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Metabolic control analysis indicates a change of strategy in the treatment of cancer

Rafael Moreno-Sánchez [a,*], Emma Saavedra [a], Sara Rodríguez-Enríquez [a], Juan Carlos Gallardo-Pérez [a], Héctor Quezada [a], Hans V. Westerhoff [b,c]

[a] Departamento de Bioquímica, Instituto Nacional de Cardiología de México, Mexico
[b] VU University Amsterdam, The Netherlands
[c] The Manchester Centre for Integrative Systems Biology, The University of Manchester, UK

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ABSTRACT

Much of the search for the "magic cancer bullet" or "block buster" has followed the expectation of a single gene or protein as "the rate-limiting step" for tumor persistence. Examples continue to abound: EGFR, VEGFR, Akt/PI3K, HIF-1α, PHD, PDK, or FAS continue to be targeted individually. However, many such attempts to block a metabolic or signal transduction pathway by targeting, specifically, a single rate-limiting molecule have proven to be unsuccessful. Metabolic control analysis (MCA) of cancer cells has generated a generic explanation for this phenomenon: several steps share the control of energy metabolism (for glycolysis: glucose transporter, hexokinase, glycogen synthesis and ATP demand; for oxidative phosphorylation: respiratory complex I and ATP demand), *i.e.*, there is no single "rate-limiting step". Targeting a type of step that does not exist is unlikely to be a successful paradigm for continued research into drug targeting of cancer.

MCA establishes how to determine, quantitatively, the degrees of control that the various enzymes in the intracellular network exert on vital flux (or function) and on the concentration of important metabolites, substituting for the intuitive, qualitative and most often erroneous concept of single rate-limiting step. Moreover, MCA helps to understand (i) the underlying mechanisms by which a given enzyme exerts high or low control, (ii) why the control of the pathway is shared by several pathway enzymes and transporters and (iii) what are the better sets of drug targets. Indeed, by applying MCA it should now be possible to identify the group of proteins (and genes) that should be modified to achieve a successful modulation of the intracellular networks of biotechnological or clinical relevance. The challenge is to move away from the design of drugs that specifically inhibit a single controlling step, towards unspecific drugs or towards drug mixtures, which may have multiple target sites in the most exacerbated, unique and controlling pathways in cancer cells. Successful nonspecific drugs should still be specific for the networks of cancer cells over those of normal cells and to establish such cell-type specificity within molecular non-specificity will continue to require sophisticated analyses. Clinical practice has anticipated the latter strategy of mixtures of drugs: combinations of anti-neoplastic drugs are already administered with encouraging results. Therefore, the most promising strategy for cancer treatment seems to be that of a multi-targeted, MCA-advised, therapy.

Abbreviations: ALDO, aldolase; Bcl-2, B-cell lymphoma-2; 3-BrPyr, 3-bromopyruvate; 2DOG, 2-deoxyglucose; EGFR, endothelial growth factor receptor; ENO, enolase; FAS, fatty acid synthase; γ-ECS, gamma-glutamyl cysteine synthetase; GDH, glutamate dehydrogenase; GLUT, glucose transporter; G6P, glucose 6-phosphate; GSH, glutathione; HIF-1, hypoxia-inducible transcriptional factor 1; HK, hexokinase; HPI, hexose phosphate isomerase; LDH, lactate dehydrogenase; MCT, monocarboxylate transporter; mTOR, mammalian target of rapamycin; OxPhos, oxidative phosphorylation; PDH, pyruvate dehydrogenase complex; PDK, pyruvate dehydrogenase kinase; PFK-1, phosphofructokinase 1; PFK-2, phosphofructokinase 2; PGAM, 3-phosphoglycerate mutase; PHD, prolyl hydroxylases; PK, protein kinases; PYK, pyruvate kinase; SDH, succinate dehydrogenase; TPI, triose phosphate isomerase; Tre6P, trehalose 6-phosphate; TRIAL, TNF-related apoptosis inducing ligand; VEGFR, vascular endothelial growth factor receptor; XIAP, X-linked inhibitor of apoptosis protein.

* Corresponding author. Instituto Nacional de Cardiología, Departamento de Bioquímica, Juan Badiano No. 1, Sección XVI, Tlalpan, México D.F. 14080, Mexico. Tel.: +52 55 5573 2911x1298, 1422.
E-mail addresses: rafael.moreno@cardiologia.org.mx, morenosanchez@hotmail.com (R. Moreno-Sánchez).

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1. Introduction

1.1. The concept of the rate-limiting step

Hans Krebs proposed that, in order to begin understanding how a pathway is regulated its "pacemaker" enzyme or "rate-limiting step" has to be identified (Krebs, 1970). Since then various criteria have been implemented in attempts to identify the rate-limiting step, which were indirect and qualitative. These included the assumption that the rate-limiting step had to be the first step in the pathway, the slowest step in the pathway, the irreversible step in the pathway, the step that was most displaced from equilibrium, or the most regulated step in the pathway (Rolleston, 1972; Newsholme and Start, 1973). Once a site in a metabolic pathway had thus been identified as "the rate-limiting step", researchers have frequently concluded that such enzyme or transporter is the only step limiting the metabolic flux through that pathway and extended this conclusion to the same pathway in all cell types and under all conditions. Herman (1980) proposed that the "basic principle of metabolic control was the regulation of at least one rate-limiting step in a given metabolic pathway". This concept of the identifiable single rate-limiting step to which regulation is confined, is not limited to the world of metabolic pathways. The concept also pervades thinking about gene expression regulation, signal transduction and oncogenic transformation.

It is worth noting that the experimental approaches usually yielded more than a single proposed rate-limiting step: glucose transporter (GLUT), hexokinase (HK), phosphofructokinase 1 (PFK-1) or pyruvate kinase (PYK) for glycolysis; isocitrate dehydrogenase or citrate synthase for the Krebs cycle; cytochrome c oxidase (COX), the ATP/ADP translocator or the Krebs cycle Ca^{2+}-sensitive dehydrogenases for oxidative phosphorylation (OxPhos) (Rolleston, 1972; Newsholme and Start, 1973; Moreno-Sánchez and Torres-Márquez, 1991; Moreno-Sánchez et al., 2008). These steps were then seen as alternatives and extensive discussions on which proposed step was the actual rate-limiting step ensued. Indeed, there was no way in which to accommodate that two or more steps in a pathway could be rate-limiting at the same time. Neither as there was a way to understand that the most limiting step of the pathway was not necessarily the step that was used by the organism to regulate pathway flux. And, there was no way to acknowledge the possibility that the flux through a pathway was actually controlled by transcription of a gene rather than by the activity of one of the metabolic enzymes.

Because the concept of the rate-limiting step assumes that there is only one single enzyme controlling the metabolic pathway flux (or the concentration of the final product), it should also be noted that in this approach, full control of the pathway flux or cellular process is assigned to the "key" step and, in consequence, assigns values of zero to the control exerted by the other enzymes and transporters. We shall now confirm that all these concepts were intuitive at best and misleading for sure (Morandini, 2009).

2. Metabolic control analysis (MCA)

2.1. Exit the rate-limiting step concept, enter the control coefficient concept

The work of three research groups has ruled out the usefulness of the concept of the single rate-limiting step, even though this has not always reached researchers that begin to consider aspects of regulation and control of cell function: there has been a dearth of informative publications on the subject, in the literature that is standard to cell biologists. First, Kacser and Burns (1973) and Heinrich and Rapoport (1973) felt the need not to have to assume that a step was either fully rate-limiting or not at all rate-limiting. They introduced a more subtle idea to capture the concept of rate-limitation, *i.e.* the concept of flux control coefficient (Burns et al., 1985). Although this new concept has been defined in more precise but also more mathematical terms (Kholodenko et al., 1995), it here suffices to use the following definition: the extent to which an enzyme in a pathway controls the flux corresponds to the percentage decrease in flux caused by a 1% decrease in the activity of that enzyme (Bruggeman et al., 2009).

This definition had the advantage that it allowed for the possibility that one pathway step would be the rate-limiting step, but also for the possibility that a pathway had no, or multiple such steps. In the former case there would be one enzyme the inhibition of which by 1% would equally decrease the flux, whilst the inhibition of no other enzyme would affect the flux. In the latter case there could be more than one enzyme in the pathway for which the inhibition would affect the flux. Herewith the issue of whether or not a pathway had a single rate-limiting step became an issue to be decided by experimentation. Of course, this left open the possibility that for some reason of evolutionary optimization in all metabolic pathways there is always a single rate-limiting step.

