cochet *et al* have employed an anti-Ras sFv derived from the neutralizing Mab Y13-259, which recognize wild-type and mutated forms of Ras, to inhibit Ras-dependent functions.⁷⁹ This group demonstrated that a lung cancer cell line (H460) undergoes apoptosis upon microinjection of a Ras sFv in the absence of any apoptotic stimulus.⁷⁹ In addition, s.c. injections of an adenoviral vector encoding the Ras sFv promoted tumor regression in pre-established subcutaneous tumor nodule in nude mice probably due to an increased apoptotic cell death.⁷⁹ Constitutive expression of the c-Ha-Ras oncogene inhibits doxorubicin-induced apoptosis and promotes cell survival in the R2T24 rat rhabdomyosarcoma cell line.⁸⁰ In this context, intracellular Ras targeting could be combined with chemotherapy to sensitize tumor cells in which Ras overexpression/mutation is associated with increased drug resistance.

P-glycoprotein

The most common alteration in drug transport is increased expression of the *MDR-1* gene that encodes the P-glycoprotein (PGP). An increase in PGP is one of the mechanisms responsible for the emergence of multidrug-resistant tumor cells to chemotherapy.⁶⁻¹⁴ PGP expression is also frequently increased in some tumors following relapse after drug treatment.⁸¹ The construction and the crystal structure of an anti-MDR-1 sFv were recently reported.⁸² The sFv was constructed from the murine monoclonal antibody C219 that recognizes a continuous peptide epitope present in both cytoplasmic domains of PGP. Although no data were provided about the functionality of this sFv, there is no doubt that the elucidation of the crystal structure of C219 sFv will facilitate the development of sFvs with higher affinity and specificity that might be used intracellularly to inhibit PGP function.

Cyclin D1

It has recently become apparent that the effect of anticancer drugs may also be modulated to a certain extent by alterations in the expression of genes that control progression through the cell cycle. Cyclins and cyclin-dependent kinases (CDK) are proteins involved in the regulation of the cell cycle progression.⁸³ Overexpression of cyclin D1 contributes to the loss of cell cycle control and to the enhancement of tumorigenesis.⁸⁴ Cyclin D1 is overexpressed in a variety of cancers including breast, pancreatic, parathyroid and squamous cell carcinomas of the head and neck.⁸⁴⁻⁸⁷ In addition, gene transfer-mediated overexpression of cyclin D1 has been associated with an increase in drug resistance that was specific for methotrexate.²⁴ An anti-cyclin D1 sFv was recently reported to exhibit an inhibitory effect in stably transduced MCF-7 breast cancer cells.⁸⁸ In this preliminary study, the effect of the anti-cyclin D1 sFv on the drug sensitivity of tumor cells was not evaluated, but based on the chemosensitizing effects of cyclin D1 antisense (see below), it would not be surprising if anti-cyclin D1 sFvs could induce a similar chemosensitizing effect.

Antisense oligonucleotides

The use of antisense is another approach that has been employed to modulate protein expression levels in tumor

cells. AS utilize the specificity of Watson–Crick base pairing to block gene expression in a specific manner. Therapeutic AS have been used in two forms: short oligonucleotides (15 to 20 acid bases long) or expressed nucleotides (a few dozen bases to several thousands). Stable intracellular expression is currently the most efficient method whereby AS can be used to inhibit gene expression.

erbB-2

One study has specifically determined the effects of *erbB-2* antisense and chemotherapy treatment in ovarian cancer cells. A retroviral vector encoding a 3.8 kb *erbB-2* antisense cDNA fragment was transfected into SKOV3 cells, a human ovarian cancer cell line. When compared with parental cells and control cells transfected with the neomycin gene, the *erbB-2* antisense-transfected SKOV3 cells were more sensitive to 5-FU and cisplatin.⁸⁹ These data are consistent with the enhanced antitumor effect obtained with intracellular anti-*erbB2* sFVs in ovarian cancer cells as described previously.

