



Cancer: A Systems Biology disease

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Abstract

Cancer research has focused on the identification of molecular differences between cancerous and healthy cells. The emerging picture is overwhelmingly complex. Molecules out of many parallel signal transduction pathways are involved. Their activities appear to be controlled by multiple factors. The action of regulatory circuits, cross-talk between pathways and the non-linear reaction kinetics of biochemical processes complicate the understanding and prediction of the outcome of intracellular signaling. In addition, interactions between tumor and other cell types give rise to a complex supra-cellular communication network. If cancer is such a complex system, how can one ever predict the effect of a mutation in a particular gene on a functionality of the entire system? And, how should one go about identifying drug targets?

Here, we argue that one aspect is to recognize, where the essence resides, i.e. recognize cancer as a Systems Biology disease. Then, more cancer biologists could become systems biologists aiming to provide answers to some of the above systemic questions. To this aim, they should integrate the available knowledge stemming from quantitative experimental results through mathematical models. Models that have contributed to the understanding of complex biological systems are discussed. We show that the architecture of a signaling network is important for determining the site at which an oncologist should intervene. Finally, we discuss the possibility of applying network-based drug design to cancer treatment and how rationalized therapies, such as the application of kinase inhibitors, may benefit from Systems Biology.

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1. Introduction

During the micro-evolutionary process of malignant transformation, cancer cells accumulate multiple genetic alterations that provide them with several capabilities (Cahill et al., 1999). The latter include the escape from normal growth control, evasion of the suicidal apoptotic program, induction of sustained angiogenesis, the

ability to metastasise, and to invade healthy tissues (Hanahan and Weinberg, 2000). In the past decades, cancer researchers have collected an enormous amount of information about the differences between cancer cells and their healthy counterparts, with the ultimate goal of identifying drug targets. This has, for instance, led to the identification of genes that are causally implicated in human cancer and to the discovery of the mutations in those genes. A cancer gene census was recently compiled (Futreal et al., 2004), which currently contains 300 genes. The vast majority of these genes function in signal transduction processes within or between cells, govern

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cell cycle progression, apoptosis, angiogenesis, and infiltration (Vogelstein and Kinzler, 2004).

Although knowledge of the molecular cell biology of cancer is enormous, at the same time, the emerging complexity of the entire ‘cancer system’ overwhelms us, leaving an enormous gap in our understanding and predictive power. In this paper, we will discuss aspects of this complexity, and how one can deal with it to answer questions that are relevant for the treatment of cancer.

2. **Zooming in: complex signaling networks**

Many signaling molecules (proteins, lipids, and ions) have been identified, and for many, the way they communicate with each other through signal transduction pathways has been elucidated. Signaling pathways consist of multiple sequential events, including covalent modifications (e.g. phosphorylation), recruitment, allosteric activation or inhibition, and binding of proteins (Alberts et al., 2002). The kinetics of these reactions are often non-linear, as a result of the properties of the enzymes carrying out these reactions (e.g. kinases or phosphatases), causing the output of a signaling pathway to depend non-linearly on the input (Ferrell, 1996; Huang and Ferrell, 1996; Kholodenko et al., 1999).

As more interactions between signaling pathways were identified, it became apparent that signaling does not necessarily occur in an independent fashion through parallel linear pathways, but rather, through a large and complex network of interacting signaling pathways (Weng et al., 1999). These interactions between pathways occur at many hierarchical levels (Kolch, 2000). Signaling proteins from different pathways may interact directly (e.g. by phosphorylation) or influence each other indirectly (e.g. via regulation of gene expression). One component may also act in more than one pathway (Campbell et al., 1998). Such ‘cross-talk’ events can result in unexpected behavior (Bhalla and Iyengar, 1999; Hornberg et al., 2004).

A recurrent theme in the regulation of biological systems is the topology of the regulatory networks (Milo et al., 2002). Feedback loops, in which a component of a pathway positively or negatively regulates the activity of upstream components of the pathway, can dramatically influence the output of a network (Bhalla and Iyengar, 1999; Ferrell, 1999, 2002; Kholodenko, 2000; Asthagiri and Lauffenburger, 2001). The identification of the structure and the intrinsic properties of frequently occurring regulatory motifs (sometimes referred to as ‘network motifs’) is a challenge and may give us a functional view on the organization of signaling networks rather than a molecular view (Yeger-Lotem et al., 2004). The complex

architecture of signaling networks can then be thought to consist of interacting network motifs, which endow the network as a whole with specific properties, much like electronic circuits built of flip-flops, capacitors, etc.