Recognizing the operational nature of the above definition of flux control coefficient, Groen and coworkers (1982) provided the *coup de grâce* to the concept of "the rate-limiting step". Considering the case of mitochondrial OxPhos (see above) they used specific inhibitors to inactivate each of a number of the participating enzymes. They found that no flux control coefficient equaled 1, that there were various enzymes for which the flux control coefficients ranged between 0.1 and 0.8, and the magnitudes of the flux control coefficients depended on the work load imposed on the mitochondria, *i.e.,* on how much ATP they were asked to synthesize (Groen et al., 1982). They also showed that the enzyme with the highest flux control coefficient was not the one that was irreversible in practice (*i.e.* COX), nor the first step in the pathway of mitochondrial respiration. The possibility that two steps could be completely limiting the flux through a pathway was demonstrated experimentally a few years later (Westerhoff and Arents, 1984).

This demonstration that flux control does not reside in a single step has since been extended to multiple other systems, also using a method differing from the above, *i.e.* that of computation in realistic mathematical models of biochemical networks (Bakker et al., 1999), with most recent applications to the heart (Cortassa et al., 2009b) and the cell cycle (Conradie et al., 2010).

2.2. Exit the far-from equilibrium consideration: Control distribution is determined by new but well-defined properties of pathway components

In pathways of chemical (and physical) reactions, flux control resides in the first irreversible step, which is thereby the rate-limiting step. What is it that makes biochemical pathways different? One obvious difference is that all steps in biochemical pathways are catalyzed by enzymes and transporters. The substrate binding site of enzymes (and transporters) also binds the product and consequently, reactions tend to be inhibited by high product concentrations. A second difference is that evolution may have acted on biochemical pathways so as to make use of this property and bestow the downstream 'demand' reactions with most of the flux control (Hofmeyr and Cornish-Bowden, 2000). More in general, the distribution of flux and concentration control over the enzymes (and transporters) in a pathway is determined by the relative extents to which the enzymes (and transporters) respond to changes in the concentrations of the metabolites that surround them (Kacser and Burns, 1973; Westerhoff and Chen, 1984). These extents correlate only loosely with distance from equilibrium and position in the pathway, and even inversely with extent to which an enzyme is regulated (Westerhoff and van Dam, 1987).

2.3. Control and regulation are not the same thing

For a long time, the concepts of regulation and control were used interchangeably. Only recently, it was realized that there are actually

two different features at play, and that it should be useful to distinguish between these by giving them two different names. The word 'control' continued to be associated with the extent of limitation as expressed by the control coefficients (for pathway flux, metabolite concentration). The word 'regulation' was proposed to indicate whether the organism or cell type under study was actually changing the amount of an enzyme (by transcriptional, post-transcriptional, translational, and/or post-translational modification processes) when trying to change the flux through the corresponding process (ter Kuile and Westerhoff, 2001; Bevilacqua et al., 2008). The corresponding quantitative measure of regulation was termed the hierarchical regulation coefficient. It was defined as the percentage change in the activity of the enzyme divided to the percentage change in flux.

This regulation coefficient has been determined for a number of cases where the yeast *Saccharomyces cerevisiae* regulated its carbon metabolism to adjust to carbon or nitrogen starvation. The hierarchical regulation coefficients differed widely between the various glycolytic enzymes in a way that did not correspond with the suspected distribution of flux control amongst the glycolytic enzymes (see below). The implication is that when the yeast cell regulates itself it does not always regulate the steps with the highest control coefficients, neither does it regulate all steps by the same factor (Rossell et al., 2006; van Eunen et al., 2009). Hence, control and regulation are not the same.

2.4. More theory for metabolic control

MCA is an operational framework, in part theoretical (Kacser and Burns, 1973) in part experimental (Groen and Westerhoff, 1990), which rationalizes the quantitative determination of the degree of control that a given enzyme (or cellular process) exerts on flux (or biological function) and on the concentration of metabolites. It helps to identify and design experimental strategies for the molecular manipulation of a given physiological process in an organism (reviewed in Fell, 1997; Moreno-Sánchez et al., 2008; Westerhoff et al., 2009; 2010). Different approaches have been developed to designing what has to be done and measured in order to identify and understand why an enzyme exerts significant or negligible control on flux and metabolite concentration. Thus, the application of MCA avoids the "trial and error" experiments for identifying and manipulating the conceptually wrong and misleading "rate-limiting step" concept and can readily explain results observed in enzyme over-expression and down-regulation experiments. To understand how a metabolic pathway is controlled and could be manipulated, its control structure has to be evaluated.

The control structure of a pathway is constituted by (i) the collection of flux control coefficients ($C^J{}_i$), each of which is the degree of control that the activity (a) of a given enzyme i exerts on flux J; (ii) the collection of the concentration control coefficients ($C^X{}_i$), each of which is the degree of control that a given enzyme i exerts on the concentration of a metabolite (X); and (iii) the elasticity coefficients (Burns et al., 1985). The control coefficients are systemic properties of the pathway enzymes and transporters that, in turn, are mechanistically determined by their elasticity coefficients ($\varepsilon^{vi}{}_X$ *or* $\varepsilon^{ai}{}_X$), which are defined as the degree of sensitivity of a given enzyme rate v_i *or* a_i to variations in the concentration of any ligand X (*e.g.*, the enzyme's ability to change its rate if the concentration of any of its ligands, *i.e.* substrate, products or allosteric modulators, is varied at constant concentration of all other ligands).

The flux control coefficient is defined as:

$$C_i^J = \frac{\delta J}{\delta a_i} \cdot \frac{a_{io}}{J_o} \approx \frac{\%\ \text{change in flux}}{\%\ \text{change in activity of enzyme}\ i}$$

in which the expression $\delta J/\delta a_i$ describes the variation in flux (J) when an infinitesimal change is effected in the activity (V_{max}) of enzyme i (Burns et al., 1985; Kholodenko et al., 1995). Any other biological function can also be considered as "flux" whereas a metabolic pathway may become the "enzyme activity". In practice, infinitesimal changes J are undetectable, and hence measurable, non-infinitesimal changes are determined, with extrapolation to very small changes. If a small change in a_i promotes a significant variation in J, then this enzyme exerts an elevated flux control (Fig. 1, curve A, position 1). In contrast, if a rather small or negligible change in flux is observed when a_i is greatly varied, then the enzyme does not exert significant flux control (Fig. 1, curve B, position 2). To obtain dimensionless and normalized values of ($C^J{}_i$), the scaling factor a_o/J_o is applied, which represents the ratio between the initial magnitudes of enzyme activity and flux (and at which point the slope $\delta J/\delta a_i$ is calculated). If all ($C^J{}_i$)s of all enzymes and transporters in the total network (*i.e.* also including enzymes in the network but outside the pathway) are added up, the sum comes to one when J represent flux and to 0 when J represent the concentration of a metabolite, a membrane potential or the phosphorylation potential of ATP (summation theorem) (Heinrich and Rapoport, 1973; Kacser and Burns, 1973; Westerhoff and van Dam, 1987).

MCA clearly distinguishes between the control exerted by a given enzyme on flux (flux control coefficient) and on the metabolite concentration (concentration control coefficient). Thus, an enzyme can have significant control on a metabolite concentration but not on the pathway flux or *vice versa*. This distinction is important for biotechnological and clinical purposes: The qualitative rate-limiting step concept for manipulating metabolic pathways does not make such differentiation, which probably has contributed to the many unsuccessful experiments reported in the literature. It should be established whether the aim of the project is to increase flux or to increase a metabolite concentration since MCA establishes for each aim a different experimental design.

To determine the flux control coefficient of a given enzyme, small variations in the enzyme content, or preferentially, in activity are required, without altering the rest of the pathway, and then the changes in flux should be determined. The experimental points are plotted as shown in Fig. 1 and the slope at the reference point a_o/J_o is calculated. This experiment, apparently easy to perform, has demanded great intellectual and experimental effort (*e.g.*, Flint et al., 1980; Groen et al., 1982; Mijakovic et al., 2005). Application of MCA towards a better understanding of cancer biology and identification of the most susceptible proteins for drug targeting, may well require a deeper knowledge of biochemistry and cell biology. However, the complexity of the disease may demand such an intensified intellectual and scientific effort and its increasing incidence and epidemiology may warrant it.

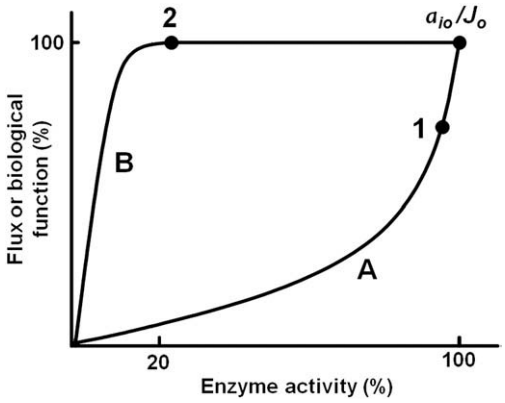

Fig. 1. Experimental determination of flux control coefficient. Small variation in the activity (a_{io}) of a controlling enzyme (or transporter) significantly affects pathway flux J_o (curve A, position 1), whereas significant variation in the activity of non-controlling enzymes (or transporters) has negligible effect on flux (curve B, position 2).

