Evolution of Resistance to Cancer Therapy

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Abstract: Acquired drug resistance is a major limitation for successful treatment of cancer. Resistance emerges due to drug exclusion, drug metabolism and alteration of the drug target by mutation or overexpression. Depending on therapy, the type of cancer and its stage, one or several genetic or epigenetic alterations are necessary to confer resistance to treatment. The fundamental question is the following: if a genetically diverse population of replicating cancer cells is subjected to chemotherapy that has the potential to eradicate it, what is the probability of emergence of resistance? Here, we review a general mathematical framework based on multi-type branching processes designed to study the dynamics of escape of replicating organisms from selection pressures. We apply the general model to evolution of resistance of cancer cells and discuss examples for diverse mechanisms of resistance. Our theory shows how to estimate the probability of success for any treatment regimen.

Key Words: Cancer Therapy; Evolution of Resistance; Evolutionary Dynamics.

1. INTRODUCTION

Drug resistance can result from two general causes [1]: (i) host factors such as poor absorption and rapid metabolism reduce the maximum achievable serum levels of the drug - this is sometimes referred to as intrinsic resistance, and (ii) specific genetic or epigenetic alterations enable resistant cancer cell clones to outgrow and escape from otherwise effective treatment. The main mechanisms of cellular resistance are depicted in Fig. (**1**). Some of these mechanisms, such as loss of a cell surface receptor or transporter, specific metabolism and an increase or alteration in the drug target, result in resistance to only a small number of related chemotherapeutic agents. Other mechanisms, however, lead to simultaneous resistance to many structurally and functionally unrelated drugs. This phenomenon is known as multidrug resistance [1, 2] and can result from changes that limit accumulation of drugs within cells by decreasing uptake, enhancing efflux, or affecting membrane lipids [3], block apoptosis which is activated by most anticancer drugs [4], induce general response mechanisms that detoxify drugs and repair DNA damage [5], and modulate the cell cycle [6] and checkpoints [7].

Many genes have been identified that contribute to diverse mechanisms of resistance to chemotherapy [8]: amplification or overexpression of the P-glycoprotein family of membrane transporters (e.g., MDR1, MRP, LRP) which decrease intracellular drug accumulation; changes in cellular proteins involved in detoxification (e.g., glutathione S-transferase pi, metallothioneins, human MutT homologue, bleomycin hydrolase, dihydrofolate reductase) or activation of the chemotherapeutic drugs (DT-diaphorase, NADP:

cytochrome P-450 reductase); changes in molecules involved in DNA repair (e.g., O6-methylguanine-DNA methyltransferase, DNA topoisomerase II, hMLH1, p21WAF1/CIP1); and activation of oncogenes such as Her-2/neu, bcl-2, bcl-XL, c-myc, ras, c-jun, c-fos, and MDM2 as well as inactivation of tumor suppressor genes like p53 can all confer resistance to therapy. The number of genetic or epigenetic alterations necessary for escape from therapy depends on the patient's genetic background, the therapy administered, and the type of cancer and its stage. Here, we will discuss mechanisms of resistance brought about by one or two genetic alterations. The following listing is not intended to be exhaustive, but serves to demonstrate the principle of escape from therapy *via* one or two alterations.

One alteration. Several genes confer cancer drug resistance if affected by a single genetic alteration. For example, single point mutations in alpha- or beta-tubulin confer resistance to hemiasterlins, which are sponge-derived tripeptides that inhibit cell growth by depolymerizing existing microtubules and inhibiting microtubule assembly [9]. Overexpression of Bcl-2 abrogates the short-term apoptotic response to chemotherapy and correlates with a poor long-term outcome [10]. Activation or overexpression of c-myc can induce and modulate drug resistance [11]. Similarly, mutation or overexpression of PI3K or Ras leads to increased radioresistance [12]. Upregulation of the transcription factor Ets-1 confers resistance to the DNA damaging agent cisplatin *via* transcriptional regulation of metallothioneins and DNA repair enzymes [13]. Several N-terminal and core-domain mutations have been identified in human topoisomerase II alpha, each of which is sufficient to confer bisdioxopiperazine resistance [14]. Overexpression of glutathione-S-transferases, a family of detoxification enzymes, is also implicated in the development of resistance toward chemotherapy agents [15]. Increased expression of the transcription factor NF- B induces drug resistance through MDR1 expression in cancer

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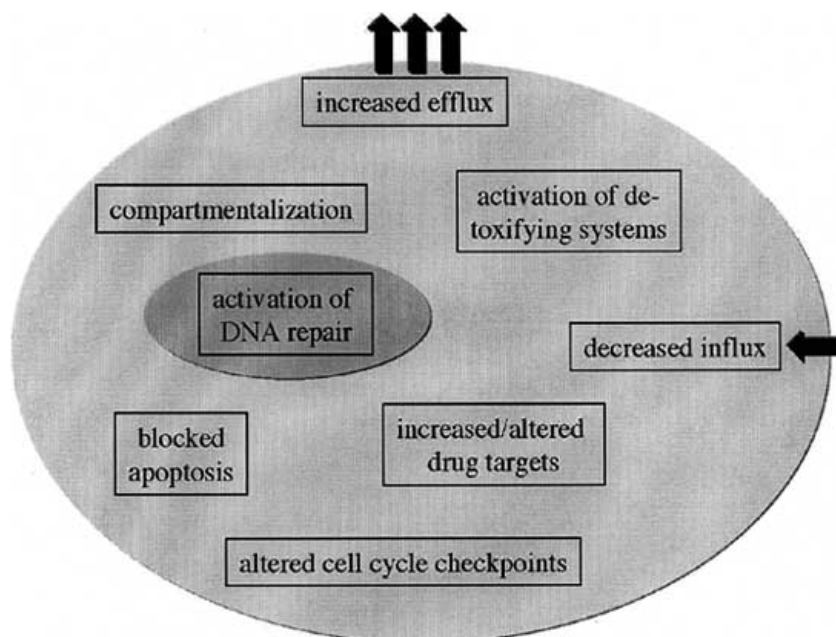
Fig. (1). Cellular factors that cause drug resistance. Cancer cells become resistant to anticancer drugs by several mechanisms. One way is to pump drugs out of cells by increasing the activity of efflux pumps, such as ATP-dependent transporters. Alternatively, resistance can occur as a result of reduced drug influx - a mechanism reported for drugs that enter on intracellular carriers or by means of endocytosis. Through compartmentalization, drug concentrations in the cytosol can be limited. In cases in which drug accumulation is unchanged, activation of detoxifying proteins can promote drug resistance. Cells can also activate mechanisms that repair drug-induced DNA damage, and disruptions in apoptotic pathways allow cells to become resistant to drug-induced cell death. Finally, alterations of cell cycle checkpoints or drug targets confer resistance to cancer therapy.

cells [16]. Overexpression of HER-2 represents a resistance mechanism to hormonal therapy in breast cancer [17]. Amplification or increased expression of p-glycoprotein confers multidrug resistance [18].

Two alterations. Other mechanisms of escape from therapy require two genetic alterations, either because of haplosufficiency of a gene such that one recessive mutation cannot confer resistance, or because of the use of combination therapy targeting two different positions in the cancer genome. For example, homozygous or compound heterozygous inactivation of p53 leads to acquired resistance to gamma irradiation and chemotherapy [4, 19]. Similarly, inactivation of both alleles of ATM confer resistance to therapy [20]; so does homozygous inactivation of Rb [21]. The cyclin dependent kinase inhibitors p16 and p18 can also be involved in resistance if inactivated in both alleles [22]. Loss of DNA mismatch repair due to hMLH1 hypermethylation or inactivation causes resistance to 5-fluorouracil in colorectal cancer [23].