Additional complexity arises from the spatiotemporal organization of signaling pathways, such as the fact that many signals travel between different cellular compartments, such as the cytoplasm and the nucleus (Pouyssegur and Lenormand, 2003; Swameye et al., 2003). This type of complexity may give rise to waves of covalently modified signaling proteins as has already been observed for calcium signaling (Brown and Kholodenko, 1999; Kholodenko, 2000; Peletier et al., 2003).

In short, many cancer genes are known, as is the composition of many of the pathways in which their gene products function and some of the ways the pathways interact (Hanahan and Weinberg, 2000). This enables the drawing of large ‘road maps’ of information that are often different in cancer cells (Fig. 1). The complexity resulting from the large number of interacting molecules, cross-talk between pathways, feedback circuitry, the non-linear relations between the interacting molecules and the spatiotemporal resolution of signaling make it difficult, if not impossible to predict the altered outcome of signaling on the basis of the changes in such an interaction map alone.

3. **Zooming out: complexity increases**

The architecture of signal transduction pathways is not where the complexity of cancer ends. Being parts of the cell, signaling networks are affected by additional levels of organization, for instance, as many proteins are not uniformly distributed over the cell. Areas with high protein concentrations might lead to macromolecular crowding (Ellis and Minton, 2003) and cause steep spatial gradients of activated signaling proteins (Brown and Kholodenko, 1999).

Numerous interactions at the supra-cellular level make the cancer system even more complex (Fig. 2A). Within a tumor, the cancer cells interact with each other, both by direct cell–cell contact and by indirect communication (e.g. binding of growth factors produced by other cancer cells). Different cancer cells may also respond in different ways to the same signal, as a result of heterogeneity (cells slightly differing from each other in terms of size, shape, the concentration of macromolecules and metabolites they comprise, and the stage of the cell cycle they are in).

Further ‘zooming out’ reveals interactions between cancer cells and other cell types (Fig. 2B). A well-studied