2.5. A case study of control: How fermentative metabolism may be enhanced by yeast

For many applications of yeast in biotechnology it should be useful to enhance the rate at which its ferments sugar to alcohol and carbon dioxide. In order to increase the flux to ethanol one should increase the activities of the rate-limiting step, in case there was one such step. Computationally, *i.e.,* using a model for *S. cerevisiae* glycolysis based on the best known kinetic data, most control resides in GLUT (Teusink et al., 2000), which has been established experimentally for average flux at steady state (Ye et al., 1999), in a dynamic setting (Reijenga et al., 2001) and for flux in *S. bayanus* (Diderich et al., 1999). Researchers have not waited for this information and have attempted to increase the ethanol production rate by over-expressing each of the presumed limiting enzymes (HK, PFK-1, PYK), separately or in combination (Schaaff et al., 1989; reviewed in Moreno-Sánchez et al., 2008). There was little if any effect on the steady state flux through the pathway, confirming the information that most control resides in the transport.

Why was it that the over-expression of the irreversible steps of the pathways did not increase the flux in many if not all the cases tested? The prime reason may have been that these steps were not rate-limiting, that the reason why they were assumed to be the rate-limiting steps was invalid, that a different step was rate-limiting, that the rate-limitation was distributed such that no single step had much flux control, or that indeed the most controlling step resides outside the metabolic pathway. The latter explanation has been established for the glycolytic flux in *E. coli* (Koebmann et al., 2002). In the case of the human parasite *Trypanosoma brucei*, control was distributed over various processes with the strongest control residing in transport and with control in the irreversible enzymes being very small (Bakker et al., 1999), whereas in the parasite *Entamoeba histolytica* control mainly resided in GLUT, HK and PGAM (Saavedra et al., 2007).

Even if the control of the over-expressed step is substantial, the extent to which an enzyme or transporter controls the pathway flux decreases with increasing expression level of that protein (Flint et al., 1980; Jensen et al., 1993). Consequently, over-expression even of enzymes with substantial control may have limited effects on pathway flux (Small and Kacser, 1993; reviewed in Moreno-Sánchez et al., 2008). In the glycolytic enzyme over-expression experiments, the strong inhibitory effect of G6P (or Tre6P in *S. cerevisiae*) on HK, and of citrate and ATP on PFK-1, have been neglected. This regulatory mechanism does not disappear in cells over-expressing the putative controlling enzymes but, on the contrary, it is exacerbated; thus, an increased HK activity without a concomitant increase in the demand of its product, G6P, will lead to its accumulation and to stronger enzyme inhibition, explaining why the enzyme may still have significant control on flux. Accordingly, over-expression of HK, PFK-1, PYK, or any other allosteric or strongly product-inhibited enzyme, may have to be accompanied by parallel manipulation of the enzymes involved in the synthesis and consumption of the regulatory metabolites; alternatively, over-expression of mutated enzymes insensitive to allosteric modulation may help to achieve an increased production. To deal with this aspect in the context of biotechnology all relevant controlling steps may have to be over-expressed (Kacser and Acerenza, 1993), thus reproducing what natural selection has already successfully accomplished.

In accordance with this suggestion, *S. cerevisiae* exposed to high glucose (>2%; 0.11 M) shows an enhanced activity of several glycolytic genes and enzymes (0.5–3 fold) as well as 5–20 fold increased glycolytic flux, in comparison with cells grown on low glucose concentrations or on non-fermentable carbon sources (van den Brink et al., 2008; Müller et al., 1995; Elbing et al., 2004; reviewed in Moreno-Sánchez et al., 2008). Under anaerobiosis (*i.e.* at less than 5 ppm O_2) at glucose excess or limitation, the activities (1.5–2 times) of ALDO, TPI, PGAM, ENO, PYK and pyruvate decarboxylase as well as the fermentative flux (≈16–20 times) of *S. cerevisiae* increase substantially (van den Brink et al., 2008; Wiebe et al., 2008; Jouhten et al., 2008); in this case of regulation, the organism does not alter the activities of GLUT, HK and PFK-1.

2.6. Another case study: Increased production of glutathione

The feedback inhibition of γ-glutamylcysteine synthetase (γ-ECS) has led several researchers to propose that this enzyme is the rate-limiting step of GSH synthesis (reviewed in Mendoza-Cózatl et al., 2005), and this proposal has been automatically extended to all organisms and to any environmental condition. Thus, many research groups have tried to increase the rate of synthesis and concentration of GSH in plants and yeasts, with the aim of increasing resistance to oxidative stress, by over-expressing γ-ECS or some other pathway enzymes. However, only a rather marginal increase in the GSH level has been achieved with no correlation between enzyme levels and GSH concentration (reviewed in Moreno-Sánchez et al., 2008). Another problem for the eventual manipulation of GSH biosynthesis is that the pathway has been analyzed considering only the GSH-synthetic reactions without taking into account the GSH-consuming reactions. The analysis of an incomplete pathway conducts to misleading conclusions about the control of flux and metabolite concentration. Metabolic modeling has illustrated that only with the incorporation of the consuming reactions of the pathway end-products, a true steady state can be established (Hofmeyr and Cornish-Bowden, 2000). In conclusion, without a solid theoretical framework, the over-expression of only one enzyme (the presumed "rate-limiting step"), or of many arbitrarily selected enzymes, cannot solve the problem of increasing the flux or metabolite concentrations.

2.7. And another case study: Increased production of amino acids

One successful story is the notable amino acid (Glu, Trp, Phe, Tyr, Ile, Lys, Val, Thr) production achieved in *Corynebacterium glutamicum*. Researchers working on this biotechnological and commercial relevant subject started out by following the rate-limiting step concept. Later on they realized that several different and simultaneous manipulations were to be implemented to succeed. In general, to increase amino acid production the following strategies have been implemented (Niederberger et al., 1992; Katsumata and Ikeda, 1993; Morbach et al., 1995; Radmacher et al., 2002; Simic et al., 2002; Koffas and Stephanopoulos, 2005; Ikeda, 2006; Asakura et al., 2007; Moon et al., 2008):

(a) Several pathway enzymes must be simultaneously over-expressed;
(b) Some of the over-expressed enzymes must contain mutations that confer insensitivity to feedback inhibition;
(c) The pathway leaks must be attenuated or fully blocked;
(d) To avoid side-effects by end-product accumulation, the specific transporters that expel the respective amino acids must be over-expressed as well. In these multi-transformed bacteria, the end-products are indeed overproduced and their excretion is accelerated; and
(e) Optimization of culture conditions, elimination or attenuation of transcription and genetic controls, increment of balanced supply of precursors, and prevention of uptake and utilization of the produced amino acid.

Summarizing, the fundamental premises of the rate-limiting step concept are that (i) for a step to be somewhat rate-limiting, specific activation or over-expression of such step should increase flux (or metabolite concentration or biological function) and inhibition or down-regulation of the key step should decrease flux, concentration or function; (ii) the extent to which this step is rate-limiting should be determined experimentally and not guessed on the basis of weakly associated features; (iii) rate-limitation needs not be confined to a single step; (iv) the steps that exert more flux control need not be the steps

that are used by the cell to regulate the flux; and (v) for up-regulating the flux, over-expression of all enzymes by the same factor may be a good strategy.

3. The quintessence: Because cells are not a library, they require integrative systems biology

Still it is paradoxical. Why is it that a molecular process that is intricately involved in a cellular function need not control its performance (flux)? Why does not the H^+-translocating ATP synthase determine its own function, *i.e.* the ATP synthesis flux it carries (Groen et al., 1982; Jensen et al., 1993)? Why is not p53 necessarily in control of cell death just because it is the node in the networks regulating cell death (Lazebnik, 2002)? The mechanistic reason is that most macromolecules that carry out the processes of life are "behaving responsibly". They adjust their functioning to signals they receive from their intracellular environment (Groen et al., 1982). The macromolecules we refer to are the enzymes that catalyze metabolic processes, the transcription factors that bind DNA and activate transcription, the enzymes that synthesize and degrade other enzymes as well as the protein kinases and phosphatases that modulate other enzymes. Their responsiveness (*i.e.* elasticity coefficients) is to changes in the activities or concentrations of other molecules in the living cell around them, such as intermediary metabolites or other, phosphorylatable proteins. These changes result from alterations in the activities of the enzymes that produce or consume them. And these activity changes are again the manifestation of the responsiveness of those enzymes, and of the transcription and translation processes setting the concentrations of those enzymes. As a consequence possibly all molecules in the cell are at least indirectly responsive to all other molecules in the cell. If one such macromolecule, say the ATP synthase is activated by a factor of two, then initially the rate at which it synthesizes ATP may increase by the same factor, but as time goes on its rate will decrease such that the final rate is in between the original rate and twice that original rate. The reason is that the increased rate of ATP synthesis will have increased the level of ATP and decrease the levels of ADP, Pi and proton motive force, and all these changes will be sensed by the enzymes and make it tone down its rate.