The accumulation of specific genetic alterations leading to acquired drug resistance is greatly accelerated by genetic instability. Genetic instability is a defining characteristic of human cancers [24]. Two main types of genetic instabilities have been identified: in a small fraction of colorectal, endometrial, gastric and some other cancers, inactivation of the mismatch repair pathway leads to an elevated point mutation rate called microsatellite instability (MIN) [25, 26]; the majority of cancers, however, has chromosomal instability (CIN) [27]. CIN refers to an increased rate of losing or gaining whole chromosomes or large parts of chromosomes

during cell division. The consequence of CIN is an imbalance in chromosome number (aneuploidy) and an increased rate of loss of heterozygosity (LOH). An elevated rate of LOH is an important property of CIN, because it accelerates the inactivation of tumor suppressors and other recessive genes [28-30].

2. CALCULATING THE PROBABILITY OF RESISTANCE

Consider a population of cancer cells that grows according to a continuous time branching process [31, 32]. At each time step, a cell either produces an offspring or dies. If each cell produces on average more than one new cell, then the basic reproductive ratio [33] is larger than one, $r > 1$, and the cancer grows over time. If a cell dies with probability a and divides with probability b per time step, then the basic reproductive ratio is given by $r = b/a$. Therapy reduces the basic reproductive ratio either by increasing the death rate, decreasing the growth rate or both. Denote the basic reproductive ratio during therapy by R. If R still exceeds one, then therapy can reduce the rate of cancer growth, but is not capable of eradicating it. If R is less than one, however, then each cell produces on average less than one new cell, and therapy can eradicate the cancer. At the time of initiating therapy, there are N cancer cells. In this first model, we assume that all these cells are genetically identical. Thus we ignore genetic heterogeneity and we do not consider the possibility of resistance mutations. Under these limiting assumptions, the probability that the cancer population is eradicated by therapy is given by

$$p = 1 \text{ for } R < 1 \qquad (1)$$
$$p = 1/R^N \text{ for } R > 1$$

Therefore, successful therapy requires the basic reproductive ratio during therapy to be less than one, $R < 1$. This means that the cancer cells are sensitive to therapy and the cancer cell population decreases over time because each cell produces on average less than one new cell. However, if R is larger than one and there is a reasonably large number of cancer cells, then treatment will certainly fail. This means that the cancer cells are resistant to therapy and the cancer cell population increases over time because each cell produces on average more than one new cell. The probability of success depends on the total number of cancer cells: the larger the population size, the less likely is extinction due to random (chance) events; in a small population, however, random extinction is possible.

2.1 One Step to Resistance

Let us now consider genetic heterogeneity. In the simplest case, there are two types of cancer cells (Fig. **2**). Type 0 cells are sensitive to therapy. Their basic reproductive ratio during therapy is less than one, $R_0 < 1$. Type 1 cells are resistant to therapy. Their basic reproductive ratio during therapy is larger than one, $R_1 > 1$. Suppose that resistant cells are not present in the cancer before the beginning of treatment. They could have a strong selective disadvantage in the absence of therapy and/or the mutation rate at which they are being produced could be very low. Hence the cancer consists of N sensitive cells at the beginning of therapy. During therapy, however, resistant cells are being produced from sensitive cells at rate u per cell division. The probability of successful therapy [34, 35] is given by

$$P = \exp\left[- Nu \, \frac{R_0}{1 - R_0} \frac{R_1 - 1}{R_1} \right] \qquad (2)$$

This probability holds in the limit of a small mutation rate, $0 < u \ll 1 - R_0$ and $0 < u \ll R_1 - 1$.
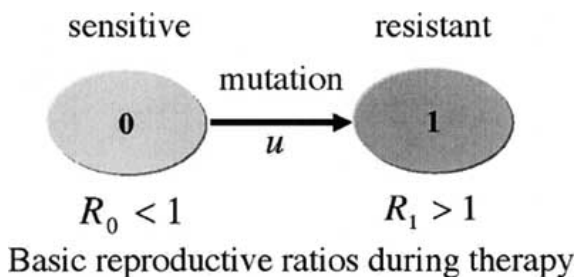

Fig. (2). One step to escape. Cells that are sensitive to cancer therapy are denoted by type 0. These cells have basic reproductive ratio less than one, $R_0 < 1$, and each such cell produces on average less than one new cell. Mutation at rate u per cell division gives rise to resistant cells denoted by type 1. These cells have basic reproductive ratio in excess of one, $R_1 > 1$, and each such cell produces on average more than one new cell.

Suppose the mutation rate conferring resistance is $u = 10^{-9}$ per cell division. Assume sensitive cells have basic reproductive ratio $R_0 = 0.9$ and resistant cells have basic repro-

ductive ratio $R_1 = 1.1$ during therapy. This means that each sensitive cell has a 90% chance of producing one new cell, which is sensitive with probability $1 - u$ and resistant with probability u. Each resistant cell produces on average 1.1 new resistant cells. If the population size at the start of therapy is $N = 10^6$, then the probability of successful therapy is very close to 100%. If, however, the initial population size is $N = 10^9$, then the probability of success drops to 44%. This means that therapy will eradicate the cancer in 44% of patients but fail in the remaining 56%. Now consider a mutation rate of $u = 10^{-7}$ per cell division. With the basic reproductive ratios being as before, the probability of success is 92% for $N = 10^6$ and essentially 0% for $N = 10^9$.

Type 1 cells, however, might preexist in the cancer prior to the onset of therapy. Assume that at the beginning of therapy, the cancer consists of N_0 cells of type 0 and N_1 cells of type 1. In this case, the probability of successful therapy [34, 35] is given by

$$P = \exp\left[-\left(N_0 u \, \frac{R_0}{1 - R_0} + N_1 \right) \frac{R_1 - 1}{R_1} \right] \qquad (3)$$

Suppose the mutation rate conferring resistance is $u = 10^{-9}$ per cell division. Assume sensitive cells have basic reproductive ratio $R_0 = 0.9$ and resistant cells have basic reproductive ratio $R_1 = 1.1$ during therapy. Consider a cancer consisting of $N_0 = 10^6$ sensitive cells. If there is one resistant cell at the start of therapy, $N_1 = 1$, then the probability of success is 91%. In contrast, if there are ten resistant cancer cells at the start of therapy, $N_1 = 10$, then the probability of success is only 40%. With the same basic reproductive ratios as before and with $N_0 = 10^9$, the probability of resistance is 40% if $N_1 = 1$ and 18% if $N_1 = 10$.

Now suppose the mutation rate conferring resistance is $u = 10^{-7}$ per cell division. Again, assume reproductive ratios of $R_0 = 0.9$ and $R_1 = 1.1$ during therapy. For $N_0 = 10^6$, the probability of success is 84% if $N_1 = 1$ and 37% if $N_1 = 10$. For $N_0 = 10^9$, however, the probability of success is 0% both if $N_1 = 1$ and if $N_1 = 10$.

Alternatively, assume that the cancer cells are in a mutation-selection balance at the start of therapy. The relative fitness values of type 0 and type 1 cells in the absence of therapy are denoted by w_0 and w_1, respectively. Without loss of generality, we set $w_0 = 1$ and $0 \le w_1 \le 1$. If $w_1 = 0$, then type 1 cells are lethal; if $w_1 = 1$, then they are neutral as compared with type 0 cells. If $w_1 < 1$, then there is a mutation-selection balance between the two cell types prior to therapy. The equilibrium number of type 1 cells in the absence of therapy is $N_1 = N_0 u/(1 - w_1)$. This equation holds in the limit of small mutation rates, $u \ll 1 - w_1$. The probability of successful therapy [34, 35] is given by

$$P = \exp\left[- N_0 u \left(\frac{R_0}{1 - R_0} + \frac{1}{1 - w_1} \right) \frac{R_1 - 1}{R_1} \right] \qquad (4)$$

for $0 \le w_1 < 1$.

