

Evolutionary Approaches to Prolong Progression-Free Survival in Breast Cancer

Ariosto S. Silva¹, Yoonseok Kam¹, Zayar P. Khin¹, Susan E. Minton², Robert J. Gillies¹, and Robert A. Gatenby¹

Abstract

Many cancers adapt to chemotherapeutic agents by upregulating membrane efflux pumps that export drugs from the cytoplasm, but this response comes at an energetic cost. In breast cancer patients, expression of these pumps is low in tumors before therapy but increases after treatment. While the evolution of therapeutic resistance is virtually inevitable, proliferation of resistant clones is not, suggesting strategies of adaptive therapy. Chemoresistant cells must consume excess resources to maintain resistance mechanisms, so adaptive therapy strategies explicitly aim to maintain a stable population of therapy-sensitive cells to suppress growth of resistant phenotypes through intratumoral competition. We used computational models parameterized by *in vitro* experiments to illustrate the efficacy of such approaches. Here, we show that low doses of verapamil and 2-deoxyglucose, to accentuate the cost of resistance and to decrease energy production, respectively, could suppress the proliferation of drug-resistant clones *in vivo*. Compared with standard high-dose-density treatment, the novel treatment we developed achieved a 2-fold to 10-fold increase in time to progression in tumor models. Our findings challenge the existing flawed paradigm of maximum dose treatment, a strategy that inevitably produces drug resistance that can be avoided by the adaptive therapy strategies we describe. *Cancer Res*; 72(24); 6362–70. ©2012 AACR.

Introduction

Disseminated breast cancer remains almost uniformly fatal largely because of evolution of resistance to available systemic therapies. While some breast cancer patients do not receive any benefit from systemic therapy, most have a good initial response but later develop resistance and the tumor recurs. Typically, response to subsequent therapies is less and less durable until finally no therapy is effective. While *de novo* resistance may be environment mediated (because of ischemia, hypoxia, or tumor–stroma interactions through adhesion or soluble factors), relapse with tumor progression is generally because of the selection for specific phenotypes that confer chemoresistance (1).

A common and well-studied mechanism of acquired resistance is increased expression of p-glycoprotein (PGP) membrane pumps, which export a number of different chemother-

apeutics from the cytoplasm. Patients treated with doxorubicin and vincristine, both PGP substrates, have shown a direct correlation of PGP detection with previous treatment (2, 3). *In vitro* selection of drug-resistant cell lines to the novel agent Carfilzomib, a proteasome inhibitor, also promoted PGP overexpression, among other mechanisms (4).

PGP is a versatile membrane pump, and its substrates include multiple chemotherapeutic and targeted agents. Thus, acquisition of resistance to one drug through PGP overexpression frequently confers cross-resistance to multiple other drugs—hence the term multidrug resistance (MDR). Importantly, PGP substrates include a number of nonchemotherapy drugs including verapamil, cyclosporin A, and sestrin (5). This property has been previously proposed to overcome MDR by adding competing molecules for PGP sites and, thus, decrease the chemotherapy efflux. *In vitro* work with PGP overexpressing cell lines (6), has shown that verapamil, at subcytotoxic levels, was capable of reverting the MDR phenotype (7, 8).

Several clinical trials have examined simultaneous administration of chemotherapy and PGP substrates (9)—the latter acting as a competing ligand to increase the intracellular concentration of the former. However, results have been disappointing, in part because of dose limitation of PGP modulators (10, 11), which typically require micromolar concentrations, which are not clinically attainable.

Recently, our group has suggested that the proliferation of chemoresistant clones could be delayed, if the minimum drug intensity necessary to maintain a stable tumor burden were used, instead of the standard maximum tolerated dose (MTD).

Authors' Affiliations: ¹Department of Cancer Imaging and Metabolism, and ²Department of Women's Oncology, Comprehensive Breast Program, H. Lee Moffitt Cancer Center, Tampa, Florida

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

A.S. Silva and Y. Kam contributed equally to this work.

Corresponding Author: Robert A. Gatenby, Department of Cancer Imaging and Metabolism, Moffitt Cancer Center, 12902 Magnolia Dr, Tampa, FL 33612. Phone: 813-745-2843; Fax: 813-745-6070; E-mail: Robert.Gatenby@moffitt.org

doi: 10.1158/0008-5472.CAN-12-2235

©2012 American Association for Cancer Research.

This approach was termed adaptive therapy (AT; ref. 12). The basic hypothesis of AT is that, while evolution of resistant strategies is inevitable, proliferation of resistant populations is not. AT seeks to suppress proliferation of resistant cells by exploiting the cost linked to the drug-resistance mechanism. That is, chemoresistant cells must synthesize and activate proteins that protect them from chemotherapy. This requires diversion of resources that would otherwise be available for proliferation. Thus, in the absence of chemotherapy, we propose that the chemoresistant cells are significantly less fit than chemosensitive cells. To exploit this difference, AT applies chemotherapy judiciously to explicitly maintain a small residual population of chemosensitive cells. These cells, during chemotherapy intervals, will proliferate at the expense of the chemoresistant cells. In effect, AT seeks to maintain a population of cells that can be controlled using them to suppress proliferation of cells that cannot be controlled by therapy.

Unlike traditional chemotherapy, which fixes drug dose and schedule, AT aims to fix the size of the tumor, and continuously adjusts the chemotherapy to achieve that goal (12). Using animal models and computational simulations, our group determined that 2 factors were decisive for the success of AT: the initial prevalence of chemoresistant cells, and the difference in the growth rate of the 2 subpopulations during chemotherapy intervals.

While the initial ratios of chemoresistant and chemosensitive cells are inherent to each patient, we hypothesized that the energetic cost of PGP pumps could be manipulated as a means of further reducing the fitness of chemoresistant cells during chemotherapy intervals. This would effectively increase the fitness advantage of the drug sensitive cells and allow them to further out-compete the chemoresistant clones, and further delay tumor progression.

This hypothesis is supported by prior studies showing that PGP+ mutants have accelerated glucose metabolism (13), and faster ATP depletion in glucose-free medium (14). Furthermore, increased glucose usage has been observed in PGP-overexpressing cells when exposed to verapamil or other PGP substrates (15). Taken together, these observations indicate that PGP+ cells grow *in vitro* at approximately the same rate as their PGP- counterparts, only because of the abundance of nutrients and oxygen. In hypoxia and low glucose, however, the basal activity of the PGP pumps taxes their growth, an effect exacerbated by the use of PGP substrates at subtoxic levels.