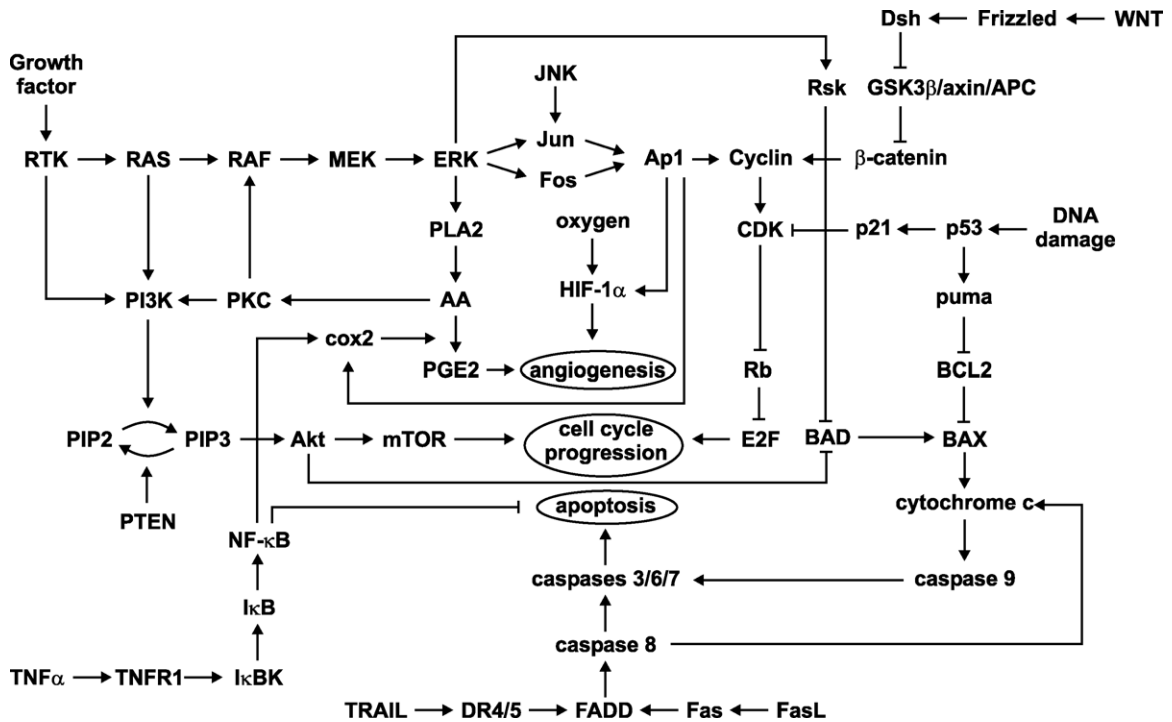


Fig. 1. Road map of cellular signaling. Depicted are the backbones of key signal transduction pathways that are involved in cell cycle progression, apoptosis, and angiogenesis. Mutations that cause cancer frequently occur directly in components of these pathways or in genes that indirectly affect the functioning of these pathways. The interconnections between the pathways give rise to large complex signaling networks. The main sources that were used for the construction of this figure are Hanahan and Weinberg (2000) and Vogelstein and Kinzler (2004).

example is the formation of new blood vessels, i.e. angiogenesis (Rak et al., 1995; Folkman, 2002; Ferrara, 2004), which is essential for a tumor to grow beyond a certain size. Between microscopic and macroscopic tumors, the micro-vessel density within, may differ by a factor of 17 (Klauber-DeMore et al., 2001). It has been proposed that a shift in the balance between pro- and anti-angiogenic factors underlies an ‘angiogenic switch’ that facilitates the outgrowth of small dormant tumors into macroscopic tumors (Hanahan and Folkman, 1996). Cancer cells may excrete growth factors, such as vascular endothelial growth factor (VEGF) or basic fibroblast growth factor (bFGF), and thereby, stimulate cell cycle progression in endothelial cells, which constitute blood vessel capillaries. Vice versa, newly formed blood vessels provide the cancer cells with more oxygen and nutrients. This process thus functions as a self-stimulatory circuit. Since positive feedbacks can cause switch-like responses (Ferrell, 2002), the interactions between tumor cells and endothelial cells may be the cause of such an angiogenic switch.

Apart from endothelial cells, there are multiple interactions with other cell types. Fibroblasts have been shown to be important for the neoplastic growth of breast

epithelial cells (Shekhar et al., 2001) and in turn, respond to several growth factors that may be excreted by tumor cells, such as epidermal growth factor (EGF). Also interactions between tumor cells and immune-derived cells can play important roles. For instance, macrophages that infiltrate tumor tissue are driven by tumor cell-derived cytokines to acquire a so-called polarized M2 phenotype and promote tumor growth and progression (Mantovani et al., 2002).

4. Cancer: a Systems Biology disease

Above, we have discussed that cancer involves a number of molecular processes at the same time. Looking back, it is clear that the interactions of these processes lead to new functionalities that would not otherwise be possible. An example is the formation of a life-threatening tumor requiring the formation of new blood vessels, which requires the cancer cells to become insensitive to growth inhibition and the endothelial cells to become activated to new blood vessel formation. Indeed, cancer is a disease based on malfunctioning of the system properties of parts of biology. We hence identify it as a Systems Biology disease. Indeed, progress