The functional reason why all intracellular processes tend to be responsible in the above sense is to make the living cell self-sustainable. Whenever an enzyme activity would get too high, it would adjust itself. In a chemical reaction pathway one would not find this phenomenon. There, the first step in the pathway is the only rate-limiting step and the concentration of the penultimate metabolite has no say. In contrast, in biology there is often at least partial control by end-product demand (Hofmeyr and Cornish-Bowden; 2000).

Because of this responsiveness, most molecules that carry out all the cellular functions engage strongly with the networks they are in. And because all those networks are again connected, the behavior of the molecules is determined by the cellular system as a whole. This is why the biology that tries to understand the functions of life in terms of molecules cannot be the biology of the molecules, but must be systems biology (Alberghina and Westerhoff, 2005). A library with the information of the individual molecules is not sufficient to understand function; we shall need a movie in which every molecule is a player.

Due to the complexity of multi-component systems such as metabolic networks, the analysis, management, integration and comprehension of the multiple variables involved in their functioning (thermodynamic equilibrium constants; kinetic constants of enzymes and transporters; pathway intermediary concentrations and fluxes; covalent regulation; protein synthesis and degradation; and genetic control and expression) seems impossible for a researcher. Systems biology is a combined theoretical–experimental approach that allows for the integrative analysis of metabolic networks functioning and helps the human mind to understand how complex biological systems work and how they can be perturbed. Thus, the researcher's expertise and scientific knowledge on cellular metabolism is potentiated, being now able to perform an integrative and dynamic analysis of cellular networks. Therefore, systems biology does not add the behaviors of the molecules but integrate them. The mathematical procedure of integration multiplies the behavior of each molecule for a very short period of time, but will then recalculate that behavior on the basis of all the new concentrations, before integrating again. The procedure is then iterated, continuously updating the mathematical behavior in the light of the development in time of the environment that they sense. This is how the biology works and this is therefore how our understanding of biology should operate, *i.e.* integratively.

Thanks to infusions from genomics and mathematical biology, integrative systems biology has been able to develop rapidly, with a significant number of scientific achievements that are relevant for the problem at hand (Westerhoff and Palsson, 2004). Consensus maps have been made of genome-wide metabolism of yeast (Herrgard et al., 2008) and soon of the human. All possible pathways through these maps can now be calculated (Schuster et al., 2002). Through flux analysis, the pathways can be measured (Isermann and Wiechert, 2003), and through so-called flux-balance analysis, the relative steady state fluxes through these can be predicted if one assumes that the system is optimal from a certain perspective, such as ATP or growth yield (Varma and Palsson, 1993).

Notably, for yeast, the systems biology predictions seem inaccurate, suggesting that other optimization criteria have been important in evolution, or that the cost of certain processes have not yet been taken into account properly (Molenaar et al., 2009; Westerhoff et al., 2010). The same issue is likely to be relevant for tumor cells. The very Warburg effect seems not to have been optimized for producing as much ATP for growth as possible, perhaps because other factors than ATP, such as oxygen supply, limit growth. The enzymes and metabolites involved in glycolysis, the Krebs cycle, and OxPhos are all part of a system. The ATP that is made almost at the end is needed almost at the beginning. The Warburg effect itself is so hard to understand because it could result from a primary mutation activating glycolysis or from a primary mutation inactivating OxPhos. And if indeed, the genotype of tumors is determined by selection for a phenotype then one may expect both cases to occur at different degrees. Considerations of metabolism, gene expression and signal transduction need to be integrated; also here the systems biology requires being integrative.

Ultimately the behavior of systems depends on the responsiveness of all their components. An ultimate version of systems biology therefore is the silicon cell, *i.e.* a precise replica of the networks in terms of kinetic equations of a component process (Snoep, 2005; Westerhoff et al., 2009). The parameters in those equations should stem from accurate experimentation. Consequently, such silicon cells are still rare whereas kinetic modeling of individual pathways has surged (for a complete list of available models see http://www.jjj.bio.vu.nl). In turn, kinetic modeling has facilitated:

(i) To quantitatively determine the control coefficients of individual enzymes (or pathways) to identifying those steps (or pathways within networks) with the highest control on flux and metabolite concentrations (or biological function). The technically difficult experiment of varying the activity of a single enzyme without perturbing the rest of the system, which is essential for determining the control coefficients, as depicted by MCA framework, is easier to perform with the use of mathematical models;
(ii) To understand the underlying kinetic mechanisms by which a pathway enzyme or transporter exerts significant or negligible control;
(iii) To identifying the molecular mechanisms by which all enzymes communicate with each other (metabolites, and coenzymes) to control their rates;

(iv) To predict pathway and network behavior under different physiological relevant conditions (individual or multiple enzyme over-expression or inhibition).

In the post-genomic era, a silicon cell seems now feasible to build with the advent of high-throughput screening methods such as genomics, transcriptomics, proteomics and metabolomics. These technologies produce a mass of information which by computational modeling allows for an integrative analysis to reconstructing the metabolic and signaling cellular networks. For heart energy metabolism a model has been created that gets close to such silicon cell (Cortassa et al., 2009b). Such silicon cells should enable us to understand ultimately how the Warburg effect occurs mechanistically, and should also enable nonlinear flux-balance analyses suggesting why it occurs functionally in tumors. In addition, an iterative process of experimentation and modeling can help us to identifying the most appropriate drug-targets with the highest therapeutic potential.

In the context of cancer, systems biology has been implemented in signal transduction (Hornberg et al., 2006). Here it was shown why it should not have been surprising that there are so many oncogenes, and why there are so many tumor suppressor genes. The oncogene product may be amongst the worst target for inhibitor action unless addiction has taken place (Hornberg et al., 2006).

Before systems biology came along, MCA was limited to pathways at steady state. It has since been developed for time dependent phenomena (Acerenza et al., 1989; Bier et al., 1996; Kholodenko et al., 1997; Westerhoff et al., 2009) and for spatial aspects (Francke et al., 2002), although the experimental applications in those fields remain rare. MCA's extension to the genome-wide network includes gene expression and signal transduction and has been called hierarchical control analysis (Snoep et al., 2002; Kahn and Westerhoff, 1991). Much systems biology beyond MCA is ready to be implemented with enormous potential.

4. The rate-limiting step concept still governs basic research on targeting cancer cells

Notwithstanding the above observations, researchers working on drug design for novel and better venues of cancer treatment (and in fact of most human diseases), have tended to assigning an essential role to their single favorite protein. This leads to a less complicated set of options for experimental testing and to clarity and simplicity of interpretation. The selection of only one "essential" protein is understandable and even justifiable when the cost of the enterprise is evaluated. However, simplicity should only be aspired to if reality itself is simple (Westerhoff et al., 2010). By now it is clear that the reality of most cancers is not simple at all, but requires the amplification of multiple oncogenes (Hanahan and Weinberg, 2000), and cannot just be seen as resulting from p53 amplification alone (Lazebnik, 2002). In addition, even the complete removal of a protein responsible for a given relevant cellular process (by knock-out of the corresponding gene, or nowadays particularly by RNA interference technology), rarely leads to the complete repression of the corresponding biological function, a phenomenon that has been called redundancy. Any concept of a single rate-limiting step that determines and should be hit by an anti-tumor drug is a liability for the success of cancer research.

The cytotoxic approach to pathological cells in a human body, such as cancer cells or parasites, should deal with the fact that the functions of both the pathological cells and the host are managed by networks rather than single rate-limiting molecules (Bakker et al., 2002). The best drug targets may not be the favorite protein or gene molecule but the networks themselves (Hornberg et al., 2006; Bakker et al., 2002; Saavedra et al., 2007; Westerhoff et al., 2008), or diffuse loops in those networks (Cortassa et al., 2009b). In fact, drugs should target the difference between the network of the pathological cell and the network of the host (Bakker et al., 2002; Saavedra et al., 2007). The approach should also reckon with the fact that oncogene amplification tends to make the cancer cell more robust with respect to perturbation of the oncogene. Therefore, inhibiting the oncoprotein itself may well harm the healthy host tissue more than the tumor (Hornberg et al., 2006), unless the phenomenon of 'addiction' kicks in.