In this work, we combined *in vitro* and computational models to investigate the possibility of maintaining a stable tumor burden by enhancing the energetic cost of therapy resistance.

Materials and Methods

Cell lines

In this study, we used the breast cancer cell lines MCF-7 and the PGP overexpressing chemoresistant MCF-7/Dox. The dose responses of these 2 cell lines were assessed for doxorubicin, and the IC_{50} values were determined to be 10.7 and 476.5 ng/mL, respectively. Both cell lines were obtained from American Type Culture Collection or Michigan Cancer Foundation (16). MCF-7/Dox cells were maintained in the presence of

0.1 to 0.5 $\mu\text{g/mL}$ doxorubicin until 1 week before experiments to ensure maintenance of the resistant phenotype.

Growth rates

The growth rates were determined using 2 methods: first, we measured the biomass increase by crystal violet staining (17). Second, we quantified the number of adherent live cells in real time (Fig. 1) in the xCelligence system (Roche) during 96 hours, for both cell lines, in normal culture conditions and in reduced glucose (2 and 0.5 g/L glucose, respectively).

To assess the fitness cost of PGP-mediated chemoresistance, we mixed and plated MCF-7 and MCF-7/Dox cells at a 2:1 ratio. Cells were initially cultured in normal media for 24 hours, when media was changed, and cells were allowed to grow for additional 72 hours in either normal or low glucose, with or without verapamil (1 $\mu\text{mol/L}$), with or without doxorubicin (Fig. 2). The dead cells were washed away and wells were

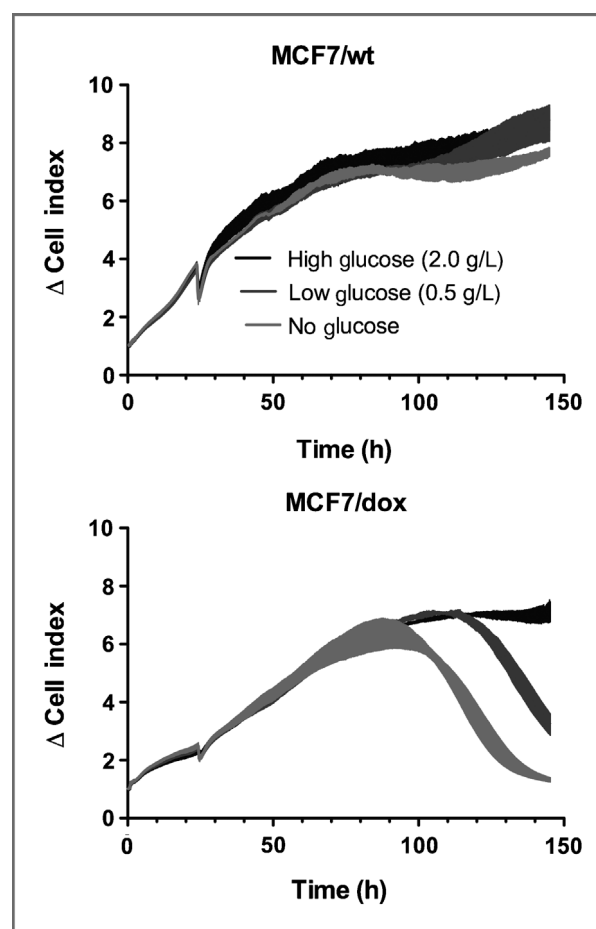


Figure 1. MCF-7 and MCF-7/Dox growth dependence on energy availability. The 2 breast cancer cell lines were plated at the same density in normal culture media (2 g/L of glucose) and grown for 24 hours when media was changed to normal glucose concentration (2 g/L), low glucose concentration (0.5 g/L), or no glucose. The PGP- cells maintained their growth in the 3 conditions, indicating that their energetic needs can be supplied from other sources in the media. The PGP+ mutant, however, showed loss of viability after 96 hours in glucose-free medium and 120 hours in low-glucose serum.