EGF receptor

Stable transfection of antisense constructs of EGFR in a variety of EGFR-expressing cancer cell lines have resulted in down-regulation of EGFR expression. For example, an EGFR-expressing clone derived from the human ovarian cancer line 2774, and then stably transfected with an antisense construct of EGFR resulted in a 40–50% reduction of EGFR expression.⁹⁰ A correlation was noted between reduced EGFR and decreased anchorage-independent growth, suggesting that EGFR is an important factor in the malignant behavior of this ovarian cancer cell line. Grandis *et al*⁹¹ have targeted EGFR mRNA using antisense oligonucleotides, and showed a decreased EGFR protein production in squamous carcinoma cell lines. In addition, inhibition of EGFR expression resulted in 86% inhibition of growth of these tumor cells. The same group showed that intratumoral injection of EGFR antisense via liposome-mediated gene transfer in subcutaneous xenografts of human squamous cell carcinomas resulted in inhibition of tumor growth and an increased rate of apoptosis.⁹² These data suggest that a combined treatment with apoptosis-inducing drugs and antisense against EGFR should have synergistic effects. However, Dixit *et al* have recently demonstrated that a reduction in EGFR causes reduced sensitivity to cisplatin. In this study, MDA-468 human breast cancer cells were stably transfected with a vector containing a 4.1 kb full-length antisense EGFR complementary DNA.⁹³ Antisense-transfected MDA-468 clones displayed a significant (>40-fold) reduction of EGFR protein levels but had a 15- to 60-fold increase of their IC₅₀ to cisplatin. Based on these results, it is clear that alterations in EGFR signal transduction pathways can influence the sensitivity of a number of tumor cells to cisplatin and other chemotherapeutic agents. However, the underlying mechanism explaining these effects has yet to be elucidated.

Cyclin D1

Alterations in cellular levels of cyclin D1 may clearly affect the sensitivity of tumor cells to chemotherapeutic agents. A few studies have specifically looked at the effect of cyclin D1 inhibition in tumor cells. In one study, chemosensitivity of cisplatin could be enhanced by exposure of pancreatic cancer cell lines to cyclin D1 anti-

sense.⁹⁴ The enhanced drug sensitivity resulted in a 10- to 25-fold reduction in the LD₂₅ of cisplatin in tumor cells treated with the antisense against cyclin D1. Driscoll *et al*⁹⁵ have derived stable transfectants from lung cancer cell lines (A549 and NCI-H441) expressing a cyclin D1 antisense construct. They demonstrated that decreased cyclin D1 expression reduced pRb stability and induced susceptibility to cell death after withdrawal of exogenous growth factors only in the antisense transfected cell lines. Two other studies have demonstrated that transfection of cyclin D1 antisense constructs can decrease the tumorigenic properties of human colon and esophageal cancer cell lines but the combined effect of chemotherapeutic drugs was not evaluated on these transfected cell lines.^{96,97}

Bcl-2

A number of recent studies have looked at the effect of antisense oligonucleotides against Bcl-2 on the drug sensitivity of tumor cells. Down-regulation of Bcl-2 expression mediated by antisense oligonucleotides in seven blast cell samples obtained from patients with acute myeloid leukemia resulted in an increase in the susceptibility of the blasts to apoptosis induced by Ara-C.⁹⁸ In another study, human melanoma grown in severe combined immunodeficient (SCID) mice were treated with continuous infusion of Bcl-2 antisense oligonucleotides for 14 days in addition to intraperitoneal injections of dacarbazine for 5 days. A significant reduction of the tumor nodules was observed in mice treated with Bcl-2 antisense compared with control oligonucleotides suggesting the potential of this approach to enhance chemosensitivity *in vivo*.⁹⁹

Other antisenses

Antisenses have been developed against a variety of other oncogenes, including *c-fos*, *c-myc* and *K-ras*.^{100,101} These antisenses have demonstrated antitumor activity both *in vitro* and in animal models. A phase I clinical study for the treatment of metastatic breast cancer uses *in vivo* infection with breast-targeted retroviral vectors expressing antisense *c-fos* or *c-myc* RNA.¹⁰² However, these antisenses have yet to be tested for the ability to increase the drug sensitivity of tumor cells.

Ribozymes

Ribozymes are RNA molecules that have catalytic activities. They function by binding to a specific RNA target through antisense sequence and inactivate it by cleaving the phosphodiester backbone at a specific site.¹⁰³

MDR-1

Kobayashi *et al*¹⁰⁴ demonstrated a reduction in MDR-1 mRNA levels when MOLT-3 cells stably expressed an anti-MDR-1 ribozyme. The modulation of MDR-1 mRNA levels was proportional to the level of ribozyme expression. Anti-MDR-1 ribozyme-transduced MOLT-3 cells displayed a 25- to 30-fold decreased resistance to vincristine compared with cells expressing a non-functional ribozyme. Liposome-mediated gene transfer of an anti-MDR-1 ribozyme into drug-resistant mesothelioma cells resulted in reduced expression of *MDR-1* gene and restored sensitivity toward chemotherapeutic drugs.¹⁰⁵