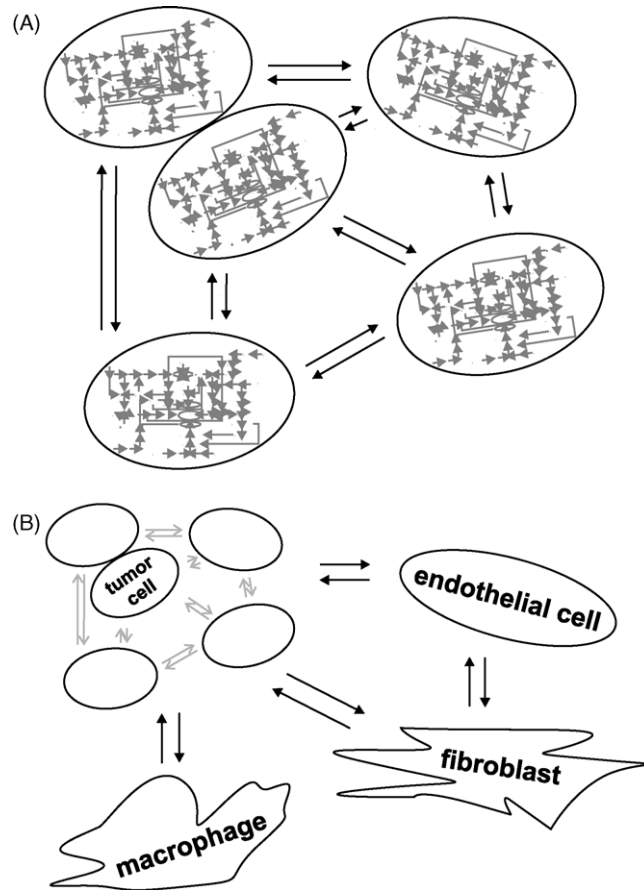


Fig. 2. Complexity at the supra-cellular level. Within a tumor, interactions take place at many levels. In addition to the large intracellular signaling networks, several supra-cellular interactions contribute to an even more overwhelming, complex system: (A) the tumor cells interact by direct physical contact or by communication via secreted signaling molecules and (B) the tumor cells also communicate with other cell types, such as endothelial cells (to provoke angiogenesis), fibroblasts (to ensure stability), and immune-derived cells (to escape immune responses).

in cancer research towards cancer therapy may develop faster if cancer is not researched only in terms of Molecular Biology but rather in terms of Systems Biology.

5. Towards integration

It appears that the many supra-cellular interactions, which add to the intra-cellular signaling, are all at least to some extent relevant for the development of cancer. As more relevant genes, proteins and interactions are being identified, the total picture will continue to grow even more complex. If we also take into account the non-linearity of the biochemical processes and the fact that different processes act on different time scales (signaling, cell cycle, and angiogenic switch), then it seems impossible that the unaided human brain will ever conceive complete understanding of such a dynamic and complex multi-cellular system just on the basis of extrapolating from the molecular-biological data alone.

Can we still expect to understand the effect of a specific mutation on the whole cancer system? And can we even begin to predict where in this complex system we should intervene to kill it? Perhaps, the only way to reach understanding at some point, is to integrate all available molecular-biological and cell-biological knowledge in such a way that our minds can still deal with the complexity. We propose that one way to solve this is by the Systems Biology approach that builds a computer replica of cancer based on quantitative experimental results. In this way, accurate experimentation and detailed modeling of network behavior in terms of molecular properties reinforce each other. Ultimately, answers to questions that remain difficult to deal with at present, by looking at interaction maps of signaling networks, could then be predicted by mathematical modeling and subsequently, those predictions should be validated by quantitative experimentation. A replica of the entire cancer system may be a little far reached at present, although we argue

that this must be the ultimate goal. In the mean time, reliable replicas of sub-systems may be closer milestones that aid in answering questions from which the understanding and treatment of cancer can benefit directly (see below).

This approach has already proven to aid in the understanding of complex cellular systems. Several detailed kinetic models ('computer replica') of metabolic systems, such as glycolysis (Schuster and Holzhütter, 1995; Teusink et al., 2000), provide rather accurate descriptions of their *in vivo* counterparts. The erythrocyte model has been important for understanding the regulatory role of 2,3-bisphosphoglycerate, as well as on how to store red blood cells. The yeast glycolysis model has led to a verification requirement that had been overlooked in metabolic biochemistry, i.e. that of testing whether the experimentally determined kinetics of the individual enzymes of a pathway could make a pathway function. When this was done, a metabolic explosion occurred. The explosion could be prevented by adding to the computer replica a regulatory loop outside the glycolytic pathway, the molecular details of which were known, but the function of which had been sought in quite different realms of yeast function. Also, for a gene expression system, a computer replica has led to the demonstration of the functionalities of molecular processes, i.e. the storage of mRNA and of histones, important in the race against time between histone-gene expression and DNA replication in early development (Koster et al., 1988). In biotechnology, a silicon cell has led to the engineering of a *Lactococcus lactis* strain with improved production capabilities (Hoefnagel et al., 2002).

The more limited, available kinetic knowledge of several mammalian signal transduction pathways has also been cast into detailed models (Bhalla and Iyengar, 1999; Kholodenko et al., 1999; Asthagiri and Lauffenburger, 2001; Schoeberl et al., 2002; Lee et al., 2003; Swameye et al., 2003; Bentele et al., 2004; Nelson et al., 2004; Stucki and Simon, 2005). These models have contributed to the understanding of the particular signaling pathways and they facilitate *in silico* experiments. With such models at hand, one could, for instance, determine what the effect of a particular mutation in (or overexpression of) a certain component would be on the functioning of the network. With the aim of integrating these computer replicas, peer-reviewed models are compiled and made web-accessible in so-called 'silicon cells' (Snoep and Westerhoff, 2004). Through this facility, anyone can experiment *in silico* with a number of pathways. The same facility can be used to assess the importance of molecular properties for the network, hence, to the determination of experimental priorities.