Suppose the mutation rate conferring resistance is $u = 10^{-9}$ per cell division. Assume sensitive cells have basic reproductive ratio $R_0 = 0.9$ and resistant cells have basic repro-

ductive ratio $R_1 = 1.1$ during therapy. Suppose resistant cells have relative fitness $w_1 = 0.9$ in the absence of therapy. If the cell population at the start of therapy is $N_0 = 10^6$, then the probability of success is close to 100%. If the cell population at the start of therapy is $N_0 = 10^9$, then the probability of success is around 18%. Table (**1a**) and Fig. (**3**) provide examples of further parameter choices.

2.1.1 Emergence Versus Pre-Existence of Resistance

A major question is whether resistance mutations preexist in the cancer or arise only after the onset of therapy. In the former case, therapy will most likely fail, unless the frequency of resistant cells at the start of therapy is very small and they have a basic reproductive ratio only marginally larger than one. In the latter case, however, therapy might succeed if the cancer size at the start of therapy is small and therapy can impose a sufficiently strong selection pressure such that sensitive cells decline quickly without producing resistance mutants. How can we quantify this statement? In eq. 4, the term $R_0/(1 - R_0)$ describes the relative contribution to treatment failure caused by resistance mutations that

emerge during therapy. The term $1/(1 - w_1)$ describes the relative contribution to treatment failure caused by mutations that preexist before therapy. Therefore, the ratio of emerging to preexisting resistance is given by

$$= \frac{R_0 \ (1 \ - \ w_1)}{1 \ - \ R_0} \tag{5}$$

For example, if $R_0 = 0.1$ and $w_1 = 0.9$, then $= 0.011$. Hence preexisting resistance contributes 90 times more to treatment failure than emerging resistance. If on the other hand $R_0 = 0.9$ and $w_1 = 0.1$, then $= 8.1$. Hence emerging resistance contributes about 8 times more to treatment failure than preexisting resistance. Similar questions have been studied in the context of HIV infection [36-40] and bacterial resistance [41].

2.1.2 The Maximum Cancer Size Compatible with Success

Is there a rule of thumb for the maximum size of a cancer that allows for a substantial probability of success? From eq. 4 we see that the critical population size is given by
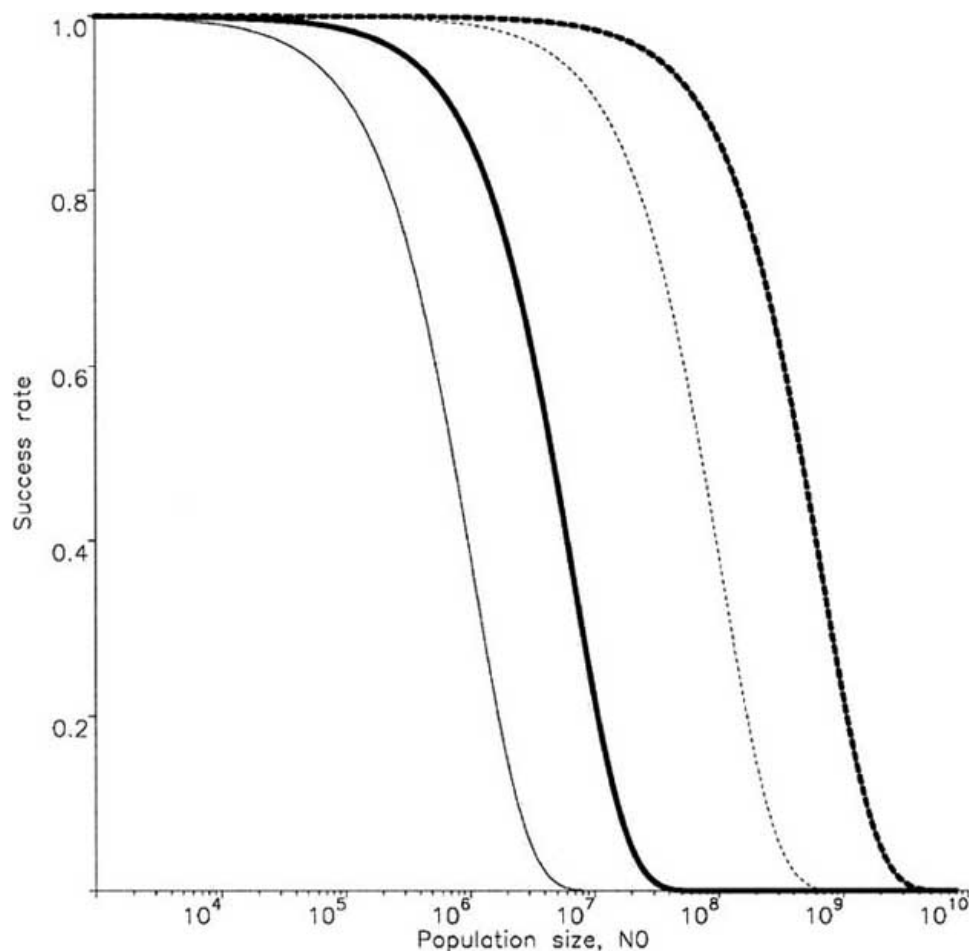

Fig. (3). Probability of successful therapy. There are two types of cells: sensitive cells, type 0, have a relative fitness value of $w_0 = 1$ in the absence of therapy and a basic reproductive ratio $R_0 < 1$ during therapy; resistant cells, type 1, have a relative fitness value of w_1 in the absence of therapy and a basic reproductive ratio $R_1 > 1$ during therapy. At the start of therapy, the cells are in a mutation-selection balance. The probability of successful therapy is given by eq 4. Parameter values are $R_1 = 2$; $R_0 = 0.9$ and $w_1 = 0.9$ (thin lines) and $R_0 = 0.5$ and $w_1 = 0.5$ (thick lines); and $u = 10^{-7}$ (solid) and $u = 10^{-9}$ (broken).

$$N_0^\star = \frac{1}{u}\left(\frac{R_0}{1 - R_0} + \frac{1}{1 - w_1}\right)^{-1} \tag{6}$$

If the cancer size by far exceeds this critical size, $N_0 \gg N_0^*$, then success is nearly impossible. However, if the cancer size is well below this critical size, $N_0 \ll N_0^*$, then success is almost certain. In the unlikely case that the size of a cancer at the start of therapy is very close the critical population size, $N_0 = N_0^*$, the probability of success is $1/e$ 37%.

Suppose the mutation rate conferring resistance is $u = 10^{-9}$ per cell division. If $R_0 = 0.1$ and $w_1 = 0.1$, then the critical population size is $N_0^* = 8 \cdot 10^8$. If the cancer greatly exceeds this size at the start of therapy, then the therapeutic outlook is dim; it is promising, in contrast, if the cancer size is well below this critical size at the start of therapy. The exact probability of resistance can be calculated using the equations outlined above. If $R_0 = 0.9$ and $w_1 = 0.9$, then the critical population size is $N_0^* = 5 \cdot 10^7$. Now suppose the mutation rate conferring resistance is $u = 10^{-7}$ per cell division. If $R_0 = 0.1$ and $w_1 = 0.1$, then the critical population size is is $N_0^* = 8 \cdot 10^6$. It drops to $N_0^* = 5 \cdot 10^5$, however, if $R_0 = 0.9$ and $w_1 = 0.9$.

We see that the total population size is decisive for the outcome of therapy. If the number of actively replicating or viable cancer cells is greater than a certain threshold, which is usually close to the inverse of the mutation rate, then treatment failure is almost certain.

