Evolutionary approaches to prolong progression-free survival in breast cancer

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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 employed 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 to standard high dose density treatment, the novel treatment we developed achieved a 2- 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 which can be avoided by the adaptive therapy strategies we describe.

Introduction

Disseminated breast cancer remains almost uniformly fatal largely due to 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 (due to ischemia, hypoxia or tumor-stroma interactions through adhesion or soluble factors), relapse with tumor progression is generally due to 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 chemotherapeutics 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 over-expression, 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 over-expression frequently confers cross-resistance to multiple other drugs – hence the term multidrug resistance (MDR). Importantly, PGP substrates include a number of non-chemotherapy drugs including verapamil, cyclosporin A, and sestamibi(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 over-expressing cell lines (6), has shown that verapamil, at sub-cytotoxic 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 due to 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). This approach was termed "adaptive therapy" (AT) (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 two

factors were decisive for the success of AT: the initial prevalence of chemoresistant cells, and the difference in the growth rate of the two sub-populations 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 demonstrating that PGP+ mutants have accelerated glucose metabolism (13), and faster ATP depletion in glucose-free medium (14). Furthermore, increased glucose utilization has been observed in PGP-over-expressing 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 sub-toxic 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 over-expressing chemoresistant MCF-7/Dox. The dose responses of these two cell lines were assessed for doxorubicin, and the IC50 values were determined to be 10.7ng/ml and 476.5ng/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 0.1-0.5 μg/ml doxorubicin until one week prior to experiments to ensure maintenance of the resistant phenotype.

Growth rates

The growth rates were determined using two methods: First, we measured the biomass increase by crystal violet staining (17). Second, we quantified the number of adherent live cells in real time (**figure 1**) in the xCelligence system (Roche) during 96h, for both cell lines, in normal culture conditions and in reduced glucose (2g/L and 0.5g/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 24h, when media was changed, and cells were allowed to grow for additional 72h in either normal or low glucose, with or without verapamil (1μM), with or without

doxorubicin (**figure 2**). The dead cells were washed away and wells were incubated with calceinAM (1 μM), a fluorescent PGP substrate, and Hoechst 33342 (1 μg/ml): the drug sensitive cells accumulate both dyes, while the PGP-positive cells efflux calceinAM. We captured fluorescent images by Axiovision (Zeiss) and quantified population sizes using CellProfiler (Broad Institute, MA).

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 (**figure 3**) (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: (a) glucose-free medium, (b) upon addition of glucose (5mM final concentration), (c) upon addition of oligomycin, which impairs mitochondrial respiration,

and (d) 2-deoxyglucose (**figure 3**), a competitor for glucose transporters, hexokinase, and blocks glycolysis due to accumulation at the phosphoglucoisomerase 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 two sub-populations of cancer cells, one chemosensitive, the other chemoresistant (**supplementary figure 1**). 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 as the effect of 2-deoxyglucose and verapamil, measured *in vitro* are depicted in **figure 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 1**.

Each computational simulation compared four different scenarios: (1) maximum tolerated dose (MTD), which halved the drug sensitive population at every bolus, arbitrarily spaced by 5 days; (2) MTD combined with chronic verapamil and 2-deoxyglucose, at concentrations of 100nM and 5mM, respectively (MTD + V + 2DG); (3) adaptive therapy (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 (4) 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 three orders of magnitude higher, 10^{12} cells (21). Considering that a sub-population 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 (**figures 4 and 5**), or indefinitely.

Verapamil levels in these simulations (100nM) were chosen due to 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: 22.4h vs. 24h. 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 over 120h, the MCF-7/Dox lose viability after 72h in absence of glucose (**figure 1**).

Energy restriction and PGP substrates synergistically reverse fitness of PGP mutants

We investigated if the addition of a glycolytic anti-metabolite, and low doses of non-chemotherapeutic PGP substrates, could be used in combination with adaptive therapy to further reduce the fitness of chemoresistant cells during therapy intervals.

When co-cultured 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 (**figure 2**).