Bcl-2

A divalent hammerhead ribozyme for the human bcl-2 mRNA was able to degrade bcl-2 mRNA rapidly *in vitro*. When this ribozyme was directly transfected into cultured prostate cancer cells (LNCap derivatives), it significantly reduced bcl-2 mRNA and protein levels within 18 h of treatment and induced apoptosis in a low bcl-2-expressing variant of LNCap, but not in a high Bcl-2-expressing LNCap line upon treatment with apoptotic agents.^{106,107}

Conclusion

Intracellular immunization represents a novel approach that can be employed to try to overcome drug resistance. In this context, intracellular immunization for cancer gene therapy should not be seen as a curative treatment in itself, but rather as an adjunct to conventional chemotherapy. This approach may be potentially used to reduce the cytotoxicity of chemotherapeutic agents, in which a similar efficacy would be reached with lower doses of drugs. Alternatively, it may also be used in conjunction with the regular dose of drugs to enhance tumor sensitivity further to those agents or even to study the contribution of a given protein to the resistant phenotype of a tumor cell. Another advantage of the intracellular immunization approach is its specificity to the target protein and its apparent lack of toxicity on normal cells that usually express low levels of the target protein.

It is clear from the data presented here that the modulation of specific molecular determinants involved in drug resistance via intracellular immunization may increase the sensitivity of a variety of tumor cell lines *in vitro*. However, the available *in vivo* data are more limited. Before intracellular immunization becomes a realistic strategy to overcome drug resistance in cancer, several problems need to be addressed. For example, one obvious requirement for this strategy to be useful in clinical situations is the efficient delivery of sFVs, antisenses and ribozymes to tumor cells. In this context, a number of gene delivery systems have been employed for cancer gene therapy, including viral (adenovirus, retrovirus, herpes virus and adeno-associated virus) and non-viral (liposome, naked DNA injection) methods. At the present time, the major limitations of gene transfer systems are their relatively low transfection efficacy and the lack of target cell specificity. The ideal situation with intracellular immunization strategies would be one whereby every tumor cell would be transduced without evidence of gene transfer in the surrounding normal tissues. This goal cannot be reached with the vector systems available at the present time. However, new concepts in viral gene transfer have recently emerged such as tropism-modified adenoviral vectors for targeted gene delivery^{108,109} and hybrid vector systems, which combine desirable properties of two different viral systems.^{110,111} These vector systems are designed to achieve significant levels of gene transfer to tumor cells while minimizing the toxicity associated with high numbers of particles. They also offer the advantage of retaining expression of the therapeutic genes not only in primarily transduced tumor cells, but also in the progeny of these tumor cells that would survive treatment. Moreover, the choice of a gene delivery system also depends on the nature of the strategy and the context of the target disease, ie local/regional versus

metastatic tumors. In the various human clinical gene therapy approaches for cancer, the disease stage considered most suitable has been loco-regional, whereby tumor is contained within an anatomical compartment. This situation potentially allows vector concentrations favoring optimal cell transduction. In this scenario, recombinant adenoviral vectors have shown the greatest potential.¹¹²

Any treatment for human malignancy that results in a bystander effect offers tremendous therapeutic advantages. This concept has been well established in HSVtk gene therapy strategies.¹¹³⁻¹¹⁵ Although intracellular immunization therapies are not toxin-based approaches, recent data suggest that apoptosis-inducing molecular therapies, such as wild-type p53 gene therapy, are capable of producing a bystander effect.¹¹⁶⁻¹¹⁸ This means that some of the strategies presented here, which are capable of inducing apoptosis, have the potential of producing a bystander effect. In such a context, increased therapeutic efficacy and efficiency might be achieved *in vivo* without the need to transduce every tumor cell.

Another factor that might potentially limit the efficacy of intracellular immunization is the multi-factorial nature of drug-resistance. For example, individual human cancers are often composed of multiple clones that are phenotypically heterogeneous, and thus may express a variety of different drug resistance genes at a given time.¹¹⁹ Moreover, the pattern of drug resistance gene expression may change over time depending on the chemotherapy regimen used. In this context, the inhibition of a single molecular target may not have a significant impact of the tumor phenotype. One would expect however, that inhibition of proteins involved in the regulation of a final common pathway such as the apoptotic cascade might have a significant effect on the majority of tumor cells given the fundamental importance of this pathway in the regulation of cell survival. An ideal strategy would be one whereby multiple pathways of drug resistance are targeted. For example, a vector co-expressing an sFv directed against Bcl-2 and a ribozyme targeted to mdr-1 RNA could be used to block both pathways in order to obtain synergistic effects.

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