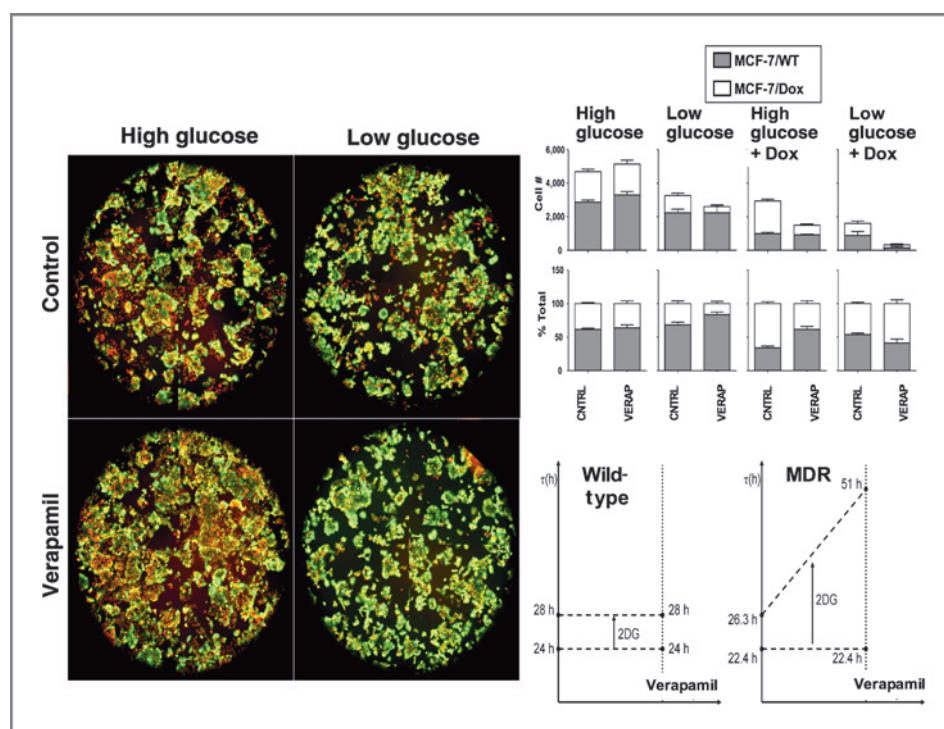


Figure 2. Competition of MCF-7 wild-type and PGP mutant cells in coculture shows reversal of fitness by energy restriction and verapamil. MCF-7 (wild-type) and the PGP mutant (MCF-7/Dox) were plated in a 2:1 ratio for 72 hours when nuclei were counted, and wild-type and PGP mutants were differentiated by calceinAM exclusion. In high-glucose medium the PGP mutant showed a shorter doubling time than the wild-type (22.4 hours vs. 24 hours), and the addition of verapamil did not alter these values. In restricted glucose conditions, however, both populations replicated more slowly (28 and 26.3 hours for the wild-type and PGP mutant, respectively). The addition of verapamil in low-glucose conditions did not affect the growth of the wild-type cells, but approximately doubled the doubling time of the PGP mutant (51 hours), indicating that the combination of energy restriction and a high-affinity PGP substrate tips the fitness advantage toward the wild-type population. The bottom right charts depict the changes in doubling time of MCF-7 and MCF-7/Dox as a function of 2-deoxyglucose and verapamil. The wild-type population (left) doubles at approximately every 24 hours in optimum conditions, but its growth is delayed upon reduction of 50% of glucose availability. The PGP mutant (right) has a faster doubling time than the wild-type and maintains its growth rate even at high rates of verapamil. However, energy restriction combined with the PGP substrate significantly delays replication. The equations that describe both curves are explained in the Supplemental Fig. S1. WT, wild-type; CNTRL, control; VERAP, verapamil; DOX, doxorubicin; 2DG, 2-deoxyglucose.

incubated with calceinAM (1 $\mu\text{mol/L}$), a fluorescent PGP substrate, and Hoechst 33342 (1 $\mu\text{g/mL}$): the drug sensitive cells accumulate both dyes, whereas the PGP-positive cells efflux calceinAM. We captured fluorescent images by Axiovision (Zeiss) and quantified population sizes using CellProfiler (Broad Institute).

Metabolism

Previous works (14, 15, 18) suggested that PGP mutants had increased glycolytic metabolism and sensitivity to energy restriction. We studied how the MCF-7 and MCF-7/Dox cell lines differed in terms of glucose consumption, and their main energetic pathway: either anaerobic or aerobic glycolysis.

The glucose uptake rate was estimated by the accumulation of the fluorescent glucose analog 2-NBDG, which is transported by facilitated transport into the cytoplasm and loses fluorescence upon phosphorylation by hexokinase (Fig. 3; ref. 19). The fluorescence in both MCF-7 and MCF-7/Dox cells was measured for 60 minutes, and the ratio between the derivatives of the curves at time 0 minutes was used to determine the fold increase in the glucose transport potential of both cell lines.

We studied the glycolytic metabolism of both cell lines using the XF Analyzer (Seahorse Bioscience), which measures pH and pO_2 changes in live cell culture in real time. For this assay we measured the acid production and oxygen consumption rates of both cell lines in: (i) glucose-free medium, (ii) upon addition of glucose (5 mmol/L final concentration), (iii) upon addition of oligomycin, which impairs mitochondrial respiration, and (iv) 2-deoxyglucose (Fig. 3), a competitor for glucose transporters, hexokinase, and blocks glycolysis because of accumulation at the phosphoglucosomerase level (20).

Computational model of clinical treatment

The clinical treatment computational model used in this article is an extension of our previous work on AT (12): the tumor burden of a patient was composed by 2 subpopulations of cancer cells, one chemosensitive and the other chemoresistant (Supplementary Fig. S1). We extended this model to include the growth rates and drug sensitivity, in optimum and energy-restricted conditions, obtained from the *in vitro* experiments with MCF-7 and MCF-7/Dox cell lines. The doubling times of the drug-sensitive and drug-resistant cell lines, as well

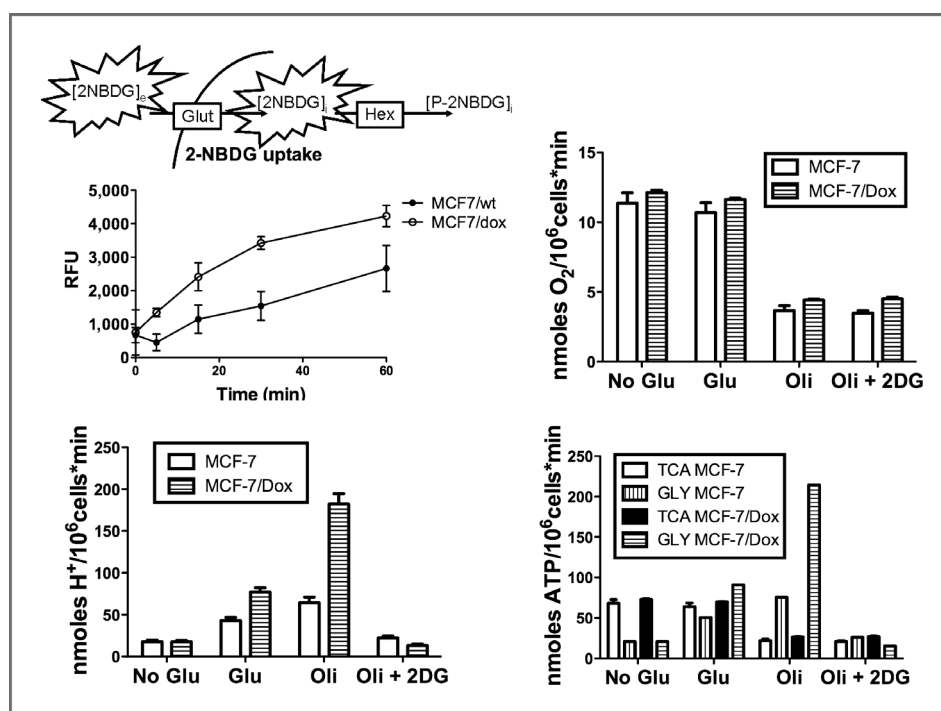


Figure 3. Differential glucose metabolism in MCF-7 and MCF-7/Dox. Top left, 2-NBDG is a fluorescent glucose analog, which once internalized and phosphorylated, loses its fluorescence. The graph depicts, in arbitrary fluorescence units, the accumulation of nonphosphorylated 2-NBDG in the first hour of incubation. Using Michaelis–Menten dynamics, V_{max} of glucose uptake for the PGP mutant is 4 times higher than for the wild-type (165 RFU/min vs. 40 RFU/min). Bottom right, in glucose-free medium (No Glu), MCF-7 cells and PGP+ mutants (MCF-7/Dox) produce most of their energy through aerobic metabolism. Once glucose is added to the medium (Glu), MCF-7 and MCF-7/Dox cells increase their energy production through glycolysis by approximately 30% and 50%, respectively. The addition of oligomycin (Oli) greatly decreases aerobic metabolism in mitochondria, forcing cells to extract energy from anaerobic glycolysis exclusively, exposing the maximum glycolytic potential of these cells, which is roughly 3 times higher in the PGP+ mutant than in the parental cell line. The addition of the nonmetabolizable glucose analog 2-deoxyglucose (2DG) greatly reduces energy production in both cell lines.