Indeed, side-effects from chemotherapy are always evident and they can be moderate, severe or lethal. Deletion or full inhibition of any enzyme or transporter, protein or gene, in a given cellular process is expected to be successful in cancer treatment only if tumor cells are targeted specifically whereas normal, healthy cells remain unaffected. Because of the extensive homology between most tumor cells and healthy cells that are important for the function of the patient, this complete inhibition strategy may well be unrealistic: at full inhibition of the tumor, the healthy tissues of the patient will also be damaged. Complete blockade of an enzyme or transporter activity that follows classical Michaelis–Menten kinetics requires the addition of more than 100 times the *Ki* value, which in practice is not achieved without compromising health. Moreover, it has been experimentally determined that, to significantly alter the pathway flux, activity of *non-controlling steps* have to be decreased beyond a threshold of 60% or more (Rossignol et al., 2003; reviewed in Moreno-Sánchez et al., 2008). Remarkably, the percentage of inhibition in protein expression normally achieved by RNA interference is around this threshold value, resulting in an over-interpretation of results when using this tool to supporting hypotheses that non-controlling transporters or enzymes are indeed the "rate-limiting step". With such a criterion of effect upon 60% inhibition, multiple steps in a pathway would become "the rate-limiting step".

Yet, the approach of identifying, and targeting, "the rate-limiting step" continues today and appears to be the dominant theoretical support for an overwhelming number of studies in drug design and cancer biology fields. Examples spread from hormone receptors and signal transduction components, transcriptional factors to "metabolic enzymes". For instance, it has been postulated that vascular endothelial growth factor receptor (VEGFR), or endothelial growth factor receptor (EGFR) or downstream protein kinases (PKs), have an "essential" or "pivotal" role in the growth of tumors and in consequence targeting of these proteins is pursued actively (reviewed in Benouchan and Colombo, 2005; Johnson, 2009). Similar arguments have been used to justifying the development of specific drugs directed to (i) the "key" or "major" transcription factor HIF-1 (Melillo, 2007; Fulda and Debatin, 2007) or (ii) the prolyl-hydroxylases (PHDs; Gottlieb and Tomlinson, 2005), the "main" enzymes involved in HIF-1 degradation, (iii) the "master metabolic regulator" Akt/PI3K/mTOR signal transduction pathway (Jones and Thompson, 2009), (iv) the "rate-limiting step metabolic enzymes" PDK (Denko, 2008), PFK-2 (Yalcin et al., 2009), LDH (Fantin et al., 2006), fatty acid synthase (FAS; Pizer et al., 2000; Mashima et al., 2009), ATP citrate lyase (ACL; Hatzivassiliou et al., 2005; Mashima et al., 2009), or aromatase (P-450) (Bhatnagar, 2007), or (v) "important" pro-apoptotic or anti-apoptotic proteins such as TRIAL and XIAP, or BCL-2, respectively (Fesik, 2005; Fantin and Leder, 2006). In none of these cases, hard, quantitative experimental data have been produced supporting the presumed rate-limitation of the above-mentioned selected proteins and processes on tumor growth.

5. Glycolysis in cancer cells

Glycolysis is enhanced in many human and animal neoplasias (reviewed in Moreno-Sánchez et al., 2007). How have these cancers up-regulated their glycolysis?: (i) Have they done this by enhancing the expression of a single rate-limiting step? (ii) Have they over-expressed all enzymes to the same extent? (iii) Or have they used a more subtle approach? The answer to the first question is clear for most cancers: Yes, all or most of the glycolytic enzymes and transporters are over-expressed in at least 70% of human cancers (Altenberg and Greulich, 2004). Gene expression of glycolytic proteins (GLUT1, GLUT3, HKI, HKII, PFK1-L, PFKFB-3, ALD-A, ALD-C,

TPI, GAPDH, PGK1, PGAM-B, ENO-α, PYK-M2, LDH-A, and MCT4) is further stimulated by hypoxia in a process mediated by the transcription factor HIF-1α (hypoxia-inducible factor 1α) (reviewed in Marín-Hernández et al., 2009). The HPI gene is also induced by hypoxia, but through a HIF-1α independent mechanism.

The *activity* of all glycolytic enzymes is concomitantly enhanced in rat AS-30D hepatoma, 2–4 fold for HPI, ALD, TPI, GAPDH, PGK, PGAM, ENO, and LDH; 8–10 fold for PYK; and 17–300 fold for PFK-1 and HK (Marín-Hernández et al., 2006), in comparison with normal rat hepatocytes, the tissue of origin. In rat Morris hepatomas, the activities of HK, PFK and PYK are 5–500 times higher than in liver (Stubbs et al., 2003). In human breast cancer, the activities of HK, ALD, PYK, and LDH are 3.7–7 times higher than in normal tissue (Balinsky et al., 1984). In human cervix HeLa cells, all enzyme activities including HK and PFK-1 are enhanced by 2–7 fold, except for PGAM and LDH (Marín-Hernández et al., 2006). Because in all these cases the expression of more than one glycolytic gene is enhanced, or the activity of more one enzyme is increased, the answer to the third question above is 'No': Most cancers have not just over-expressed a single rate-limiting step. And because the extents of over-expression differ significantly between the various enzymes in the same cancer, the answer to the second question is 'No' as well: Apparently, cancers have enhanced their glycolysis in subtler ways, perhaps as subtle as noted in yeast (Rossell et al., 2006).

In summary, the strategy selected by cancer cells to achieve an increased glycolytic flux to maintaining an adequate ATP supply is highly similar to that described for amino acid over-producing bacteria:

(a) Over-expression of most glycolytic proteins (reviewed in Moreno-Sánchez et al., 2007);
(b) Isoenzyme expression shift, from the PFK-1 isoenzyme with allosteric inhibitor-sensitivity/low allosteric activator affinity to the PFK-1 isoenzyme with allosteric inhibitor-insensitivity/high allosteric activator affinity (reviewed in Marín-Hernández et al., 2009);
(c) Decrease in the activity of relevant branching fluxes, *e.g.*, through the partial inactivation of PDH complex (Denko, 2008) thus diminishing leaks; and
(d) Over-expression of MCT4 (Gallagher et al., 2007), the plasma membrane transporter responsible for secreting lactate.

In other words, cancer cells do not over-express solely one rate-limiting step to increase glycolytic pathway flux and metabolite concentration, but they follow a variety of simultaneous strategies to achieve the desired objective. It is as with microorganisms found in a niche: they have evolved/adapted in whatever way it took to thrive. It will be interesting and important to try to understand more precisely which ways these are, perhaps in order to interfere with them, or with their selection.

6. A mishap in the Warburg hypothesis? Functional mitochondria as anti-cancer targets

We shall now present an example of an analysis of a number of cancers from a more subtle point of view than the rate-limiting step concept. Warburg (1956) originally proposed that the prime cause of cancer was an energy deficiency caused by an irreversible damage to mitochondrial function, which induced an increased glycolysis. Since then, the field of cancer biology research has assumed that the Warburg hypothesis applies to all or most cancer cell types (Pedersen, 1978; Atsumi et al., 2002; Rossignol et al., 2004; Robey et al., 2005; Xu et al., 2005; Seyfried and Mukherjee, 2005; Gottlieb and Tomlinson, 2005; Matoba et al., 2006; Denko, 2008; Kroemer and Pouyssegur, 2008; Jones and Thompson, 2009), because one of the hallmarks of many types of cancer is certainly an increased glycolytic capacity, which persists in the presence of high O_2 concentration (reviewed in Moreno-Sánchez et al., 2007, 2009).

However, contribution of glycolysis to cellular ATP supply can be as low as 10% in some cancer types and 50–70% in other cancer types (reviewed in Moreno-Sánchez et al., 2009). In consequence, OxPhos also significantly contributes to ATP supply in cancer cells. Somewhat surprisingly, this second aspect of the Warburg hypothesis ("cancer cells have impaired mitochondria") has been ignored or taken as an established fact. Because of the absence of hard experimental evidence for this part of the hypothesis, it has rather become the metabolic central dogma of tumor cells.

According to Warburg's hypothesis, the main oxidizable substrate for cancer cells in the human body should be glucose, and glycolytic inhibitors should potently block tumor growth. Substrates oxidized by mitochondria should not be metabolized by cancer cells and hence mitochondrial inhibitors should be innocuous. Glycolytic inhibitors such as gossypol, 2DOG, 3-BrPyr, oxamate are indeed effective anti-cancer drugs but at relatively high doses and only when used in combination with drugs targeting other pathways or cellular processes. In addition, they are not specific for the glycolytic enzymes and transporters, and they are also cytotoxic for non-tumor cells (reviewed in Pelicano et al., 2006; Rodríguez-Enríquez et al., 2009). On the other hand, mitochondrial inhibitors are potent tumor growth blockers when used alone or in combination with other anti-cancer drugs. Therefore, the analysis of whole set of available data indicates that glycolysis may not be the rate-limiting pathway for ATP production in some cancer cells, or that mitochondria have an essential function for tumors other than just ATP production. Hence, the usual statement that glycolysis predominates over OxPhos in terms of ATP supply in tumor cells should be re-evaluated and most probably verified experimentally for each particular type of tumor cells.