Besides, the detailed kinetic models of signal transduction pathways and networks, our understanding has also improved from the analysis of more general so-called 'core models', that describe the system of interest not in full detail, but in a simplified manner. These models have led to the discovery of ultra-sensitivity in signaling pathways (Goldbeter and Koshland, 1982; Huang and Ferrell, 1996) and to suggestions on the possibility of spatially resolved signaling (Brown and Kholodenko, 1999) and on possible functions of differential feedback regulation (Brightman and Fell, 2000). In addition, general principles on the control of signal transduction have been identified, such as how the control of the dynamic activity of signal transduction pathways is distributed over kinases and phosphatases (Kahn and Westerhoff, 1991; Heinrich et al., 2002; Hornberg et al., 2005a). Part of this has been substantiated experimentally (Hornberg et al., 2005a).

For those who dislike mathematics and theoretical biology, we highlight that mathematical modeling is not part of the definition of Systems Biology. The modeling is a mere tool for helping the human mind to appreciate how systems work and how malfunctioning systems may be cured. For sure, there are other ways of managing system understanding; extensive experience, such as present in the brain of general practitioners is extremely valuable in this respect. However, we would expect that more of the advances of functional genomics may be brought to fruition when mathematical models are used to amplify the potency of human insight and experience.

For integration of the total system, models of supra-cellular interactions will also be needed. Although many of those interactions have only been described qualitatively, such models have been reported, for instance, with respect to the functioning of the heart (Noble, 2004). Several angiogenesis models describe, for instance, the formation of (sometimes even three-dimensional) capillary networks (Chaplain and Anderson, 2004).

The combination of quantitative experimentation and mathematical modeling has already increased our understanding of a few complex biological systems. Many others remain to be analyzed, and the ultimate challenge in this field is to integrate all processes that are relevant for understanding the system cancer and to use the acquired understanding for drug development (Gatenby and Maini, 2003; Kitano, 2003; Butcher et al., 2004; Christopher et al., 2004; Khalil and Hill, 2005).

6. Network-based drug design

The inhibition of enzymes that contribute to aberrant signaling and cancer progression holds promise for the

treatment of cancer. Here, one should perhaps also think about a substantial reduction in the rate of tumor progression and metastasis instead of only about a successful total elimination of tumors. Such new therapies could benefit directly from analyzing networks and whole systems, rather than all their individual parts of systems. Which clinically relevant questions may be answered by a Systems Biology approach?

The occurrence of regulatory circuitry, such as feedback loops, for instance, may have great impact in the efficacy of an inhibitor of a certain signaling cascade. Let us consider a simple pathway of which the activity is crucial for the proliferation rate of a particular cancer cell (Fig. 3 and Appendix A). An oncologist might aim to treat a patient by applying a drug that affects one of the processes in which this protein engages. With increasing drug concentration, the activity of the pathway decreases. However, if in this pathway, a negative feedback loop inactivates an upstream component, and overlaps the site of drug action, the same drug concen-

tration becomes much less effective especially when the oncologist wishes to inhibit the pathway by at least 80% (Fig. 3A). In other words, if a negative feedback loop is active, the drug concentration that will be needed to inhibit the pathway to a certain extent increases. Interestingly, if another drug is applied, which inhibits a different protein outside of the feedback loop (in this case, functioning upstream of the action of the negative feedback loop), the presence of the feedback loop does not affect the extent to which the drug inhibits the activity of the pathway by nearly as much (Fig. 3B). In a simplistic way, this example shows that the structure of the network is important for the position in the pathway at which an oncologist should intervene. Therefore, it is essential to include possible regulatory circuits (and their strengths and connectivity) when studying a signaling pathway.