as the effect of 2-deoxyglucose and verapamil, measured *in vitro* are depicted in Fig. 2. The parameters for this model, including the effects of verapamil and 2-deoxyglucose in the doubling time and sensitivity to doxorubicin, are listed in the Supplementary Table S1.

Each computational simulation compared four different scenarios: (i) MTD, which halved the drug-sensitive population at every bolus, arbitrarily spaced by 5 days; (ii) MTD combined with chronic verapamil and 2-deoxyglucose, at concentrations of 100 nmol/L and 5 mmol/L, respectively (MTD + V + 2DG); (iii) AT, which consisted of an initial dose of half of the MTD, and adjusting the dose intensity by increases or decreases of 20%, in case of tumor growth or reduction, respectively; and (iv) AT combined with chronic verapamil and 2-deoxyglucose (AT + V + 2DG).

The detection level for tumor burden was arbitrarily established as 10^9 cells and the lethal burden as 3 orders of magnitude higher, 10^{12} cells (21). Considering that a subpopulation of drug-resistant clones is already present in all simulations, the best treatment would be the one that extended the hypothetical patient survival the longest (Figs. 4 and 5), or indefinitely.

Verapamil levels in these simulations (100 nmol/L) were chosen because of dose-limiting heart damage, previously observed in clinical trials (10).

Results

PGP mutants have accelerated glycolytic metabolism

The MCF-7/Dox cells replicate faster than MCF-7 in optimum conditions: 22.4 hours versus 24 hours. However, the energetic cost of resistance of the basal activity of the PGP pumps becomes a significant burden for these cells under energy restricted conditions: while MCF-7 cells are able to maintain their growth rate in absence of glucose for more than 120 hours, the MCF-7/Dox lose viability after 72 hours in absence of glucose (Fig. 1).

Energy restriction and PGP substrates synergistically reverse fitness of PGP mutants

We investigated if the addition of a glycolytic antimetabolite, and low doses of nonchemotherapeutic PGP substrates, could be used in combination with AT to further reduce the fitness of chemoresistant cells during therapy intervals.

When cocultured in optimum glucose conditions, the PGP+ mutant overgrows the parental cell line, but in low glucose concentrations, and in combination with verapamil, the parental cell line out-competes the drug resistant cells (Fig. 2).

Figure 2 (right) depicts how glucose restriction, or addition of 2-deoxyglucose, increases the doubling time of MCF-7 cells from 24 to 28 hours, whereas verapamil has no effect (note in the column "Low Glucose" the reduction in the number of