Figure 2 (right panel) depicts how glucose restriction, or addition of 2-deoxyglucose, increases the doubling time of MCF-7 cells from 24h to 28h, while verapamil has no effect (note in the column "Low Glucose" the reduction in the number of MCF-7 cells, in gray, when compared to "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 22.4h to 26.3h, while the verapamil alone in high glucose media had no effect. The combination of verapamil and 2-deoxyglucose, however, caused a two-fold increase in doubling time for the PGP mutant (22.4h to 51h). 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 up-regulation of glucose uptake

We studied the rate of glucose uptake in both cell lines through accumulation of the fluorescent glucose analog 2-NBDG (**figure 3**). The initial rate of uptake of the glucose analog in the PGP+ mutant (estimated as the first derivative at time 0') was four times higher than for the parental cell line (165 RFU/min versus 40 RFU/min), indicating that part of the accelerated glucose metabolism observed in the MCF-7/Dox cells is due to an increase in the glucose transporter activity.

PGP mutants are more dependent on anaerobic glycolysis

In order to estimate the actual ATP production, we made the following assumptions: (1) all oxygen consumed is used in the Krebs cycle, and every six molecules of O_2 consumed produces 36 molecules of ATP; (2) the proton production corresponds to ~85% of the lactate production in atmospheric conditions (pO_2~21%)(22); and (3) that every molecule of lactate produced corresponds to the generation of one ATP molecule through anaerobic glycolysis;

Using these assumptions, we generated the chart of **figure 3**, which suggests three main conclusions: (1) glucose restriction does not significantly affect energy production in MCF-7 cells, but causes a 20% decrease in the MCF-7/Dox, (2) the extra energy produced by the PGP+ mutants comes from anaerobic glycolysis, and (3) when aerobic metabolism is blocked, the PGP+ mutants are capable of obtaining from two- to three-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 sub-population, which allows the re-growth 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 one of two conditions were satisfied: (1) the drug resistant cells were present in a very small number or (2) 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 anti-metabolites.

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 (**figures 4 and 5**), based on Wishart et al (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 to traditional high-dose density therapy, patients with initial prevalence of 10% PGP+ cells (**figure 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 sub-population, progression-free survival (compared to MTD) is improved by a factor of 4 with AT, and by a factor of 10 with the AT + verapamil + 2-deoxyglucose combination (**figure 5**).

Discussion

We previously proposed that the growth of sub-populations of resistant clones in a heterogeneous tumor could be suppressed, if a competing chemo-sensitive population were spared. This approach, termed Adaptive Therapy, explicitly limits application of chemotherapy to maintain a stable population of chemo-sensitive 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 due to toxicity. Additionally, 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 employ 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 (~10μM for both (7),(25)). Unfortunately, conventional clinical doses of these PGP substrates cannot achieve this concentration (the maximum concentrations

observed in clinical trials were 5.5μM for quinidine (11), and 0.5μM 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 extra-cellular 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 over-expressing PGP cells during chemotherapy intervals.

Since PGP over-expressing cells consume ATP to extrude drugs, we propose that the chronic administration of non-chemotherapeutic 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 over-expressing 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 ~22h to 51h) in PGP+ mutants. These results

suggest that the combination of energy depletion and increased energy utilization through administration PGP substrates synergistically reduce the growth of MDR sub-populations.

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, while 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 (**figure 5**).

The percentage of PGP+ mutants in our simulations was estimated from three 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 (EMT) (30). The actual effect of these stromal pumps in protecting the tumor is a subject of debate, as some authors have proposed that, while 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 maximum tolerated dose (MTD), Adaptive Therapy (AT), and AT combined with non-chemotherapeutic PGP substrates and 2-deoxyglucose, show dramatic differences in progression-free survival. Compared to 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 5mM/0.1uM, 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 1.25mM for up to 7 weeks (33), while chronic administration of 2-DG at 0.18mM 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 x 10^{-6}/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 (PFS) and observed survival (OS) from the example in **figure 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 demonstrated, using computation 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 non-chemotherapy PGP substrates, between treatments, will further burden the chemoresistant clones and increase their relative fitness disadvantage, when compared to chemosensitive cells. We demonstrate, 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.

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Figure legends

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

Figure 2 Competition of MCF-7 wild type and PGP mutant cells in co-culture 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 72h, 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 (22.4h versus 24h), and the addition of verapamil did not alter these values. In restricted glucose conditions, however, both populations replicated more slowly (28h and 26.3h for the wild type and PGP mutant, respectively). The addition of verapamil in low glucose conditions did not affected the growth of the wild type cells, but approximately doubled the doubling time of the PGP mutant (51h), indicating that the combination of energy restriction and a high-affinity PGP substrates tips the fitness advantage towards the wild type population. The bottom right charts depict the changes in doubling time of MCF-7 and MC-7/Dox, as a function of 2-deoxyglucose and verapamil. The wild type population (left) doubles at approximately every 24h 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 figure 1.