Let us discuss a few concrete examples for the size of the cancer burden at diagnosis and the start of therapy. (i) Chronic myeloid leukemia (CML) is associated with the oncogenic fusion gene BCR-ABL generated by the Philadelphia (9;22) translocation in a hematopoietic stem cell [42]. This chromosomal aberration leads to a clonal expansion of the leukemic stem cell pool and consequently to a slow accumulation of immature myeloid progenitors, so-called blasts. The abundance of leukemic stem cells at diagnosis [43-45] has been estimated to be around $N = 250000$ cells. Leukemic differentiated cells, however, have reached an abundance of 10^{12} cells at the time of diagnosis. (ii) Colorectal cancer results from an accumulation of mutations in oncogenes and tumor suppressor genes such as APC, RAS, PI3K and p53 [46]. This sequence of mutations causes a clonal expansion of colorectal epithelial cells. The earliest identifiable lesion contains about $N = 10^9$ cells (1 cm cross-section) (Bert Vogelstein, personal communication). (iii) Candidate genes contributing to lung tumors include the oncogenes ras, myc, and cyclin D and the tumor suppressor genes p53, p16, Rb, FHIT, PTEN, PP2a and PP1. The tumor size at diagnosis [47] is about 3 to 6 cm in diameter. (iv) Breast cancer is the most frequently diagnosed cancer in women [46]. Sporadic breast cancers have alterations in growth factors and their receptors, intracellular signaling molecules, regulators of cell cycling, adhesion molecules and proteases. The tumor size at diagnosis is on average 1.5 cm in diameter [48]. (v) Brain tumors are classified as astrocytomas, oligodendrogliomas, ependymomas, medulloblastomas, meningiomas or schwannomas depending on the cells that give rise to the cancer. Several genes, such as p53, PTEN, CDKNA2, CDK4, EGFR, NF2, and myc, can be involved in this disease. Brain tumors are diagnosed once their diameter has reached about 8 cm [49].

Many solid tumors are detected when their diameters have reached one or a few centimeters. If this size correlates with cell numbers of about 10^9 to 10^{11}, then most of these tumors will exceed the critical size for mutation rates of about $u = 10^{-9}$ and therefore the probability of successful therapy will be low. However, not all of these cells might be viable: often, the center of a tumor is necrotic and thus the cell numbers might be lower than calculated from the diameter. Also, only some cancer cells might actively replicate - others can be dormant or dividing only a few times before undergoing terminal differentiation (see Section 3). Thus, the effective population size of a cancer can be a subset of all cancer cells.

There are two ways to combat treatment failure due to evolution of resistance mutations: development of diagnostic tools that detect cancers at smaller size or development of treatments that require multiple mutations for resistance. The latter brings us to the next section.

2.2 Two Steps to Resistance

Let us now assume that two genetic or epigenetic alterations are needed to confer resistance to a particular cancer therapy. We consider four types of cells: cells that are wild type in both positions are denoted by 00; cells that have either position mutated are denoted by 01 and 10; all these cells are sensitive to therapy. Finally, cells that have both positions mutated are denoted by 11; these cells are resistant to therapy (Fig. **4a**). The rates at which the two positions are mutated per cell division are denoted by u_1 and u_2. In the absence of therapy, the fitness values of the cells are given by w_{00}, w_{01}, w_{10} and w_{11}, respectively. Wild type cells have fitness $w_{00} = 1$, whereas all others have fitness less than one. During therapy, the basic reproductive ratios are given by R_{00}, R_{01}, R_{10} and R_{11}. Resistant cells have a basic reproductive ratio in excess of one, whereas all other cells have basic reproductive ratios less than one. The probability of successful therapy [34, 35] of a cancer of size N is given by

$$P = \exp(-NC\, u_1 u_2 z) \tag{7}$$

The parameter $z = 1 - 1/R_{11}$ denotes the probability that a cellular lineage starting from one resistant cell escapes from therapy. The risk coefficient, C, is given by

$$C = a_{00}(1 + a_{01} + a_{10}) + a_{01}b_{01} + a_{10}b_{10} + b_{11}(1 + b_{01} + b_{10})$$

Here $a_i = R_i/(1 - R_i)$ for $i = 00, 01, 10$ and $b_i = 1/(1 - w_i)$ for $i = 01, 10, 11$. The maximum population size that can be contained by therapy is given by

$$N^* = \frac{1}{Cu_1 u_2 z}$$

If the cancer size at diagnosis by far exceeds this critical size, $N \gg N^*$, then success is nearly impossible. However, if the cancer size is well below this critical size, $N \ll N^*$, then success is almost certain.

Let us discuss the individual contributions to the risk coefficient, C, in detail (Fig. **4b**). The risk coefficient is determined by the distribution of the four different cell types at the start of therapy and the probabilities that cellular lineages, each starting from a single cell of a different type, es-

cape from therapy. The initial distribution of the cell types results from the mutation-selection balance in the absence of therapy. Denote the relative abundances of the individual types at the start of therapy by x_{00}, x_{01}, x_{10} and x_{11}. These frequencies depend on the fitness values of the cells as well as the mutation rates at which they are being produced [34, 35]. The frequency of cells that are wild type in both positions, x_{00}, is close to one. This approximation holds for small mutation rates u_1 and u_2; both mutation rates must be much smaller than $1 - w_i$ for $i = 01, 10, 11$. The frequencies of cells that have one mutated position are given by $x_{01} = u_2 b_{01}$ and $x_{10} = u_1 b_{10}$, respectively. The frequency of cells that have both positions mutated is given by $x_{11} = u_1 u_2 b_{11} (1 + b_{01} + b_{10})$.
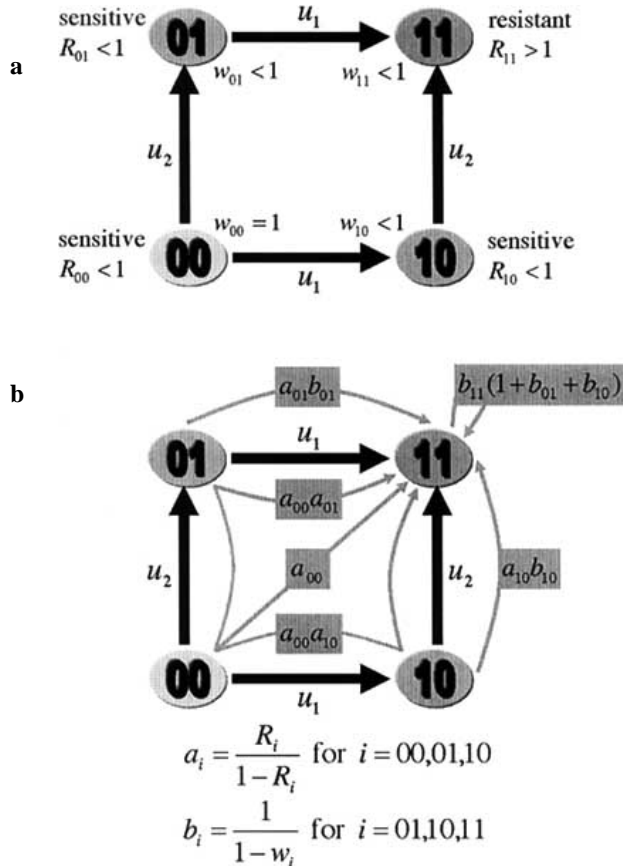

Fig. (4). Two steps to escape. (a) Two positions in the cancer genome are crucial for the evolution of resistance. Cells wild type in both positions are denoted by 00 and are sensitive to therapy; they have basic reproductive ratio $R_{00} < 1$ during therapy. Cells wild type in one position are denoted by 01 and 10, respectively, and have basic reproductive ratios $R_{01} < 1$ and $R_{10} < 1$ during therapy. Cells mutated in both positions are denoted by 11 and are resistant; their basic reproductive ratio is $R_{11} > 1$. The rates at which the two positions are mutated per cell division are denoted by u_1 and u_2 respectively. (b) The individual contributions to the evolution of resistance depend on the basic reproductive ratio during therapy and the fitness values of the different cell types in the absence of therapy. Here $a_i = R_i /(1 - R_i)$ for $i = 00, 01, 10$ and $b_i = 1/(1 - w_i)$ for $i = 01, 10, 11$.