Once a system has been characterized quantitatively, then how should one identify the best drug target(s)? A promising approach should be to determine which components and processes in the system are actually controlling its behavior, for instance, by applying metabolic or hierarchical control analysis (MCA and HCA, respectively). These are conceptual and mathematical tools to determine how much an enzyme controls a particular steady-state value (e.g. metabolite concentration, flux) (Kacser and Burns, 1973; Heinrich and Rapoport, 1974; Westerhoff and Van Dam, 1987; Heinrich and Schuster, 2001; Fell, 1997). MCA has mainly focused on steady states in metabolic systems, although it has also been applied to determine control on dynamic properties of metabolic systems (Acerenza et al., 1989; Kholodenko et al., 1997; Reijenga et al., 2002; Ingalls and Sauro, 2003). HCA has done the same with respect to steady-state concentrations of signaling molecules (Kahn and Westerhoff, 1991; Lee et al., 2003). Recently, we extended HCA to the time-dependent domain, in order to analyze the dynamic activity of signal transduction cascades, in terms of signaling amplitude, duration of signaling and the integrated response (Hornberg et al., 2005a). We found that the control on the activity of a signaling cascade is generally distributed over many proteins and processes rather than mediated by any one protein or any one process in particular. In fact, all reactions that activate a pathway (often kinases) were shown to be precisely as important for the amplitude of signaling as all inactivating reactions (often phosphatases). The duration and integrated response were found to be controlled more by phosphatases than by kinases.

Applying HCA to the dynamic properties that result from the interactions in signal transduction pathways, regulatory circuits, networks, between cells, and different cell types, will provide a quantitative answer to

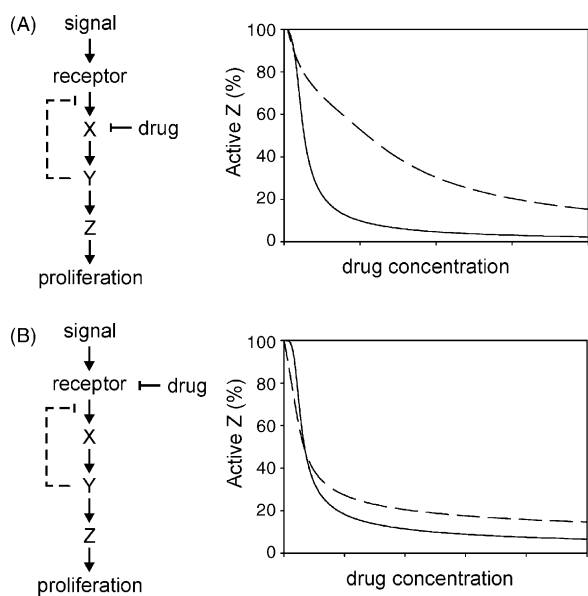


Fig. 3. Feedback circuitry may influence drug efficacy. A simple pathway is considered, which consists of a receptor and of proteins X, Y and Z, is activated by a signal, and leads to cell proliferation. A negative feedback loop is constituted by protein Y inhibiting the activation of X by the receptor. A drug inhibits the action of a pathway component as indicated. The steady-state activity of protein Z (in the presence of signal) is plotted versus the drug concentration. The solid lines represent the case where the feedback loop is inactive, the dashed lines where it is active. If a negative feedback loop is active, then inhibition within that loop (A) can be much less effective than inhibition upstream of the loop (B). See Appendix A for details on the simple core model that was used.

issues regarding how individual processes control such dynamic properties. We have recently used this approach to calculate to which extent individual reactions in the complex MAP kinase signaling network control the dynamic time profile of ERK phosphorylation (Hornberg et al., 2005b). Another question could be for example, how much control does the mutated protein X have on cell proliferation? Or, how much do individual signaling pathways contribute to the secretion of VEGF by tumor cells? How important is VEGF secretion by tumor cells for the growth of endothelial cells, and indirectly, for their own growth? Answering such questions is of vital importance not only for our understanding of these complex cellular systems, but also for drug design, since it allows for the identification of drug targets according to the magnitude of their control on cell pathology (Cascante et al., 2002). Such a strategy has, for instance, been applied to the search for drug targets against parasites (Bakker et al., 2000).

7. Kinase inhibitors: smart drugs?

Conventional cancer treatment relies on surgery, radiotherapy, and chemotherapy (DeVita et al., 2001). The development of rationalized cancer therapy, based on the knowledge of the biology of cancer, may enhance the arsenal of oncologists. Inhibition of constitutive pathways, e.g. by monoclonal antibodies or tyrosine kinase inhibitors, is a strategy that is already employed in clinical trials (Mendelsohn and Baselga, 2000; Sebolt-Leopold, 2000; Shawver et al., 2002). The success of drugs in general depends mainly on three things: (i) pharmacokinetics and drug transport into the tissue (how well can the drug reach its target?); (ii) selectivity (does the drug cause toxic side effects?); (iii) efficacy (are the cancer cells affected sufficiently by the attack on the drug target?). For some chemotherapeutics, such as doxorubicin, it was found that not all tumor cells may be reached efficiently, due to difficulties with drug diffusion through tissues (Lankelma et al., 1999; Lankelma, 2002). Such problems will also arise for rationalized therapies, but may be alleviated by mathematical modeling and rationalized dosing. Recently, we integrated the available experimental data regarding the transport of doxorubicin into a computer replica that enabled simulation of the effect towards tumor cells of a transient drug concentration (Lankelma et al., 2003).