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 non-phosphorylated 2-NBDG in the first hour of incubation. Using Michaelis-Menten dynamics, Vmax of glucose uptake for the PGP mutant is four 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

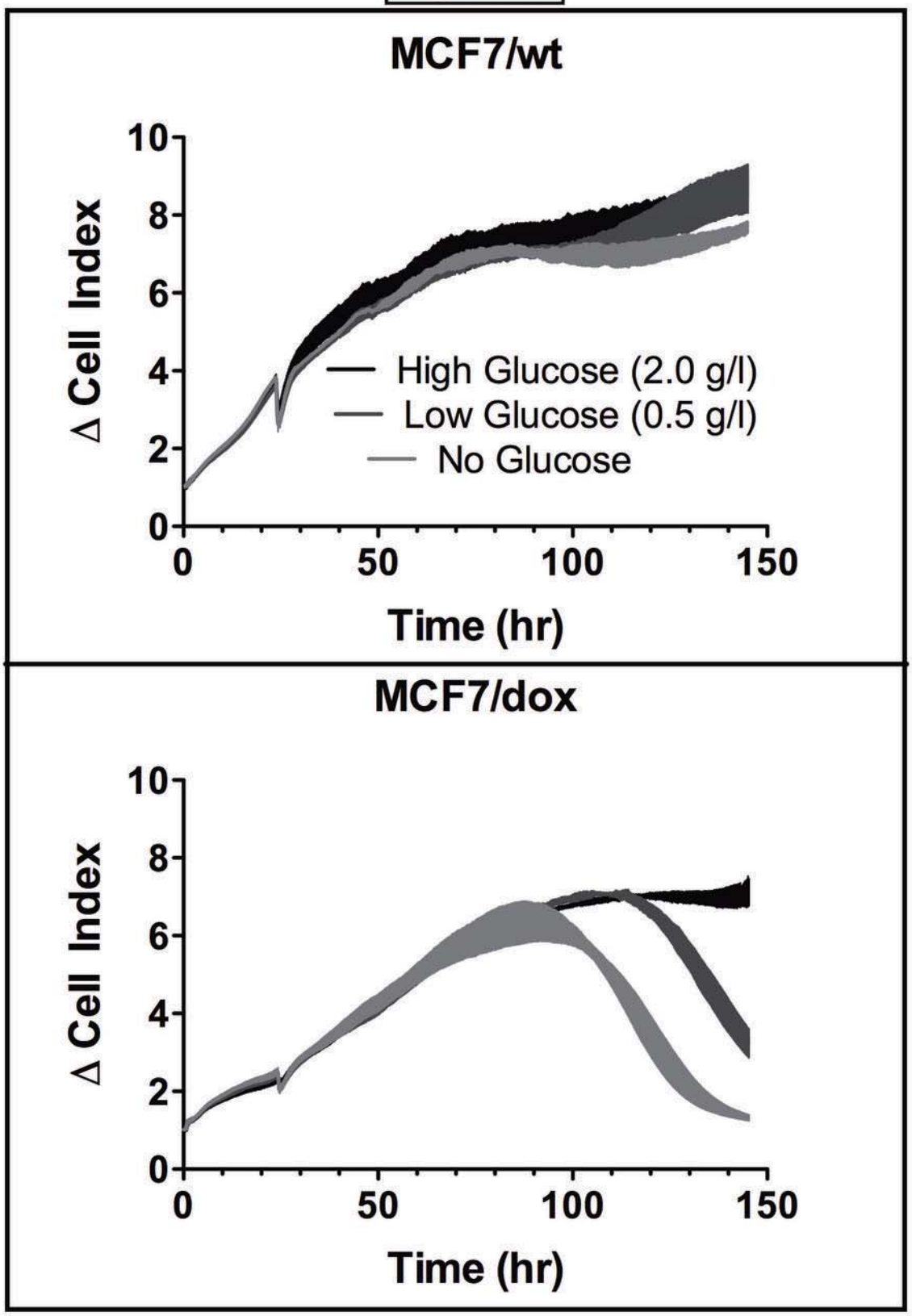
roughly three times higher in the PGP+ mutant than in the parental cell line. The addition of the non-metabolizable glucose analog 2-deoxyglucose (2DG) greatly reduces energy production in both cell lines.