The escape probability of a lineage starting with one cell of type 00, 01, 10, or 11 is denoted by p_{00}, p_{01}, p_{10} and p_{11},

respectively. These probabilities depend on the basic reproductive ratios of the cells as well as the mutation rates at which they are being produced [34, 35]. The escape probability of a lineage starting with one cell of type 00 is given by $p_{00} = u_1 u_2 a_{00} (1 + a_{01} + a_{10})z$ where $z = 1 - 1/R_{11}$. The first term in the brackets indicates the contribution of the direct mutation from 00 to 11, while the second and third terms indicate sequential mutations *via* 01 and 10, respectively. Direct mutation from 00 to 11 is more important than sequential mutation from 00 *via* 01 to 11 if $R_{01} < 1/2$. Likewise, direct mutation from 00 to 11 is more important than sequential mutation from 00 *via* 10 to 11 if $R_{10} < 1/2$. Obviously, the more deleterious cells 01 and 10 are, the more important direct mutation becomes [34, 35]. The escape probabilities of lineages starting with one cell of type 01 or 10, respectively, are given by $p_{01} = u_1 a_{01} z$ and $p_{10} = u_2 a_{10} z$. Finally, the escape probability of a lineage starting with one cell of type 11 is $p_{11} = z = 1 - 1/R_{11}$. The mutation-selection balance distribution of the cell types and the escape probabilities of single lineages give rise to the risk coefficient [34, 35].

Let us now discuss numerical examples. Assume the basic reproductive ratios during therapy are $R_{00} = R_{01} = R_{10} = 0.9$ and $R_{11} = 1.1$. Suppose the fitness values in the absence of therapy are $w_{00} = 1$ and $w_{01} = w_{10} = w_{11} = 0.9$. If the rates at which the two positions are altered per cell division are $u_1 = u_2 = u = 10^{-9}$, then the probability of success is very close to 100% for both $N = 10^9$ and $N = 10^{12}$. If the mutation rates per cell division are $u_1 = u_2 = u = 10^{-7}$, then the probability of success is very close to 100% for $N = 10^9$ and about 60% for $N = 10^{12}$. If the mutation rates are $u_1 = u_2 = u = 10^{-5}$ - either due to microsatellite instability (MIN) or DNA damaging therapy inducing higher mutation rates - then the probability of success is 0.6% for $N = 10^9$ and essentially 0% for $N = 10^{12}$.

Now assume the basic reproductive ratios during therapy are $R_{00} = R_{01} = R_{10} = 0.1$ and $R_{11} = 1.1$ and suppose the fitness values in the absence of therapy are $w_{00} = 1$ and $w_{01} = w_{10} = w_{11} = 0.1$. If $u_1 = u_2 = u = 10^{-9}$ and if $u_1 = u_2 = u = 10^{-7}$, then the probability of success is again very close to 100% for both $N = 10^9$ and $N = 10^{12}$. If $u_1 = u_2 = u = 10^{-5}$, then the probability of success is 96% for $N = 10^9$ and 0% for $N = 10^{12}$. Table (**1b**) provides examples of further parameter choices.

2.3 Tumor Suppressor Gene Inactivation

Some cancers can acquire resistance to therapy by means of the inactivation of a tumor suppressor gene (TSG). First, suppose that both alleles of the TSG are wild type, $TSG^{+/+}$, in all cells at the beginning of therapy. During therapy, $TSG^{+/+}$ cells have basic reproductive ratio $R_{00} < 1$ and are sensitive to therapy. However, they can accumulate genetic alterations inactivating both TSG alleles. The first allele is usually inactivated by a point mutation, whereas the second allele can be inactivated either by a second point mutation or a loss of heterozygosity (LOH) event (Fig. **5**). Denote the mutation rate per gene per cell division by u, and the rate of LOH by p. The first allele is inactivated at rate $2u$ per cell division, because either of the two alleles can be inactivated first. Once the first TSG allele has been inactivated, $TSG^{+/-}$,

Table 1.   **Probability of Success. (a) The table shows the probability of successful therapy if one genetic alteration is needed for resistance (Equation 4). The basic reproductive ratio of resident cancer cells is denoted by R_0 and the relative fitness of resistant cancer cells prior to therapy by w_1. Parameter values are $u = 10^{-9}$, $N_0 = 10^8$, $w_0 = 1$, and $R_1 = 2$. (b) The table shows the probability of successful therapy and the critical population size if two genetic alterations are needed for resistance (Equation 7). The basic reproductive ratio during therapy is denoted by R and can differ for types 00, 01, 10, and 11. The fitness values prior to therapy are denoted by w. Parameter values are $u_1 = u_2 = 10^{-7}$ and $N = 10^{12}$.**

Table 1a.   Probability of Success (One Alteration)

R_0 \\ W_1	0.10	0.50	0.90	0.99
0.10	94%	90%	60%	0.7%
0.50	90%	86%	58%	0.6%
0.90	60%	58%	39%	0.4%
0.99	0.7%	0.6%	0.4%	0%

Table 1b.   Probability of Success and Critical Population Size (Two Alterations)

type	00	01	10	11	P	N^*
R	0.00	0.00	0.00	1.10	100%	$4 \cdot 10^{14}$
w	1.00	0.00	0.00	0.00		
R	0.10	0.50	0.50	1.10	99%	$1 \cdot 10^{14}$
w	1.00	0.50	0.50	0.10		
R	0.90	0.90	0.90	1.10	84%	$6 \cdot 10^{12}$
w	1.00	0.10	0.10	0.10		
R	0.10	0.10	0.10	1.10	83%	$5 \cdot 10^{12}$
w	1.00	0.90	0.90	0.90		
R	0.90	0.90	0.90	1.10	60%	$2 \cdot 10^{12}$
w	1.00	0.90	0.90	0.90		
R	0.99	0.99	0.99	1.10	0%	$2 \cdot 10^{10}$
w	1.00	0.99	0.99	0.99		

cells have basic reproductive ratio $R_{01} < 1$ and are sensitive to therapy. If their basic reproductive ratio is the same as the basic reproductive ratio of wild type cells, $R_{00} = R_{01}$, then the TSG is strictly recessive and inactivation of one allele does not alter the reproductive capabilities or death rates of cells during therapy. If $R_{00} < R_{01} < 1$, then the TSG is haploinsufficient and inactivation of one allele can increase the growth rate or decrease the death rate of cells during therapy. The remaining allele is inactivated at rate $u + p$ per cell division. Once both TSG alleles have been inactivated, $TSG^{-/-}$, the cells are resistant to therapy and have basic reproductive ratio $R_{11} > 1$. At the beginning of therapy, the cancer consists of N wild type cells, $TSG^{+/+}$. The probability of successful therapy [34, 35] is given by

$$P = \exp\left[- N2u\,(u+p)\frac{R_{00}}{1 - R_{00}}\left(1 + \frac{R_{01}}{1 - R_{01}}\right)\frac{R_{11} - 1}{R_{11}} \right]$$

(8)