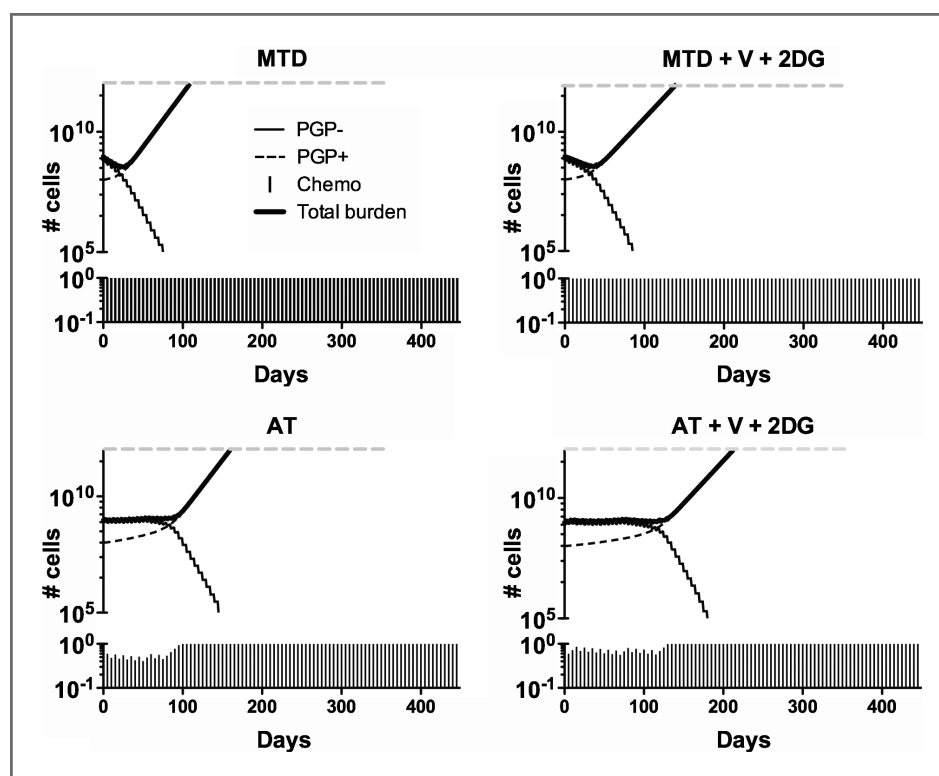


Figure 4. Tumor burden simulation of a hypothetical patient carrying a subpopulation of 10% PGP mutants treated with standard or AT regimens combined with verapamil and 2DG. MTD (upper left) promptly reduces the total tumor burden, but the patient soon relapses and stops responding. The combination of MTD with an antimetabolite (2DG) and a PGP substrate (verapamil) increases patient survival in approximately 30% (upper right). AT (bottom left) is capable of maintaining the resistant subpopulation under control for longer (increase in overall survival by 50%). The best results are obtained when 2DG and verapamil are administered together with AT, resulting in a 4-fold increase in progression-free survival. Progression-free survival was considered as the interval between the beginning of the treatment and the moment when the tumor burden resumes growth in presence of therapy. In this example, progression-free survival was 35 days for MTD and 135 days for AT + verapamil + 2DG. The simulations considered 2 subpopulations of cancer cells: the first corresponded to 90% of the tumor burden and had growth rate and drug response modeled from *in vitro* experiments with MCF-7 cells. The second subpopulation represented 10% of the total tumor burden and had the same growth and drug response properties as the PGP mutant MCF-7/Dox. The description of the computational model and parameter values (doubling time and drug sensitivity) are described in the Supplementary Fig. S1 and Supplementary Table S1. The IC_{50} for the chemosensitive subpopulation was arbitrarily set to 1, whereas for the PGP+ mutant, it was considered to be 100-fold higher. The standard therapeutic dose was also set as one arbitrary unit, and the initial dose of the AT algorithm was set as half this value.

MCF-7 cells, in gray, when compared with "High Glucose," but no additional difference is observed in the final number of MCF-7 when Verapamil is added to the low-glucose media). Similarly, MCF-7/Dox responded to glucose restriction with an increase in the doubling time from 22.4 to 26.3 hours, whereas the verapamil alone in high-glucose media had no effect. The combination of verapamil and 2-deoxyglucose, however, caused a 2-fold increase in doubling time for the PGP mutant (22.4–51 hours). When the chemotherapeutic agent doxorubicin is added to the high-glucose media, the PGP mutant became fitter (more surviving cells than the wild-type). This dominance was reversed by addition of verapamil or by energy restriction alone.

PGP mutants show upregulation of glucose uptake

We studied the rate of glucose uptake in both cell lines through accumulation of the fluorescent glucose analog 2-NBDG (Fig. 3). The initial rate of uptake of the glucose analog in the PGP+ mutant (estimated as the first derivative at time 0') was 4 times higher than for the parental cell line (165 RFU/min

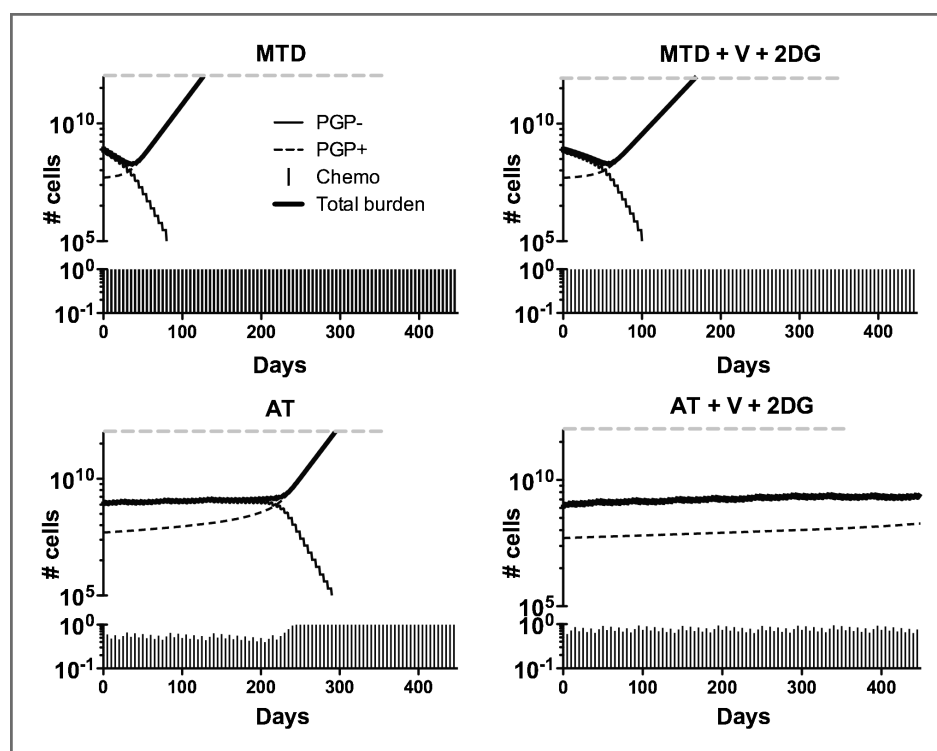
vs. 40 RFU/min), indicating that part of the accelerated glucose metabolism observed in the MCF-7/Dox cells is because of an increase in the glucose transporter activity.

PGP mutants are more dependent on anaerobic glycolysis

To estimate the actual ATP production, we made the following assumptions: (i) all oxygen consumed is used in the Krebs cycle, and every 6 molecules of O_2 consumed produces 36 molecules of ATP; (ii) the proton production corresponds to approximately 85% of the lactate production in atmospheric conditions ($pO_2 \sim 21\%$; ref. 22); and (iii) that every molecule of lactate produced corresponds to the generation of 1 ATP molecule through anaerobic glycolysis.

Using these assumptions, we generated the chart of Fig. 3, which suggests three main conclusions: (i) glucose restriction does not significantly affect energy production in MCF-7 cells, but causes a 20% decrease in the MCF-7/Dox, (ii) the extra energy produced by the PGP+ mutants comes from anaerobic glycolysis, and (iii) when aerobic metabolism is blocked, the

Figure 5. Tumor burden simulation of a hypothetical patient carrying a subpopulation of 5% PGP mutants treated with standard or AT regimens combined with verapamil and 2DG. In a hypothetical untreated patient with an average PGP+ subpopulation (5%), the combination of AT, 2DG, and verapamil (bottom right) is capable of keeping the patient stable for over 500 days in contrast to the observed survival of 122 days of MTD (top left). Similarly to Fig. 4, these simulations consisted in a mix of chemosensitive and PGP-positive chemoresistant cells, but this time at a proportion of 95% and 5% of the total tumor burden, respectively. These 2 subpopulations were modeled based on *in vitro* data from the MCF-7 and MCF-7/Dox cell lines. The competition between these subclones was modeled as described in Supplementary Fig. S1, with growth rate and dose response parameters listed Supplementary Table S1.



PGP+ mutants are capable of obtaining from 2- to 3-fold more energy anaerobically than the parental cell line.