Figure 4 Tumor burden simulation of a hypothetical patient carrying a sub-population of 10% PGP mutants treated with standard or Adaptive Therapy regimens combined with verapamil and 2DG. Maximum tolerated dose (MTD, upper left) promptly reduces the total tumor burden, but the patient soon relapses and stops to respond. The combination of MTD with an anti-metabolite (2DG) and a PGP substrate (verapamil) increases patient survival in ~30% (upper right). Adaptive therapy (AT, bottom left) is capable of maintaining the resistant sub-population under control for longer (increase in overall survival by 50%). The best results are obtained when 2DG and verapamil are administered together with adaptive therapy resulting in a 4-fold increase in progression free survival (PFS). 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, PFS was 35 days for MTD and 135 days for AT + verapamil + 2DG. The simulations considered two sub-populations 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 sub-population 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 figure 1** and **supplementary table 1.** The IC50 for the chemosensitive sub-population was arbitrarily set to 1, while 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 adaptive therapy algorithm was set as half this value.

Figure 5 Tumor burden simulation of a hypothetical patient carrying a sub-population of 5% PGP mutants treated with standard or Adaptive Therapy regimens combined with Verapamil and 2DG. In a hypothetical untreated patient with an average PGP+ sub-population (5%), the combination of adaptive therapy, 2DG and Verapamil (bottom right) is capable of maintaining the patient stable for over 500 days in contrast to the observed survival of 122 days of maximum tolerated dose (MTD, top left). Similarly to the

Figure 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 two sub-populations were modeled based on *in vitro* data from the MCF-7 and MCF-7/Dox cell lines. The competition between these sub-clones was modeled as described in **supplementary figure 1**, with growth rate and dose response parameters listed **supplementary table 1**.