Assume that the mutation rate is about $u = 10^{-7}$ per allele per cell division; the point mutation rate has been measured to be around 10^{-10} per base per cell division [50], and a typical TSG allele might be inactivated by any one of 1000 point mutations. The rate of LOH might be about $p = 10^{-6}$ in genetically stable cells; in genetically unstable cells, however, the rate of LOH has been determined to be $p = 10^{-2}$ per cell division [51]. Suppose the basic reproductive ratios of both wild type cells and cells with one inactivated TSG allele are $R_{00} = R_{01} = 0.1$, and the basic reproductive ratio of cells with two inactivated TSG alleles is $R_{11} = 1.1$. Then the probability
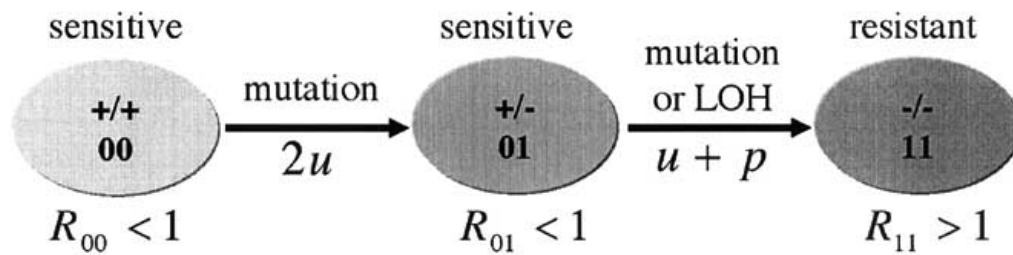
Fig. (5). Inactivation of a tumor suppressor gene (TSG). First, both alleles of the TSG are wildtype, +/+. The first allele is inactivated at rate $2u$ per cell division; the mutation rate per allele per cell division is denoted by u, and either of the two alleles can be inactivated first. Once the TSG is heterozygously inactivated, +/–, the second allele of the TSG is inactivated at rate $u+p$, where p denotes the rate of loss of heterozygosity (LOH). Then the TSG is homozygously or compound heterozygously inactivated, –/–.

of successful therapy is very close to 100% if the cancer initially consists of $N = 10^9$ or $N = 10^{12}$ genetically stable cells wild type with respect to the TSG. If the cancer initially consists of $N = 10^9$ genetically unstable cells, however, then the probability of successful therapy is 98%. This chance drops to 0% if $N = 10^{12}$. Now assume basic reproductive ratios of $R_{00} = R_{01} = 0.9$ and $R_{11} = 1.1$. In that case and with genetically stable cells, the probability of successful therapy is close to 100% for $N = 10^9$ and 17% for $N = 10^{12}$. With genetically unstable cells, the probability is 0% both for $N = 10^9$ and $N = 10^{12}$. Finally, consider $R_{00} = 0.1$, $R_{01} = 0.5$ and $R_{11} = 1.1$. Now the probability of success is close to 100% for $N = 10^9$ and 98% for $N = 10^{12}$ genetically stable cells, and 82% for $N = 10^9$ and 0% for $N = 10^{12}$ genetically unstable cells.

Cells with one or two inactivated TSG alleles, however, can preexist in the cancer before the onset of therapy. Suppose that at the start of therapy, the cancer consists of N_{00} cells wild type with respect to the TSG, $TSG^{+/+}$, N_{01} cells with one inactivated TSG allele, $TSG^{+/-}$, and N_{11} cells with two inactivated TSG alleles, $TSG^{-/-}$. In that case, the probability of successful therapy [34, 35] is given by

$$P = \exp\left[\left(- 2N_{00}u\,(u+p)\,\frac{R_{00}}{1-R_{00}}\left(1 + \frac{R_{01}}{1-R_{01}}\right) - N_{01}(u+p)\,\frac{R_{01}}{1-R_{01}} - N_{11}\right)\frac{R_{11}-1}{R_{11}}\right] \tag{9}$$

This probability holds in the limit of small mutation rates, $(u+p) \ll 1 - R_{00}, 1 - R_{01}, R_{11} - 1$.

Again, assume that the mutation rate is $u = 10^{-7}$ per allele per cell division, and the rate of LOH is $p = 10^{-6}$ in genetically stable cells and $p = 10^{-2}$ in genetically unstable cells. Suppose the basic reproductive ratios are $R_{00} = R_{01} = 0.9$ and $R_{11} = 1.1$. First, assume all cells are genetically stable. Suppose the cancer initially consists of $N_{00} = 10^9$ cells with two wild type TSG alleles, $N_{01} = 100$ cells with one wild type TSG allele and $N_{11} = 1$ cell with zero wild type TSG alleles. Then the probability of successful therapy is 91%. If $N_{00} = 10^{12}$, $N_{01} = 1000$ and $N_{11} = 0$, then the probability of success is 17%. If $N_{00} = 10^{12}$, $N_{01} = 1000$ and $N_{11} = 1$, however, then the probability of success is 15%. Now assume all cells are

genetically unstable. Then the probability of success is 0% for all combinations of parameter values used above.

What is the critical population size that correlates with a substantial probability of success if two alterations are needed for escape from therapy? Assume that at the beginning of therapy, the cancer consists of N wild type cells, $TSG^{+/+}$. Then the maximum population size that can be contained by therapy is given by

$$N^* = \left[2u(u+p)\,\frac{R_{00}}{1-R_{00}}\left(1 + \frac{R_{01}}{1-R_{01}}\right)\frac{R_{11}-1}{R_{11}}\right]^{-1}$$

For example, if $R_{00} = R_{01} = 0.9$, $R_{11} = 1.1$, $u = 10^{-7}$, and $p = 10^{-6}$, then the critical population size is $N^* = 6 \cdot 10^{11}$. If the number of cancer cells in a patient is below this threshold, then the therapy will be successful; if it is above, failure is likely. If the cancer cells have chromosomal instability, however, then $p = 10^{-2}$ and the critical population size reduces to $N^* = 6 \cdot 10^7$. Thus the response to therapy crucially depends on whether or not a particular cancer has already evolved some form of genetic instability.

2.4 *n* Steps to Resistance

Now suppose that n genetic alterations in specific positions of the genome are necessary for resistance to cancer therapy. Without loss of generality, the genomes of the population can be described as binary strings of length n. There are $m = 2^n$ possible cell types, enumerated by $i = 0, ..., m$. We refer to the cell type unmutated in all positions, $0...0$, as 'wild type' cancer cells with index 0; cells mutated in all positions, $1...1$, are resistant mutants with index m. In the absence of therapy, cell types i have fitness values w_i with $0 \le w_i \le 1$. Hence, the distribution of cancer cells at the beginning of therapy is determined by their fitness values, w_i. Therapy reduces the proliferation capabilities of sensitive cell types such that their reproductive ratios, R_i, are less than one; resistant cell types, however, have reproductive ratios in excess of one.

First, assume that all cells except the wild type, 0, have the same fitness, $w < 1$, in the absence of treatment and assume that all cells except escape mutants, m, have the same basic reproductive ratio, $R < 1$, during treatment. The rate at which any position is mutated per cell division is given by u.