Computational models

The principle of AT is that the competition between chemosensitive and chemoresistant tumor cells would reduce the growth rate of both populations. Conventional high-dose therapy sharply decreases the sensitive tumor subpopulation, which allows the regrowth of drug-resistant clones. To avoid this negative effect, AT proposes to treat patients with the minimum drug concentration required to maintain tumor burden below a disease-inducing level.

Our simulations using AT showed that the drug-resistant population could be maintained stable, if at least 1 of 2 conditions were satisfied: (i) the drug resistant cells were present in a very small number or (ii) the drug-resistant cells were less fit in absence of therapy (slower replication). As shown for the MCF-7 breast cancer cell line, the cost of chemoresistance can be masked in optimum growth conditions, as is the case *in vitro*. However, in the inhospitable tumor microenvironment, there is a clear fitness disadvantage for the PGP+ cells, which can be significantly enhanced by combining PGP substrates and glycolytic antimetabolites.

We simulated the combination of AT with chronic administration of low concentrations of verapamil and 2-deoxyglucose during chemotherapy intervals. The simulated patients had an initial prevalence of PGP+ of 10% or less (Figs. 4 and 5), based on Wishart and colleagues (23), who examined a cohort of 29 untreated breast cancer patients, and found that PGP expression was observed in less than 10% of cells in 28 of 29 patients. Similar results were obtained in examining the PGP

expression in multiple myeloma specimens from untreated patients, with reported median cell expression of 3% to 7% (24).

Our simulations showed that, compared with traditional high-dose-density therapy, patients with initial prevalence of 10% PGP+ cells (Fig. 4) have a 2- to 3-fold increase in progression-free survival using AT, and a 4-fold increase when AT was enhanced using verapamil and 2-deoxyglucose.

In patients with a smaller chemoresistant subpopulation, progression-free survival (compared with MTD) is improved by a factor of 4 with AT, and by a factor of 10 with the AT + verapamil + 2-deoxyglucose combination (Fig. 5).

Discussion

We previously proposed that the growth of subpopulations of resistant clones in a heterogeneous tumor could be suppressed, if a competing chemosensitive population were spared. This approach, termed AT, explicitly limits application of chemotherapy to maintain a stable population of chemosensitive cells that, through their fitness advantage in the absence of therapy, suppress proliferation of resistant phenotypes.

Although this initially appears to be a radical departure from conventional high-dose-density therapy, the concept of AT, which uses a lower dose and more tolerable chemotherapy, is in agreement with the clinical goals for the treatment of metastatic breast cancer. That is, chronic long-term treatment with intermittent breaks and/or frequent dose reductions occurs regularly because of toxicity. In addition, quality of life is an important aspect of therapy because the treatment is ongoing and not curable. Therefore, treatment at the MTD, especially in the long term, is difficult to maintain. Thus, the AT

strategy seeks to use conventional practice but use evolutionary principles to obtain optimal long-term tumor control.

Traditional attempts to circumvent cancer cell resistance to chemotherapy have focused on using PGP substrates to compete for membrane efflux pumps, and increase intracellular accumulation of chemotherapy. *In vitro* studies have shown that verapamil and quinidine cause intracellular chemotherapy accumulation only when present in high concentrations [$\sim 10 \mu\text{mol/L}$ for both (7, 25)]. Unfortunately, conventional clinical doses of these PGP substrates cannot achieve this concentration [the maximum concentrations observed in clinical trials were $5.5 \mu\text{mol/L}$ for quinidine (11), and $0.5 \mu\text{mol/L}$ for verapamil (10)]. Thus, it is not surprising that clinical results for combining chemotherapy and verapamil were disappointing. Furthermore, PGP is only one of several intra- and extracellular drug-resistance mechanisms (26).

Here, we propose an extension of this strategy that exploits the many available PGP substrates. We hypothesize that non-chemotherapeutic PGP-substrate drugs might be used to increase the metabolic cost of MDR, reducing the fitness of the overexpressing PGP cells during chemotherapy intervals.

As PGP overexpressing cells consume ATP to extrude drugs, we propose that the chronic administration of nonchemotherapeutic PGP substrates will exhaust resources in the chemoresistant population, reducing their ability to proliferate. Thus, our goal, rather than inhibiting chemotherapy efflux, is to increase futile PGP activity during chemotherapy intervals. Unlike previous MDR-modulating therapies, our approach does not require that PGP be the sole mechanism of drug resistance, but simply *one* of the mechanisms.

To test our hypothesis, we built a computational model of hypothetical patients with different initial prevalence of PGP mutant clones. We parameterized the model using *in vitro* studies of the breast cancer cell MCF-7 and a related chemoresistant line overexpressing PGP pumps (MCF-7/Dox). We find MCF-7/Dox consumes more energy than its parental cell line (MCF-7), as previously determined for other PGP mutant cell lines (14, 15, 18), and that this higher energetic need is supplemented by anaerobic glycolysis. Our results also confirm that glucose restriction, in combination with the PGP substrate verapamil, increases doubling time (from ~ 22 to 51 hours) in PGP+ mutants. These results suggest that the combination of energy depletion and increased energy usage through administration PGP substrates synergistically reduce the growth of MDR subpopulations.

Our simulations show that, in patients with a PGP+ tumor burden lower than 10%, AT combined with a glycolytic anti-metabolite (2-deoxyglucose), and a PGP substrate (verapamil) significantly increases observed survival, whereas in patients with smaller drug resistant burdens, this evolutionary double bind strategy can stabilize tumor burden for a period 10-fold longer than standard MTD (Fig. 5).

The percentage of PGP+ mutants in our simulations was estimated from 3 clinical trials with breast cancer patients, which have estimated PGP+ prevalence in untreated patients from undetectable (27) to below 10% (23, 28). Moreover, these pumps were shown to be functional and to confer drug resistance *ex vivo* (28). The presence of PGP+ cells was also

detected in the surrounding desmoplastic stroma of breast cancer patients (29), and in combination with positive PGP tumor cells, correlated with poor prognosis. It is unclear if the stroma actually produces these pumps by influence of the tumor, if they are produced and shed by the PGP+ tumor cells, or if the mesenchymal cells are actually cancer phenotypes that have undergone epithelial-to-mesenchymal transformation (30). The actual effect of these stromal pumps in protecting the tumor is a subject of debate, as some authors have proposed that, whereas PGP expression reduces the intracellular drug accumulation, it actually increases its penetration into tissue and tumor (31, 32). The effect of stromal PGP expression in the competition of PGP+ and PGP- cells should be subject of investigation in a more suitable spatial model in the future.