Figure 1



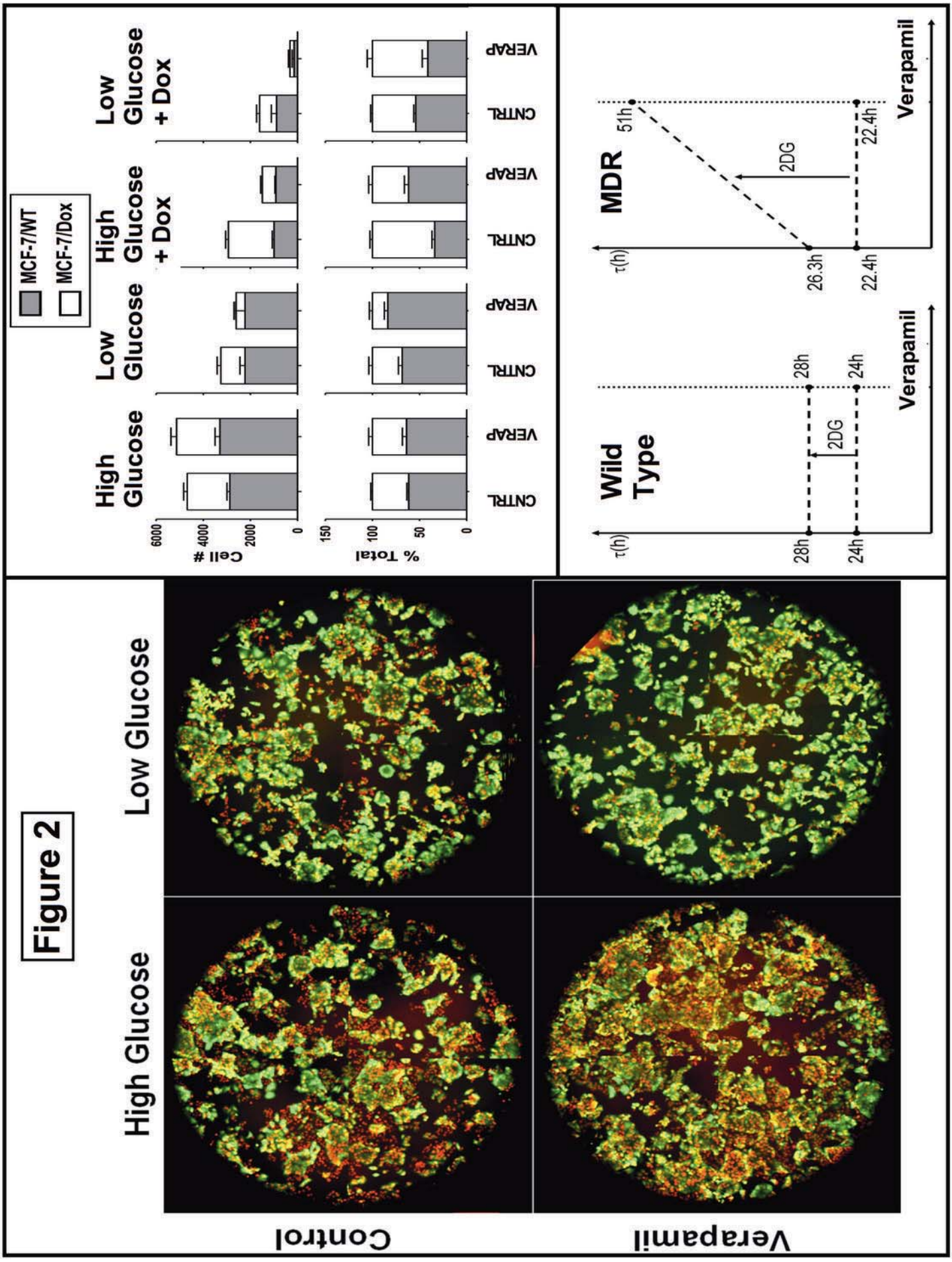


Figure 3

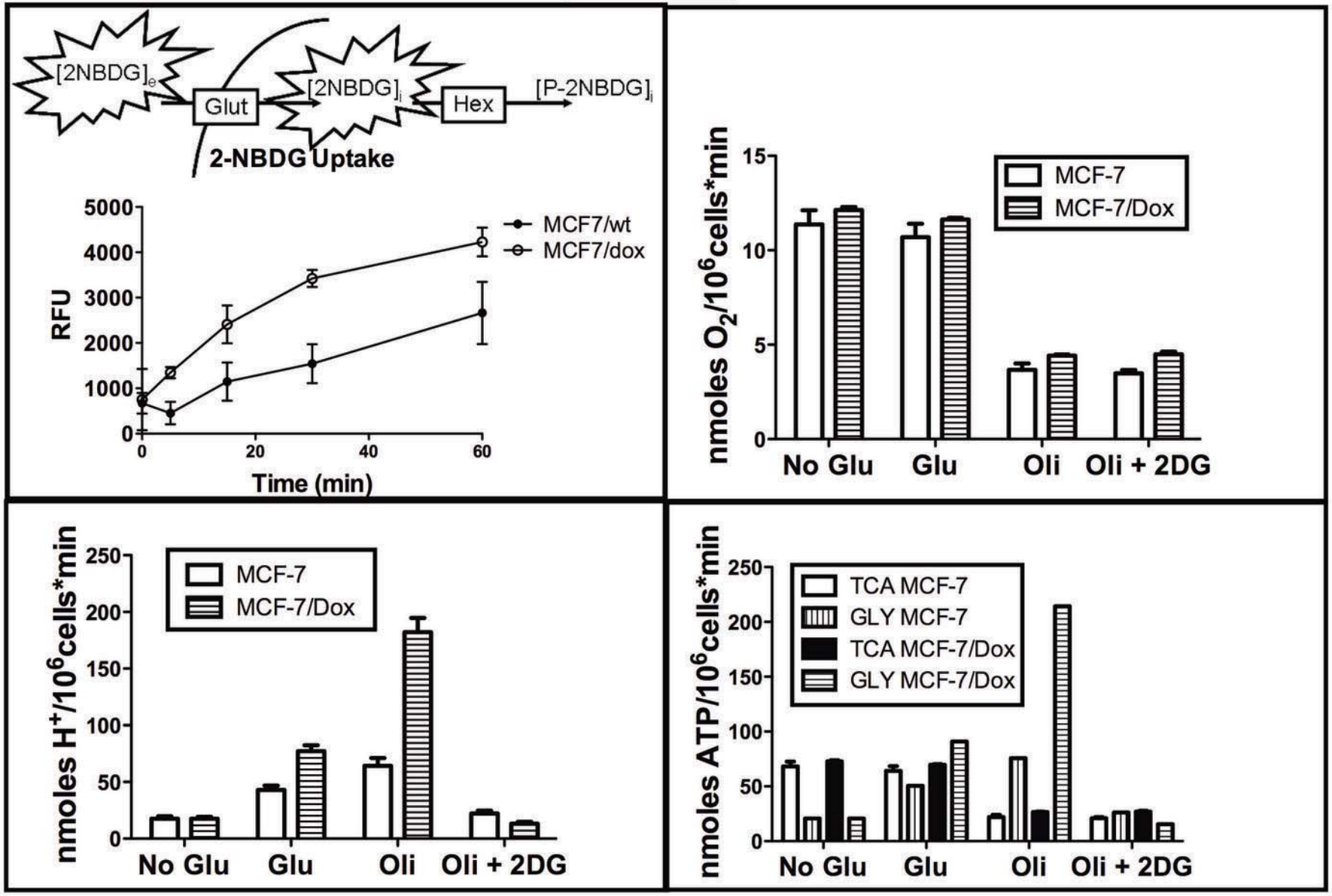


Figure 4