7. Energy metabolism in mitochondria-deficient cells (rho cells) and stem cells

It has been demonstrated that rho cells survival depends on an enhanced glycolytic activity (60% more *vs.* parental) which is coupled to an efficient plasma membrane redox system (Hyun et al., 2007), to maintaining the cytosol redox status. In consequence, rho cells viability is drastically abolished by glycolytic inhibitors (2DOG, iodoacetamide, oxamate) at doses 5–10 times lower than those used in wild type cells, whereas significant higher doses (50-times *vs.* wild type) of mitochondrial inhibitors (rhodamine 123, safranine O) are required to abolish cellular growth (Hu et al., 2000; Liu et al., 2001; Hyun et al., 2007). The lactate overproduction and the anti-glycolytic drugs hypersensitization observed in rho cells is similar to that observed in parental counterpart cells after OxPhos inhibitors (rhodamine 123, rotenone, antimycin) treatment and in solid tumor hypoxic micro-regions (Rodríguez-Enríquez et al., 2009). These observations indicate that, only when OxPhos is fully abolished, cells switch over to glycolysis (Liu et al., 2001). On the other hand, the lack of mitochondria, and hence the absence of a mitochondrial inner membrane electrical gradient (negative inside), diminishes the ability of rho cells to uptake and accumulate delocalized lipophilic cation drugs (such as rhodamine 123, 6G, MKT077 and other positively charged anti-neoplastic drugs) (Hu et al., 2000; reviewed in Rodríguez-Enríquez et al., 2009), thus limiting their anti-proliferation effect.

The absence of mtDNA in rho cells also brings about (i) higher resistance towards classical anti-neoplastic drugs (paclitaxel, doxorubicin, daunomycin) decreasing apoptosis incidence; (ii) development of a more invasive cancer phenotype in prostate carcinoma and osteosarcoma (Ferraresi et al., 2008; Moro et al., 2008; Mineri et al., 2009); (iii) decrease in the cellular ROS concentration compared to their parental counterpart cells (Hyun et al., 2007; Cuperus et al., 2009).

As stem cells are now considered as the precursors of cancer, it is relevant to analyze how these cells deal with the problem of ATP

supply/demand. Normal human mesenchymal stem cells exhibit (i) higher glycolytic enzymes levels and lactate production rates, (ii) higher sensitivity to glycolytic drugs; and (iii) lower sensitivity to OxPos inhibitors. Upon induction to differentiation, these cells develop: (i) higher number of mtDNA copies, content of respiratory chain enzymes, ATP content and sensitivity to OxPhos inhibitors; (ii) accelerated oxygen consumption rate; and (iii) lower lactate production and sensitivity to glycolytic inhibitors (Chen et al., 2008). Thus, these observations suggest an energy metabolism transition from glycolysis to OxPhos in normal stem cells with differentiation (Chen et al., 2008).

No changes in the expression of the majority of the glycolytic gene, together with significant decrease in lactate production and glucose uptake, is observed in monolayer-cultured oncogenic-transformed mesenchymal stem cells in comparison with their parental stem line. In parallel, oncogenic transformation of stem cells also induces up-regulation of some genes of the Krebs cycle and higher mitochondrial ATP-dependence (Funes et al., 2007). In strikingly contrast, tumors derived from the same cancer stem cells show up-regulation of glycolytic genes and down-regulation of Krebs cycle genes (probably induced by hypoxia), although the glycolytic flux does not significantly change. Thus, these observations suggest that normal stem cells are predominantly glycolytic but they shift towards oxidative energy metabolism when experiencing oncogenic transformation, which in turn questions the assumption that aerobic glycolysis is part of the neoplastic transformation (Funes et al., 2007). The complete characterization of the energy metabolism setting in cancer stem cells will facilitate application of MCA and systems biology for target identification and design of appropriate drugs.

8. Control analysis of cancer energy metabolism

MCA of tumor energy metabolism has been undertaken. Elasticity control analysis of glycolysis in AS-30D hepatocarcinoma (Marín-Hernández et al., 2006) showed that the main flux control (71%) resided in the first part of the glycolytic pathway (*i.e.* GLUT and HK). The rest of the control (29%) was localized in the ALD–LDH segment, with a negligible contribution of PFK-1 (<6%). Despite its exacerbated over-expression (100–500 fold in AS-30D hepatocarcinoma), tumor HK was strongly inhibited by its product G6P and hence only an 8–10 fold increase in flux was achieved. On the other hand, PFK-1 was moderately over-expressed, but the tumor isoenzyme was highly activated by F2,6BP and AMP, which surpassed the inhibition by citrate, ATP, and low pH. These findings provided a mechanistic explanation for the respective high and low flux control exerted by HK and PFK-1 in tumors. The study also showed that a massive over-expression of glycolytic enzymes does not lead to uncontrolled flux, but rather invokes strict regulatory mechanisms (potent product-inhibition, allosteric modulation), which persist in the tumor cells. Kinetic modeling of glycolysis in AS-30D and HeLa tumor cells has revealed that indeed GLUT and HK together with HPI are the main flux-controlling steps in both tumors.

An integrated modeling of OxPhos (including Krebs cycle, respiratory chain, membrane potential, ATP/ADP exchange, and ATP synthase) and mitochondrial Ca^{2+} dynamics in heart showed that the control of the ATP synthesis was distributed among several steps (Cortassa et al., 2009a,b). This feature of distributed control appears to be conserved in tumor cells: Control analysis of OxPhos in AS-30D hepatocarcinoma showed that the respiratory chain site 1 (30%) and the ATP-consuming enzyme block (protein and nucleic acid synthesis; ion ATPases) (34%) were the main controlling sites (Rodríguez-Enríquez et al., 2000). The latter observation shows that the flux control may reside in part, outside the pathway, supporting the proposal by Hofmeyr and Cornish-Bowden (2000) that the end-product demand (which is usually overlooked in studies of metabolism because these metabolites are frequently not considered as part of the pathway) also exerts significant flux control (see also above).

Control analysis of tumor energy metabolism establishing the main sites of control in glycolysis and OxPhos, and Flux Analysis assessing the predominant energy pathway (*cf.* Westerhoff et al., 2010), may provide a more rational and quantitative approach to the identification and design of more specific therapeutic strategies. Therefore, it should be desirable to apply MCA of energy metabolism (and of other relevant pathways and cellular processes) to many other different types of cancer, avoiding misleading generalizations such as "ATP supply in cancer cells is mainly provided by glycolysis".

MCA could also be used to improve the selectivity and effectiveness of anti-cancer drugs (Hornberg et al., 2006). For instance, analysis of the control distribution of a common metabolic pathway in both tumor and normal cells, in order to identify the steps with the highest control in the former but low control in the latter may allow for the identification of drug-targets with higher therapeutic potential. Thus, selectivity towards a given anti-neoplastic drug can be determined as follows:

$$\text{drug selectivity} = \frac{R^{J(tumor)}_{drug}}{R^{J(host)}_{drug}} = \frac{C^{J(tumor)}_{i} \cdot \varepsilon^{i(tumor)}_{drug}}{C^{J(host)}_{i} \cdot \varepsilon^{i(host)}_{drug}}$$

Where R^{J}_{drug} are the response coefficients of the flux J (or biological function) towards the drug (enzyme inhibitor), C^{J}_{i} are the flux control coefficients of enzyme i from tumor and normal cells and ε^{i}_{drug} are their corresponding enzyme elasticity coefficients toward the drug. Whether the tumor and host enzymes have the same elasticities for the drug and are confronted to the same drug concentration, then the selectivity will only depend on the ratio of the flux control coefficients exerted by the tumor and host enzymes.

The effectiveness of a drug to affect a metabolic pathway in cancer cells depends on the response coefficient of the pathway flux to the drug which in turn, does not only depend on how efficient is a drug to inhibits a particular enzyme, but also on the control coefficient that the latter has on the entire pathway (Hornberg et al., 2006). Thus, an anti-neoplastic drug may exhibit potent (in the nanomolar concentration) inhibition on isolated enzyme activity but, if this enzyme has low control on the entire pathway flux (or cellular function), then negligible perturbation of the tumor metabolic flux (or function) can be expected and thus, no effect on the disease treatment. In this regard, a target identification computational program has been introduced for the systematic search of drug targets, effective inhibitor concentrations and type of inhibitor in kinetic models (Schulz et al., 2009).