Computational simulations of hypothetical patients treated with standard MTD, AT, and AT combined with nonchemotherapeutic PGP substrates and 2-deoxyglucose, show dramatic differences in progression-free survival. Compared with traditional high-dose-density therapy, patients with initial prevalence of 10% PGP+ cells will have a 2- to 3-fold increase progression-free survival using AT, and a 4-fold increase when AT was combined with verapamil and 2-deoxyglucose. When 5% or fewer drug-resistant cells are initially present, progression-free survival is improved by a factor of 4 with AT, and by a factor of 10 with the AT/verapamil/2-deoxyglucose combination.

The chronic combined doses of 2-DG/verapamil proposed in this manuscript were 5 mmol/L per $0.1 \mu\text{mol/L}$, respectively, which would be sufficient to mimic in blood the results from the low glucose-culture media. Clinical trials with glioblastoma patients have reached weekly boluses as high as 1.25 mmol/L for up to 7 weeks (33), whereas chronic administration of 2-DG at 0.18 mmol/L was achieved in a clinical trial with patients with solid tumors (34). Similar to chemotherapeutic agents, the chronic administration of glycolytic inhibitors may cause toxicity to healthy tissues. In the case of 2-DG the most affected would be the brain, retinae, and other organs with known high glucose consumption (35). Dietary interventions, such as reduction in carbohydrate uptake, as well as increases in the doses of verapamil, can be envisioned as alternative ways of reaching the same goal of cost of resistance of PGP+ mutants.

For the sake of simplicity, the computational model assumed that the conversion rate of chemosensitive cells into PGP+ chemoresistant cells was negligible. While it is difficult to determine the spontaneous and drug-induced mutation rate in patients, *in vitro* experiments have suggested this conversion rate is about $1.8 \times 10^{-6}/\text{generation}$ (36). When this spontaneous rate of conversion from a sensitive to a resistant state is added to the original simulations, the progression-free survival and observed survival from the example in Fig. 5 are reduced from 500 and 590 days, respectively, to 475 and 560 days, respectively.

As a next step, the approach here proposed will be tested in mouse models (Nu/Nu) using the same cell lines (MCF-7 and MCF-7/Dox) with doxorubicin as chemotherapeutic agent in combination with verapamil and 2-deoxyglucose. A mixed population of tumor cells will be injected in the mammary

fat pad and the tumor volume will be monitored by caliper measurements and ultrasound.

In summary, we investigate an alternative cancer treatment strategy that is explicitly designed to maintain a residual population of therapy-sensitive cancer cells so that they can use their fitness advantage to suppress proliferation of resistant phenotypes. Essentially, we accept the continued presence of cells that we can potentially kill so that they can reduce the growth of populations that we cannot, and could ultimately lead to patient death. We have previously shown, using computational models and *in vivo* experiments, that this approach prolongs survival, while using lower drug doses, so that quality of life is high. Here, we hypothesize that the addition of nonchemotherapy PGP substrates, between treatments, will further burden the chemoresistant clones and increase their relative fitness disadvantage, when compared with chemosensitive cells. We show, using computational models parameterized by *in vitro* studies with clinically available drugs, that this approach can potentially increase the time to tumor recurrence in breast cancer patients by 4- to 10-fold.