Then the probability of successful therapy [34, 35] is given by

$$P = \exp(-NC_n u^n z). \tag{10}$$

Here $z = 1 - 1/R_m$. If the n mutations can occur in any order, then the risk coefficient, C_n, is given by

$$C_n = \sum_{i=0}^{n} \binom{n}{i} f_{n-i}(a) f_i(b)$$

where $a = R/(1 - R)$ and $b = 1/(1 - w)$. The function f is recursively defined as

$$f_0(x) = 1$$

$$f_i(x) = x \sum_{j=0}^{i-1} \binom{i}{j} f_j(x),$$

This scenario accounts for all possible transitions from cell type 0 to cell type m, including single and multiple mutation steps. Multiple simultaneous mutations cannot be neglected in the calculation because intermediate mutants have frequencies of the order of the mutation rate [31]. If, however, the n mutations must occur in a particular order, then the risk coefficient is given by

$$C_n = [a^2(1 + a)^{n-1} - b^2(1 + b)^{n-1}]/[a - b].$$

The general mathematical framework can be applied to any mutation-selection network describing arbitrary mutation and fitness landscapes. We can include multiple resistant mutants and neutral networks. If all intermediate mutants are deleterious, then the probability of successful therapy [34, 35] is given by

$$P = \exp(-NC_n z \prod_{i=0}^{n} u_i).$$

Here position i is mutated with probability u_i per cell division. In a special case, all mutation rates are the same, $u_i = u$ for all i. Let us define the critical population size $N^* = 1/(C_n u^n z)$. If $N = N^*$, then the probability of success is $1/e$. If $N \gg N^*$, success is nearly impossible. If $N \ll N^*$, success is almost certain.

3. DISCUSSION

In this paper, we have presented how to calculate evolutionary dynamics of resistance to any treatment regimen. We have outlined how to determine the probability of successful therapy if one or two genetic or epigenetic alterations are needed to confer resistance. We have discussed different assumptions for the distribution of resident and resistant cancer cells at the start of therapy: initially, the cancer can entirely consist of sensitive cells; alternatively, partial or fully resistant mutants can already be present in the cancer when therapy is started. These cells can be in a mutation-selection balance with the resident cancer cells. We have discussed different mutation rates as well as different fitness landscapes and their effects on the risk of resistance mutations. We have analyzed the inactivation dynamics of tumor suppressor genes and have discussed the effect of haploinsufficiency on resistance to cancer therapy. Our theory can determine the probability of success for arbitrary networks of n mutations necessary for resistance.

We presented a rule of thumb for estimating the risk of resistance from the cancer size at diagnosis. If the number of viable, actively replicating cancer cells by far exceeds a critical threshold size, N^*, then the therapeutic outlook is dim. If the number of cancer cells is well below this threshold, however, then therapy is likely to succeed. The critical population size is approximately given by the inverse of the mutation rate - therefore, the critical size is much smaller for a cancer with genetic instability than for a cancer with normal mutation rates. The chance of successful therapy is thus much lower for cancers with genetic instability.

Our theory represents a simple approach to the complex phenomenon of cancer resistance. We consider resident and (intermediate as well as fully) resistant cancer cells but exclude the complicated interplay of cancer cells with endothelial cells, stromal cells, immune system cells and normal cells residing in the tissue. The analysis of the situations arising under these circumstances requires frequency-dependent fitness and cannot be approached using our framework. Our calculations are based on multi-type branching processes that describe the accumulation of mutations in independent lineages.

Human cancers are highly heterogeneous. Although some of the heterogeneity in cancers arises as a result of continuing mutagenesis, it can also be due to the aberrant differentiation of cancer cells. Many types of tumors contain cancer cells with heterogeneous phenotypes reflecting differentiation hierarchies that normally occur in healthy tissues. Thus, both normal stem cells and tumorigenic cells give rise to phenotypically heterogeneous cells that exhibit various degrees of differentiation. There is some evidence that only a subset of cancer cells, so called cancer stem cells, are capable of extensive proliferation [52]. For example, human AML stem cells could be identified and purified as $CD34^+$ $CD38^-$ cells from patient samples [53]. Despite the fact that these cells represented a small proportion of AML cells (0.2% in one patient), they were the only cells capable of transferring AML from human patients to NOD/SCID mice in the vast majority of cases. It has also been shown for solid tumors that the cells are phenotypically heterogeneous and that only a small proportion of cells are clonogenic *in vitro* and *in vivo* [54-56]. For example, only 1 in 1000 to 1 in 5000 lung cancer, ovarian cancer or neuroblastoma cells were found to form colonies in soft agar [57].

Conventional therapies may shrink cancers by killing mainly cells with limited proliferation potential. Cancer stem cells, however, might be less sensitive to therapies and may remain viable throughout treatment to re-establish the cancer. For example, Philadelphia chromosome-positive hematopoietic stem cells are insensitive to imatinib therapy [58]. Initially successful therapies are thus doomed. In contrast, if therapies can be targeted against cancer stem cells, then they might render the cancers unable to maintain themselves or expand. Thus, even if cancer stem cell-directed therapies do not deplete cancer cells initially, they might eventually lead to cures.

REFERENCES

References 59-61 are related articles recently published in Current Pharmaceutical Design.

[1]   Gottesman MM. Mechanisms of cancer drug resistance. Annu Rev Med 2002; 53: 615-627.

[2]   Gottesman MM, Fojo T, Bates SE. Multidrug resistance in cancer: role of atp-dependent transporters. Nat Rev Can 2002; 2: 48-58.

[3]   Liu YY, Han TY, Giuliano AE, Cabot MC. Ceramide glycosylation potentiates cellular multidrug resistance. FASEB J 2001; 15: 719-730.

[4]   Lowe SW, Ruley HE, Jacks T, Housman DE. p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. Cell 1993; 74: 957-967.

[5]   Synold TW, Dussault I, Forman BM. The orphan nuclear receptor sxr coordinatetely regulates drug metabolism and efflux. Nat Med 2001; 7: 584-590.

[6]   Shah MA. Cell cycle-mediated drug resistance: an emerging concept in cancer therapy. Clin Cancer Res 2001; 7: 2168-2181.

[7]   Henning W, Sturzbecher HW. Homologous recombination and cell cycle checkpoints: Rad51 in tumour progression and therapy resistance. Toxicology 2003; 193: 91-109.

[8]   Clynes M. Multiple drug resistance in cancer 2: molecular, cellular and clinical aspects. Boston: Kluwer Academic 1998.

[9]   Poruchynsky MS, Kim JH, Nogales E, Annable T, Loganzo F, Greenberger LM, *et al.* Tumor cells resistant to a microtubule-depolymerizing hemiasterlin analogue, hti-286, have mutations in alpha- or beta-tubulin and increased microtubule stability. Biochemistry 2004; 43: 13944-13954.

[10]   Schmitt CA, Rosenthal CT, Lowe SW. Genetic analysis of chemoresistance in primary murine lymphomas. Nat Med 2000; 6: 1029-1035.

[11]   Chiang CS, Sawyers CL, Mcbride WH. Oncogene expression and cellular radiation resistance: A modulatory role for c-myc. Mol Diagn 1998; 3: 21-27.

[12]   Gupta AK, Bakanauskas VJ, Cerniglia GJ, Cheng Y, Bernhard EJ, Muschel RJ, *et al.* The ras radiation resistance pathway. Cancer Res 2001; 61: 4278-4282.

[13]   Wilson LA, Yamamoto H, Singh G. Role of the transcription factor ets-1 in cisplatin resistance. Mol Cancer Ther 2004; 3: 823-832.

[14]   Jensen LH, Wessel I, Moller M, Nitiss JL, Sehested M, Jensen PB. N-terminal and core-domain random mutations in human topoisomerase II alpha conferring bisdioxopiperazine resistance. FEBS Lett 2000; 480(2-3): 201-207.

[15]   Townsend DM, Tew KD. The role of glutathione-s-transferase in anticancer drug resistance. Oncogene 2003; 22: 7369-7375.

[16]   Bentires-Alj M, Barbu V, Fillet M, Chariot A, Relic B, Jacobs N, *et al.* Nf-kappab transcription factor induces drug resistance through MDR1 expression in cancer cells. Oncogene 2003; 22: 90-97.

[17]   Dowsett M. Overexpression of her-2 as a resistance mechanism to hormonal therapy for breast cancer. Endocr Relat Cancer 2001; 8: 191-195.