9. Clinical treatment of cancer

9.1. Current chemotherapeutic regimes

The majority of chemotherapeutic drugs perturb nuclear DNA, cytoskeletal dynamics or essential metabolic and signal transduction pathways (Table 1). The initial effects are followed by the induction of apoptosis and eventually, by cell death (Guzman et al., 2002; Xing and Orsulic, 2005). For most cancers however, single-agent treatment with a variety of such chemotherapeutic drugs have low response rates (<15%). Part of this may be caused by selection of tumor cells that are resistant against the therapeutic. In view of our above analysis we contend that part of this is also because each drug was aimed at a single target only. A better approach to cancer treatment should be the drug combination therapy, in which several different proteins in different pathways and cellular processes are targeted simultaneously, in addition to DNA itself (Table 2).

Indeed, oncologists have improved treatment protocols by combining several drugs: Current clinical treatment of cancer patients usually combines three or more drugs for attaining high (>20–30%) healing rates or increased index of survival of the five major groups of

Table 1
Molecular targets of anti-cancer drugs.

Drug	Target
	DNA synthesis
– Pt-based drugs: cisplatin; carboplatin; oxaliplatin	DNA adducts formation
– Cyclophosphamide	
– Anthracyclines: doxorubicin (adriamycin); epirubicin; daunorubicin	DNA intercalation
– Methotrexate	Dihydrofolate reductase inhibitor
– 5-Fluorouracil; capecitabine	Thymidylate synthetase inhibitor
– Gemcitabine; 6-mercaptopurine	Ribonucleotide reductase inhibitor
– Bleomycin	DNA strand break induction
– Dacarbazine; temozolomide	DNA mehylation
	Topoisomerase inhibitors:
– Topotecan; irinotecan; campthotecina; kaemferol	Isoform I
– Etoposide; doxorubicin; mitoxantrone	Isoform II
	Mitotic spindle microtubule assembly
Taxanes: paclitaxel; docetaxel	Depolymerization inhibitor
– Vinorelbine; vinblastine, vincristine	Polymerization inhibitor
	Signal transduction
– Tamoxifen; raloxifene	Estrogen receptor (ER) inhibitor
– Flutamide	Androgen uptake inhibitor
Small molecule PK inhibitors:	
– Imatinib (gleevec)	Inhibitor of Bcr-Abl; PDGFR (platelet derived growth factor receptor); c-Kit-stem cell factor receptor; EGFR; VEGFR.
– Lapatinib	Inhibitor of EGFR (ErbB1) and HER-2 ErbB2 tyrosine kinase activities
– Gefitinib; erlotinib	EGFR tyrosine kinase inhibitor
– Sorafenib	Raf-1, VEGFR and PDGFR inhibitor
– Rapamycin	mTOR kinase inhibitior
Monoclonal antibodies	
– Trastuzumab (herceptin)	Inhibitor of human epidermal growth factor receptor-2 (HER2)/neu or ErbB-2 receptor in the Raf/MEK/ERK and PI3K/PDK/Akt signal transduction pathways
Cetuximab (erbitux)	EGFR inhibitor
Bevacizumab	VEGFR inhibitor
Infliximab	TNF-alfa inhibitor
	Aromatase inhibitors
– Steroidal: exemestane	Irreversible binding
– Non-steroidal: anastrozole; letrozole	Enzyme activity inhibitor

Data taken from Potier (1989), Navolanic and McCubrey (2005), Kostova (2006), and Johnson (2009).

tumors: lung, breast, prostate, ovarian and colorectal. For instance, by adding the monoclonal antibody trastuzumab to conventional adjuvant chemotherapy (see Table 1), a significant reduction in deaths in surgically treated HER-2 positive breast cancer patients, is achieved (Romond et al., 2005). Combination therapies for other types of cancer such as aggressive B-cell non-Hodgkin lymphoma, choriocarcinoma, metastatic carcinomas (lung, breast, colon and prostate), testicular cancer, ovarian germ cell tumors, Hodgkin lymphoma, and small-cell lung cancer have also achieved higher survival rates and retarded relapse, although for some of them cure has remained elusive (Savage et al., 2009). Perhaps, this multi-site therapy can be improved by targeting the truly controlling steps of the most relevant cellular processes in tumors, thus decreasing the risk for undesired effects on healthy tissues.

Most current chemotherapeutic drugs are rather unspecific as they also perturb other sites in both normal and cancer cells. For instance, doxorubicin is not only inhibiting the division of tumor cells and inducing their apoptosis (Rabbani et al., 2005), it is also cardiotoxic and induces oxidative stress by affecting the mitochondrial respiratory chain. Many anti-cancer drugs targeting nuclear DNA also affect mtDNA (Table 1). Topotecan is also a HIF-1 inhibitor. 5-fluorouracil induces additional inhibition of RNA splicing and mRNA translation. Capecitabine alters ribonucleotide reductase and PKs involved in apoptosis. Gemcitabine up or down regulates multiple membrane transporters. Topoisomerase and microtubule assembly inhibitors affect the DNA synthesis or cytoskeleton of all cells in an organism. Monoclonal antibodies like trastuzumab or cetuximab neutralize growth factors indispensable for homeostasis of normal cells. Etoposide and cyclophosphamide have inhibition of glycolysis as a side effect. Pt-based drugs are nephrotoxic and cause membrane lipid peroxidation (for detailed descriptions of anti-cancer drugs effects on energy metabolism see Rodríguez-Enríquez et al., 2009).

9.2. Alternative drug design for cancer treatment

9.2.1. Simultaneous administration of drugs targeting controlling sites of essential cellular processes

This approach is exactly what oncologists have ended up doing for clinical treatment of cancer patients. However, most of the anti-cancer drugs used in chemotherapy are rather unspecific for cancer cells. They may also affect non-cancer cells by acting on the same molecular target in those cells, or they may affect other targets in healthy cells (reviewed in Rodríguez-Enríquez et al., 2009). Drugs should be targeted at the difference between the intracellular network that is vital for the survival of tumor cells (or that the tumor has in store for its survival) and the network that ensures function of normal host cells. This is particularly clear by tumors having drug resistance mechanisms in their armor. To avoid the surge of drug resistance in solid tumors, simultaneous targeting of (i) P-glycoprotein and other similar multidrug resistance pumps that are over-expressed in cancer cells and efficiently expel xenobiotics such as doxorubicin, daunorubicin, paclitaxel, vinblastine, vincristine, etoposide and epirubicin (Balimane and Chong, 2005), and (ii) relevant or unique cellular processes such as energy metabolism or apoptosis (which is usually blocked by over-expressed resistance factors; Gallardo-Pérez et al., 2009), may have substantial success. Protein network-based analysis has also concluded that, to damage a complex biological system, a higher efficiency is achieved by multiple, moderate attacks on selected targets than by the complete suppression of a single, equally well-selected, target (Csermely et al., 2005; Kell, 2006).

Some types of cancer cells generate a trans-membrane electrical gradient (negative inside) across both plasma and inner mitochondrial membranes of a higher magnitude (15–50 mV) than that of normal cells (reviewed in Rodríguez-Enríquez et al., 2009). This situation provides the required driving force for a greater cytosolic and intra-mitochondrial accumulation of lipophilic cationic molecules in cancer cells over normal tissues. Indeed, specific targeting of these cancer cells seems promising with lipophilic drugs that are weak bases with a delocalized net positive charge when they are in their protonated state. These would accumulate more into the mitochondria and could cause more damage there than in the mitochondria of normal cells because of their higher concentration. In addition, these molecules should be expected to be able to leave the mitochondria upon deprotonation, a proton-gradient dissipating cycle resulting through which they will act as uncouplers of OxPhos. Examples of this type of anti-cancer drugs are daunorubicin, doxorubicin as well as the rhodamines and casiopeinas (copper-based phenanthroline or bipyridene-coordinated molecules). Unfortunately, rhodamines have shown high nephro-toxicity, indicating that new-generation rhodamine derivatives should be more specific for cancer cells and exhibit lower toxicity towards healthy tissues; casiopeinas are in the initial stages of development and except for an attenuated cardiotoxicity in comparison to doxorubicin (Hernández-Esquivel et al., 2006), their toxicity has not been evaluated as yet.

Cells resistant against this type of mitochondrial anti-cancer drug, may have decreased the mitochondrial H^+ electrochemical gradient

Table 2
Current clinical protocols for cancer treatment.