References

- Meads MB, Gatenby RA, Dalton WS. Environment-mediated drug resistance: a major contributor to minimal residual disease. *Nat Rev Cancer* 2009;9:665–74.
- Grogan TM, Spier CM, Salmon SE, Matzner M, Rybski J, Weinstein RS, et al. P-glycoprotein expression in human plasma cell myeloma: correlation with prior chemotherapy. *Blood* 1993;81:490–5.
- Sonneveld P, Schoester M, de Leeuw K. Clinical modulation of multidrug resistance in multiple myeloma: effect of cyclosporine on resistant tumor cells. *J Clin Oncol* 1994;12:1584–91.
- Gutman D, Morales AA, Boise LH. Acquisition of a multidrug-resistant phenotype with a proteasome inhibitor in multiple myeloma. *Leukemia* 2009;23:2181–3.
- Troutman MD, Thakker DR. Novel experimental parameters to quantify the modulation of absorptive and secretory transport of compounds by P-glycoprotein in cell culture models of intestinal epithelium. *Pharm Res* 2003;20:1210–24.
- Dalton WS, Durie BG, Alberts DS, Gerlach JH, Cress AE. Characterization of a new drug-resistant human myeloma cell line that expresses P-glycoprotein. *Cancer Res* 1986;46:5125–30.
- Bellamy WT, Dalton WS, Kailey JM, Gleason MC, McCloskey TM, Dorr RT, et al. Verapamil reversal of doxorubicin resistance in multidrug-resistant human myeloma cells and association with drug accumulation and DNA damage. *Cancer Res* 1988;48:6365–70.
- Shen F, Chu S, Bence AK, Bailey B, Xue X, Erickson PA, et al. Quantitation of doxorubicin uptake, efflux, and modulation of multidrug resistance (MDR) in MDR human cancer cells. *J Pharmacol Exp Ther* 2008;324:95–102.
- McHugh K, Callaghan R. Clinical trials on MDR reversal agents. In: Colabufo NA, editor. *Biological and pharmaceutical advance in the antitumor treatment*. Kerala, India: Research Signpost, 2008. p. 321–53.
- Dalton WS, Grogan TM, Meltzer PS, Scheper RJ, Durie BG, Taylor CW, et al. Drug-resistance in multiple myeloma and non-Hodgkin's lymphoma: detection of P-glycoprotein and potential circumvention by addition of verapamil to chemotherapy. *J Clin Oncol* 1989;7:415–24.
- Wishart GC, Bissett D, Paul J, Jodrell D, Harnett A, Habeshaw T, et al. Quinidine as a resistance modulator of epirubicin in advanced breast cancer: mature results of a placebo-controlled randomized trial. *J Clin Oncol* 1994;12:1771–7.
- Gatenby RA, Silva AS, Gillies RJ, Frieden BR. Adaptive therapy. *Cancer Res* 2009;69:4894–903.
- Millon SR, Ostrander JH, Brown JQ, Raheja A, Seewaldt VL, Ramanujam N. Uptake of 2-NBDG as a method to monitor therapy response in breast cancer cell lines. *Breast Cancer Res Treat* 2011;126:55–62.
- Broxterman HJ, Pinedo HM, Kuiper CM, Kaptein LC, Schuurhuis GJ, Lankelma J. Induction by verapamil of a rapid increase in ATP consumption in multidrug-resistant tumor cells. *FASEB J* 1988;2:2278–82.
- Broxterman HJ, Pinedo HM, Kuiper CM, Schuurhuis GJ, Lankelma J. Glycolysis in P-glycoprotein-overexpressing human tumor cell lines. Effects of resistance-modifying agents. *FEBS Lett* 1989;247:405–10.
- Raghunand N, Martinez-Zaguilan R, Wright SH, Gillies RJ. pH and drug resistance. II. Turnover of acidic vesicles and resistance to weakly basic chemotherapeutic drugs. *Biochem Pharmacol* 1999;57:1047–58.
- Gillies RJ, Didier N, Denton M. Determination of cell number in monolayer cultures. *Anal Biochem* 1986;159:109–13.
- Kaplan O, Navon G, Lyon RC, Faustino PJ, Straka EJ, Cohen JS. Effects of 2-deoxyglucose on drug-sensitive and drug-resistant human breast cancer cells: toxicity and magnetic resonance spectroscopy studies of metabolism. *Cancer Res* 1990;50:544–51.
- Yamada K, Saito M, Matsuoka H, Inagaki N. A real-time method of imaging glucose uptake in single, living mammalian cells. *Nat Protoc* 2007;2:753–62.
- Wick AN, Drury DR, Nakada HI, Wolfe JB. Localization of the primary metabolic block produced by 2-deoxyglucose. *J Biol Chem* 1957;224:963–9.
- Moreno E. Is cell competition relevant to cancer? *Nat Rev Cancer* 2008;8:141–7.
- Schornack PA, Gillies RJ. Contributions of cell metabolism and H⁺ diffusion to the acidic pH of tumors. *Neoplasia* 2003;5:135–45.
- Wishart GC, Plumb JA, Going JJ, McNicol AM, McArdle CS, Tsuruo T, et al. P-glycoprotein expression in primary breast cancer detected by immunocytochemistry with two monoclonal antibodies. *Br J Cancer* 1990;62:758–61.
- Hokanson JA, Brown BW, Thompson JR, Drewinko B, Alexanian R. Tumor growth patterns in multiple myeloma. *Cancer* 1977;39:1077–84.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: A.S. Silva, Y. Kam, R.J. Gillies, R.A. Gatenby
Development of methodology: A.S. Silva, Y. Kam, R.A. Gatenby
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y. Kam, Z.P. Khin, R.A. Gatenby
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A.S. Silva, Y. Kam, Z.P. Khin, R.J. Gillies, R.A. Gatenby
Writing, review, and/or revision of the manuscript: A.S. Silva, Y. Kam, S.E. Minton, R.J. Gillies, R.A. Gatenby
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Y. Kam, R.A. Gatenby
Study supervision: A.S. Silva, R.A. Gatenby

Grant Support

This work was funded by the James S. McDonnell Foundation 21st Century Science Initiative Grant "Cancer Therapy: Perturbing a Complex Adaptive System" as well as NIH/NCI 1U54CA143970-01 and NIH/NCI R01CA170595.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received June 9, 2012; revised September 14, 2012; accepted October 6, 2012; published OnlineFirst October 12, 2012.

25. Tsuruo T, Iida H, Kitatani Y, Yokota K, Tsukagoshi S, Sakurai Y. Effects of quinidine and related compounds on cytotoxicity and cellular accumulation of vincristine and adriamycin in drug-resistant tumor cells. *Cancer Res* 1984;44:4303–7.
26. Meads MB, Hazlehurst LA, Dalton WS. The bone marrow microenvironment as a tumor sanctuary and contributor to drug resistance. *Clin Cancer Res* 2008;14:2519–26.
27. Schneider J, Bak M, Efferth T, Kaufmann M, Mattern J, Volm M. P-glycoprotein expression in treated and untreated human breast cancer. *Br J Cancer* 1989;60:815–8.
28. Sanfilippo O, Ronchi E, De Marco C, Di Fronzo G, Silvestrini R. Expression of P-glycoprotein in breast cancer tissue and *in vitro* resistance to doxorubicin and vincristine. *Eur J Cancer* 1991;27:155–8.
29. Linn SC, Giaccone G, van Diest PJ, Blokhuis WM, van der Valk P, van Kalken CK, et al. Prognostic relevance of P-glycoprotein expression in breast cancer. *Ann Oncol* 1995;6:679–85.
30. Bebawy M, Combes V, Lee E, Jaiswal R, Gong J, Bonhoure A, et al. Membrane microparticles mediate transfer of P-glycoprotein to drug sensitive cancer cells. *Leukemia* 2009;23:1643–9.
31. Tunggul JK, Melo T, Ballinger JR, Tannock IF. The influence of expression of P-glycoprotein on the penetration of anti-cancer drugs through multicellular layers. *Int J Cancer* 2000;86:101–7.
32. Patel KJ, Tannock IF. The influence of P-glycoprotein expression and its inhibitors on the distribution of doxorubicin in breast tumors. *BMC Cancer* 2009;9:356.
33. Singh D, Banerji AK, Dwarakanath BS, Tripathi RP, Gupta JP, Mathew TL, et al. Optimizing cancer radiotherapy with 2-deoxy-d-glucose dose escalation studies in patients with glioblastoma multiforme. *Strahlenther Onkol* 2005;181:507–14.
34. Racz LE, Rosenblatt J, Schlesselman J, Langmuir V, Tidmarsh G, Rocha-Lima C, et al. Combining glycolytic inhibitors with chemotherapy: phase I trial of 2-deoxyglucose and docetaxel in patients with solid tumors. *J Clin Oncol* 2005;23:3190.
35. Pelicano H, Martin DS, Xu RH, Huang P. Glycolysis inhibition for anticancer treatment. *Oncogene* 2006;25:4633–46.
36. Chen GK, Duran GE, Mangili A, Beketic-Oreskovic L, Sikic BI. MDR 1 activation is the predominant resistance mechanism selected by vinblastine in MES-SA cells. *Br J Cancer* 2000;83:892–8.