The fact that kinase inhibitors are designed against targets that are mutated or overactive in cancer cells provides some intuitive confidence that they will be working more selectively against cancer cells than conventional chemotherapeutics (Sebolt-Leopold, 2000). On the other

hand, one could argue that healthy cells also depend on the activity of the pathways that are inhibited by those drugs, and that they will also suffer from targeting those pathways.

Related to this issue is the question whether inhibiting deregulated pathways will indeed affect the cancer cell such that the disease goes into remission. Mutated enzymes and overactive (or impaired) signaling pathways are responsible for the malignant phenotype, which makes them obvious targets for cancer therapy. This does, however, not necessarily mean that targeting these same enzymes or pathways will have the reverse effect on the tumor cells. It may be that, e.g. 80% inhibition of the activity of a mutated kinase is not enough to block signaling through the pathway, whereas 80% inhibition of the normal counterpart of that kinase is enough to do so. Then, maybe, the mutated protein is not the best target. Indeed, when a kinase has been mutated to become overactive, it will usually exert less control on signal transduction and is thereby a bad target. In this example, it could be better to inhibit another protein in the cascade.

To resolve such issues, it is necessary to determine how important different enzymes are for signaling through a pathway, and how important different pathways are for, e.g. cell survival and growth. Comparing such quantifications for normal cells and for cancer cells would reveal which enzymes or pathways make the most effective targets for cancer treatment.

Appendix A

The data used for Fig. 3 were derived with a simple core model of a linear signaling pathway. Signaling is initiated by an active receptor (Ra) that is inactivated (to become R) and can be reactivated over time. Ra causes activation of a cascade of three kinase/phosphatase monocycles. First Ra causes phosphorylation of X (to become Xp). Xp then activates phosphorylation of Y (to Yp), which, in turn, leads to phosphorylation of Z (to Zp). A negative feedback loop was modeled by Yp activating the formation of inactive X (Xi). As Xi does not participate in phosphorylation of Y, Yp, thus, negatively influences its own production.

The model contains the following irreversible reactions:



The reaction velocities are given by Michaelis Menten kinetics: $v = \frac{V_{\max} \times S}{K_m + S}$

Explicitly, the equations used for each reaction are:

$$v1 = \frac{Ra}{0.1 + Ra}, \quad v2 = \frac{0.3 \times R}{1 + R},$$

$$v3 = \frac{Ra \times X}{0.1 + X}, \quad v4 = \frac{0.3 \times Xp}{1 + Xp}$$

$$v5 = \frac{Xp \times Y}{0.1 + Y}, \quad v6 = \frac{0.3 \times Yp}{1 + Yp},$$

$$v7 = \frac{Yp \times Z}{0.1 + Z}, \quad v8 = \frac{0.3 \times Zp}{1 + Zp}$$

$$v9 = \frac{Yp \times X}{0.1 + X}, \quad v10 = \frac{0.3 \times Xi}{1 + Xi}$$

The initial concentrations (in mM) at $t = 0$ are:

$Ra = 1$	$Xp = 0$	$Z = 1$
$R = 0$	$Y = 1$	$Zp = 0$
$X = 1$	$Yp = 0$	$Xi = 0$

*Reactions $v9$ and $v10$ were added to model the case with an active feedback loop (dashed line in Fig. 3) and omitted to model the case without a feedback loop.

The model was analyzed using Gepasi software (Mendes, 1997; Mendes and Kell, 1998). The effect of a drug on the steady-state Zp concentration was modeled by using the Scan option in Gepasi; the V_{max} of the affected reaction ($v5$ in Fig. 3A and $v3$ in Fig. 3B) was varied between 0.01 and 2 (density: 1000). The steady-state Zp concentration in the non-inhibited case ($V_{max} = 2$) was scaled to 100% (in order to compare the case, where the feedback loop was active with the case where it was not active).

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