Carcinoma	Combination therapy	References
Lung	– Surgery + cyclophosphamide + cisplatin + doxorubicine; radiation.	– NSCLC Collaborative Group (1995)
	– Cisplatin or carboplatin + vinblastin or fluorouracil or paclitaxel or docetaxel + radiation, or ± bevacizumab.	– Schiller et al. (2002)
	– Gefitinib; gemcitabine + Pt-based.	– Ohyanagi et al. (2009)
	– Cisplatin + vinorelbin; cituximab (InCan).	
	– Docetaxel + infliximab.	
Breast	– Doxorubicin or epirubicin + cyclophosphamide; paclitaxel; ± trastuzumab.	Banerjee et al. (2007),
	– Docetaxel or paclitaxel + carboplatin; ± trastuzumab.	Dinh et al. (2008),
	– Fluorouracil + doxorubicin + cyclophosphamide; paclitaxel (InCan).	Jones et al. (2009)
	– Docetaxel; fluorouracil + epirubicin + cyclophosphamide.	
	– Cyclophosphamide + methotrexate + fluorouracil + tamoxifen.	
	– Bevacizumab + capecitabine or paclitaxel or cyclophosphamide + metrhotrexate or doxorubicin + docetaxel.	
Prostate	– Surgery + radiation ± hormonotherapy (InCan).	– Ahmed et al. (2007)
	– Flutamide + goserelin or leuprolide (gonadotropin releasing hormone agonists)	– Seruga and Tannock (2008)
	– Docetaxel + prednisone.	– Tannock et al. (2004)
Ovary	– Cyclophosphamide or erlotinib or capecitabine + bevacizumab.	– Spannuth et al. (2008)
	– Irinotecan + fluoruracil or carboplatin + paclitaxel ± bevacizumab.	– Ray-Coquard et al. (2009)
	– Doxorubicin or topotecan + paclitaxel.	– Kumaran et al. (2009)
	– Carboplatin + topotecan + doxorubicin (InCan).	
	– Gemcitabine + oxaliplatin.	
Colorectal	– Bevacizumab + fluoruracil ± irinotecan.	– Chau and Cunningham (2009)
	– Irinotecan + cetuximab (InCan) or oxaliplatin or fluorouracil ± bevacizumab.	– Vasile et al. (2009)
	– Fluoruracil or capecitabine + irinotecan or oxaliplatin.	

The + symbol indicates simultaneous administration whereas a semicolon mark indicates a subsequent round of treatment. InCan, standard first line treatment protocol followed in the Instituto Nacional de Cancerología de México (Mohar et al., 2009).

by over-expressing uncoupling protein 2 (UCP2), which acts as a mitochondrial H^+ channel, collapsing the trans-membrane electric potential generated by the respiratory chain, reducing the accumulation of weak bases (which are electrically positive in their protonated form at neutral pH) such as daunorubicin and doxorubicin into the inner mitochondrial membrane, the mitochondrial matrix space and the mitochondrial DNA. By relying less on mitochondrial OxPhos in the first place, these cells would be less sensitive to the uncoupling effects of the drugs. Possibly because of greater dependence on Krebs cycle substrate-level phosphorylation catalyzed by succinylCoA synthetase, these drug-resistant cells also increase the utilization of alternative oxidizable substrates such as glutamine, fatty acids and ketone bodies (reviewed in Moreno-Sánchez et al., 2007; Ralph et al., 2010) offering interesting second drug targets. Here, it is useful to remember that OxPhos requires coupled respiratory activity (*e.g.*, electron transport and H^+ pumping catalyzed by the respiratory chain to build up a H^+ electrochemical gradient across the inner mitochondrial membrane that drives ATP synthesis), whereas oxidation of alternative substrates in the presence of an over-expressed UCP2 may only require uncoupled respiratory activity (*e.g.*, electron transport with no H^+ gradient generation) to oxidize the reducing equivalents produced by the Krebs cycle enzymes, avoiding their accumulation and the inhibition of the pathway flux.

From the perspective of flux control analysis, it appears that GLUT and HK, but not PFK-1 and PYK, provide the best targets for therapeutic intervention at the level of energy metabolism in at least some hypoxic and glycolytic tumors. For OxPhos in well-oxygenated, oxidative fast-growing tumors, respiratory complex I and the ATP demand seem to be the best targets. Thus, specific, potent and cell-permeant inhibitors of these controlling steps of glycolysis and OxPhos may prove to be suitable targets, along with specific inhibitors for other cancer cell processes. Also here similar tumor phenotypes, such as that of an increased activity of glycolysis, may be achieved by many more than a single combination of oncogenic mutations. We would recommend the establishment of the molecular and then systems biological basis of the phenotype and then engage in tumor (not necessarily patient) specific treatment with a cocktail of inhibitors.

It may also be possible to exploit the more acidic intracellular pH in certain tumors because some compounds such as α-tocopheryl-succinate become more potent anti-cancer drugs at lower pH than at neutral pH (Neuzil et al., 2002). For potency, preferred compounds will be those with low nanomolar range *Ki* values and drug design should consider that the compound has to penetrate into the core of solid tumors, for which a hydrophobic moiety may prove beneficial.

9.2.2. Multi-site drugs

3-BrPyr, an alkylating agent, is effective in killing tumor cell lines either with high respiratory activity (human HL-60 leukemia; human lymphoma Raji) or that are mitochondria-deficient (C6F leukemia and Raji/C8 rho cells), with slightly higher potency. The compound is also effective against cancer cells that express a multidrug-resistant phenotype (HL-60/AR), as well as in treatment of tumors implanted in rabbits (Ko et al., 2001; Geschwind et al., 2002; Xu et al., 2005). 3-BrPyr also inhibits the glycolytic enzymes GAPDH, PGK, mitochondria-bound HK-II, the mitochondrial enzymes PDH complex, SDH, GDH and the pyruvate transporter, thus also inhibiting OxPhos and inducing apoptosis (Jones et al., 1995; Ko et al., 2004; Pereira da Silva et al., 2009; Chen et al., 2009). Notwithstanding their low cytotoxic potency against cancer cells ($IC_{50} \sim 50\ \mu M$) (Ko et al., 2004; Xu et al., 2005), drugs like BrPyr that affect several different proteins in a variety of pathways and cellular processes are a rational and encouraging alternative for cancer treatment and warrant further experimentation: they fulfill the requirement that they hit the network much more than individual target molecules. What may still be needed is that they hit the network in the tumor more than in the healthy cells of the patients (Bakker et al., 2002; Hornberg et al., 2007). In this regard, multi-functional drug design can be another way to tackle a multi-factorial disease.

The vitamin E analogue α-tocopheryl-succinate (α-TOS) may specifically target (and freely diffuse) tumor cells by virtue of the more acidic extra- and intracellular pH. Once inside the cell, α-TOS induces apoptosis in mesothelioma, bladder, lung and several other cancer cell types and tumors, apparently without much affecting normal cells, through a variety of effects: by promoting an increased oxidative stress through the selective inhibition of SDH (Neuzil et al., 2004; Dong et al., 2008, 2009); by acting as a BH3 mimetic (Shiau et al., 2006; Dong et al., 2008); and by causing lysosomal destabilization (Neuzil et al., 1999, 2002).

Also the copper-based casiopeinas seem multi-site anti-cancer drugs because they (i) affect DNA synthesis by intercalating with the DNA (Rivero-Müller et al., 2007), (ii) induce apoptosis through a caspase-mediated mechanism (Trejo-Solís et al., 2005), (iii) increase oxidative stress (Alemón-Medina et al., 2008), (iv) block OxPhos and decrease cellular ATP content by inhibiting mainly 2-OGDH, PDH and SDH (Marín-Hernández et al., 2003; Rodríguez-Enríquez et al., 2006), and (v) also perturb glycolysis by specifically inhibiting HK. Drugs that target glycolysis and OxPhos simultaneously can be advantageous for cancer treatment because of network targeting, but also because it has been described that there may be cellular subpopulations within solid tumors, some with a predominant glycolytic phenotype localized away from blood vessels in hypoxic regions, and others with a predominant mitochondrial metabolism localized near to blood vessels (Sonveaux et al., 2008). Of course here the problem persists, that non-tumor cells in the body of the patient may also be targeted by the drugs.

10. Concluding remarks

The frequently recurring idea of only blocking the presumed key enzyme or rate-limiting step of a pathway or cellular process to stop tumor growth has not yielded the pursued results, except for a very few examples (imatinib). Therefore this notion is not a good paradigm for anti-tumor drug targeting. The signal transduction drugs (see Tables 1 and 2) have shown some success in the treatment of some cancers (leukemia) perhaps because they display potent inhibition of several protein kinases, and because they also affect other cellular targets. Administration of drugs targeting single key gene products or pathways yields low rates of response and should not be expected to cure cancer (Hayden, 2008). MCA and the oncologic clinical practice, have both demonstrated that control of function is shared by multiple steps. To achieve some degree of success in the clinical treatment of cancer, a combination therapy appears mandatory involving, for instance, conventional drugs such as cisplatin and doxorubicin (Shoshan and Linder, 2008), or the multi-site drugs α-TOS and casiopeinas, for simultaneous inhibition of multiple cellular targets (see Table 2). In consequence, basic research regarding improved cancer treatment protocols should focus on identifying the controlling steps of metabolic pathways and cellular networks and on designing specific potent drugs targeting such controlling sites, pathways and networks. These drugs should be administered simultaneously to achieve higher success rates, or potentially in a dynamic correlation that is designed by sophisticated systems biological analyses.

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