[18]   Ling V. P-glycoprotein: its role in drug resistance. Am J Med 1995; 99: 31S-34S.

[19]   Lowe SW, Bodis S, McClatchey A, Remington L, Ruley HE, Fisher DE, *et al.* p53 status and the efficacy of cancer therapy *in vivo.* Science 1994; 266: 807-810.

[20]   Westphal CH, Rowan S, Schmaltz C, Elson A, Fisher DE, Leder P. ATM and p53 cooperate in apoptosis and suppression of tumorigenesis, but not in resistance to acute radiation toxicity. Nat Genet 1997; 16: 397-401.

[21]   Volm M, Stammler G. Retinoblastoma (rb) protein expression and resistance in squamous cell lung carcinomas. Anticancer Res 1996; 16: 891-894.

[22]   Diccianni MB, Chau LS, Batova A, Vu TQ, Yu AL. The p16 and p18 tumor suppressor genes in neuroblastoma: implications for drug resistance. Cancer Lett 1996; 104: 183-192.

[23]   Arnold CN, Goel A, Boland CR. Role of hmlh1 promoter hypermethylation in drug resistance to 5-fluorouracil in colorectal cancer cell lines. Int J Cancer 2003; 106: 66-73.

[24]   Lengauer C, Kinzler KW, Vogelstein B. Genetic instabilities in human cancer. Nature 1998; 396: 643-649.

[25]   Kinzler KW, Vogelstein B. Lessons from hereditary colon cancer. Cell 1996; 87: 159-170.

[26]   Perucho M. Cancer of the microsatellite phenotype. Biol Chem 1996; 377: 675-684.

[27]   Rajagopalan H, Nowak MA, Vogelstein B, Lengauer C. The significance of unstable chromosomes in colorectal cancer. Nat Rev Can 2003; 3: 675-701.

[28]   Nowak MA, Komarova NL, Sengupta A, Jallepalli PV, Shih IeM, Vogelstein B, *et al.* The role of chromosomal instability in tumor initiation. Proc Natl Acad Sci USA 2002; 99(25): 16226-16231.

[29]   Nowak MA, Michor F, Komarova NL, Iwasa Y. Evolutionary dynamics of tumor suppressor gene inactivation. Proc Natl Acad Sci USA 2004; 101: 10635-10638.

[30]   Michor F, Iwasa Y, Nowak MA. Dynamics of cancer progression. Nat Rev Can 2004; 4: 197-205.

[31]   Seneta E. Population growth and multi-type galton-watson processes. Nature 1970; 225: 776.

[32]   Athreya KB, Ney PE. Branching processes. New York: Springer 2004.

[33]   Anderson RM, May RM. Infectious diseases of humans. Oxford: Oxford University Press 1992.

[34]   Iwasa Y, Michor F, Nowak MA. Evolutionary dynamics of escape from biomedical intervention. Proc Roy Soc Lond B 2003; 270: 2573-2578.

[35]   Iwasa Y, Michor F, Nowak MA. Evolutionary dynamics of invasion and escape. J Theor Biol 2004; 226: 205-214.

[36]   Nowak MA, May RM. Virus Dynamics. Oxford: Oxford University Press 2000.

[37]   Bonhoeffer S, Nowak MA. Pre-existence and emergence of drug resistance in HIV-1 infection. Proc Ry Soc Lond B 1997; 264: 631-637.

[38]   Bonhoeffer S, May RM, Shaw GM, Nowak MA. Virus dynamics and drug therapy. Proc Natl Acad Sci USA 1997; 94: 6971-6976.

[39]   Ribeiro RM, Bonhoeffer S, Nowak MA. The frequency of resistant mutant virus before antiviral therapy. AIDS 1998; 12: 461-465.

[40]   Arnaout RA, Shaw GS, Borrow P, Nowak MA, Iwasa Y. Epitope targeting for optimal vaccines. Submitted 2005.

[41]   Balaban NQ, Merrin J, Chait R, Kowalik L, Leibler S. Bacterial persistence as a phenotypic switch. Science 2004; 305 1622-1625.

[42]   Sawyers CL. Chronic myeloid leukemia. N Engl J Med 1999; 340: 1330-1340.

[43]   Holyoake TL, Jiang X, Drummond MW, Eaves AC, Eaves CJ. Elucidating critical mechanisms of deregulated stem cell turnover in the chronic phase of chronic myeloid leukemia. Leukemia 2002; 16: 549-558.

[44]   Holyoake TL, Jiang X, Eaves C, Eaves A. Isolation of a highly quiescent subpopulation of primitive leukemic cells in chronic myeloid leukemia. Blood 1999; 94: 2056-2064.

[45]   Holyoake TL, Jiang X, Jorgensen HG, Graham S, Alcorn MJ, Laird C, *et al.* Primitive quiescent leukemic cells from patients with chronic myeloid leukemia spontaneously initiate factor-independent growth *in vitro* in association with up-regulation of expression of interleukin-3. Blood 2001; 97: 720-728.

[46]   Vogelstein B, Kinzler KW. The genetic basis of human cancer. New York: McGraw-Hill 2002.

[47]   Sandler HM, Curran WJ Jr, Turrisi AT 3rd. The influence of tumor size and pre-treatment staging on outcome following radiation therapy alone for stage I non-small cell lung cancer. Int J Radiat Oncol Biol Phys 1990; 19: 9-13.

[48]   Chagpar AB, Martin RC, Hagendoorn LJ, Chao C, McMasters KM. Lumpectomy margins are affected by tumor size and histologic subtype but not by biopsy technique. Am J Surg 2004; 188: 399-402.

[49]   Rodriguez-Galindo C, Billups CA, Kun LE, Rao BN, Pratt CB, Merchant TE, *et al.* Survival after recurrence of Ewing tumor. Cancer 2002; 94: 561-569.

[50]   Kunkel TA, Bebenek K. DNA replication fidelity. Annu Rev Biochem 2000; 69: 497-529.

[51]   Lengauer C, Kinzler KW, Vogelstein B. Genetic instability in colorectal cancer. Nature 1997; 386: 623-627.

[52]   Reya T, Morrison SJ, Clarke MC, Weissman IL. Stem cells cancer and cancer stem cells. Nature 2001; 414: 105-111.

[53]   Bonnet D, Dick JE. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. Nat Med 1997; 3: 730-737.

[54]   Fidler IJ, Kripke ML. Metastasis results from preexisting variant cells within a malignant tumor. Science 1977; 197: 893-895.

[55]   Fidler IJ, Hart IR. Biological diversity in metastatic neoplasms: origins and implications. Science 1982; 217: 998-1003.

[56]   Southam CM, Brunschwig A. Quantitative studies of autotransplantation in human cancers. Cancer 1961; 14: 971-978.

[57]   Hamburger AW, Salmon SE. Primary bioassay of human tumor stem cells. Science 1977; 197: 461-463.

[58]   Michor F, Iwasa Y, Hughes TP, Branford S, Shah NP, Sawyers CL, *et al.* Dynamics of chronic myeloid leukemia. Nature 2005; 435: 1267-1270.

[59]   Berenguer B, La Casa C, de la Matta MJ, Martin-Calero MJ. Pharmaceutical care: past, present and future. Curr Pharm Design 2004; 10(31): 3931-46.

[60]   Bergmann-Leitner ES, Duncan EH, Leitner WW. Identification and targeting of tumor escape mechanisms: a new hope for cancer therapy? Curr Pharm Design 2003; 9(25): 2009-23.

[61]   Tortora G, Melisi D, Ciardiello F. Angiogenesis: a target for cancer therapy. Curr Pharm Design 2004; 10(1): 11-26.