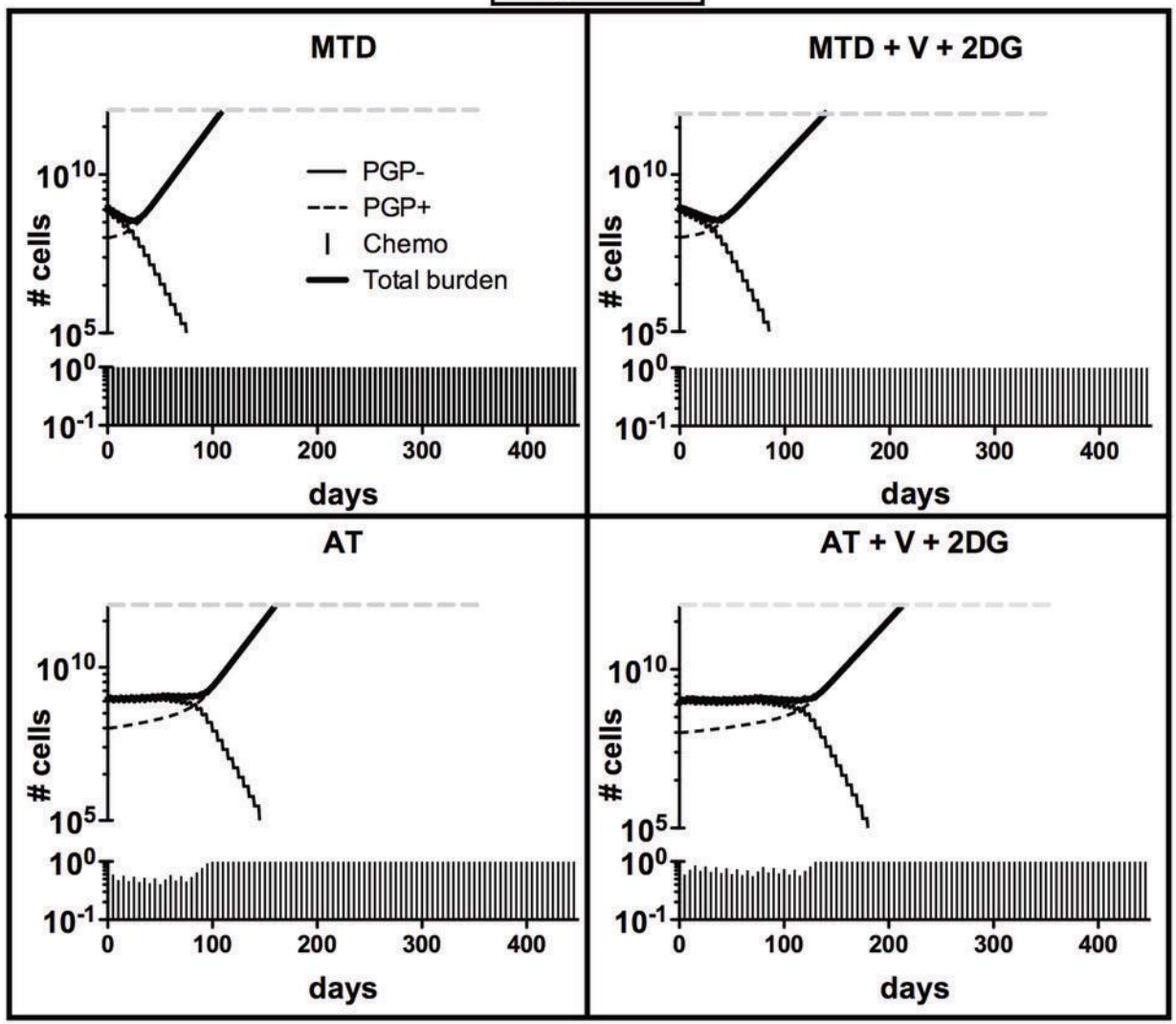
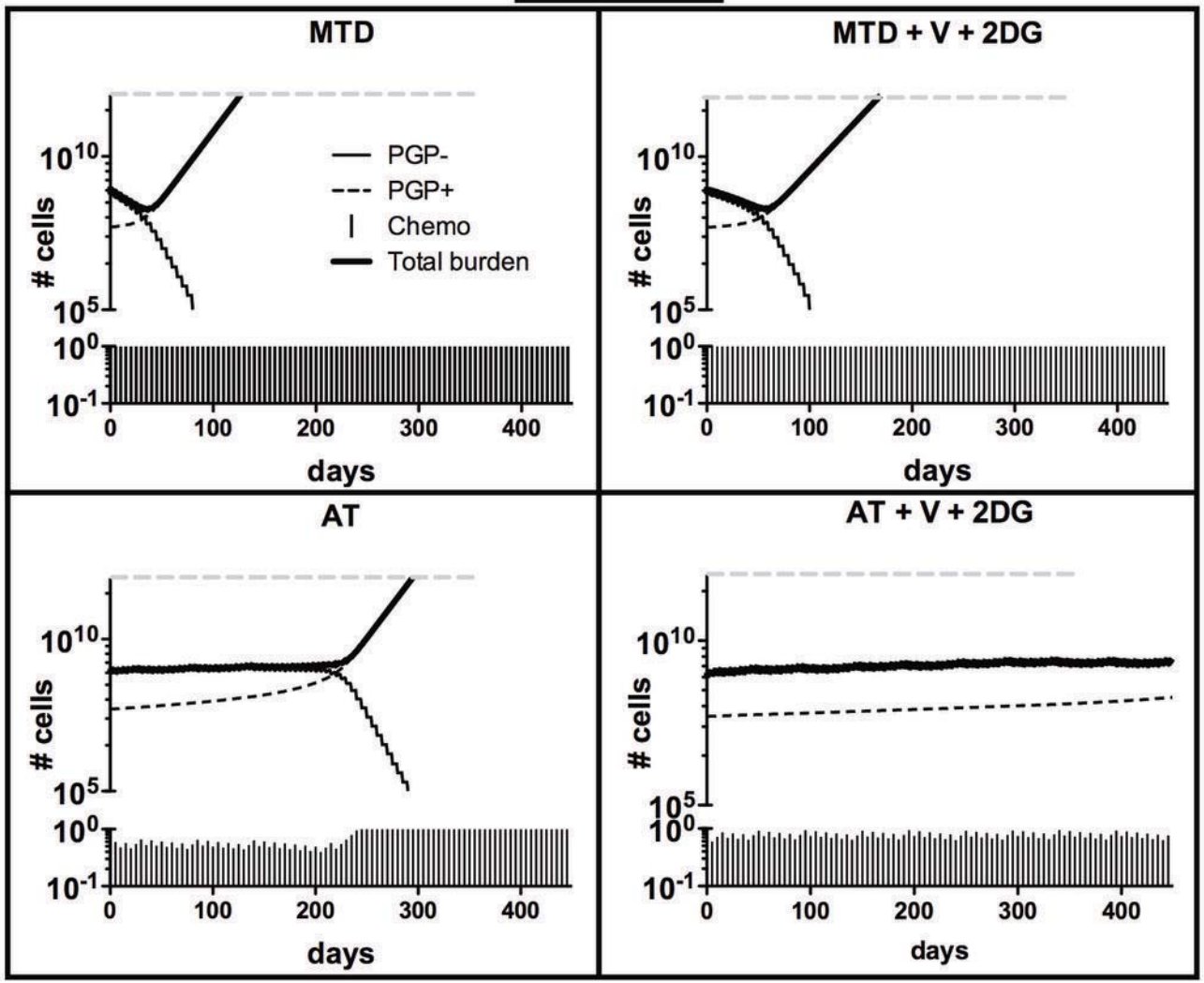


Figure 5



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Evolutionary approaches to prolong progression-free survival in breast cancer

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