Targeting molecular addictions in cancer

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Cancer cells depend on a finite number of critical signals for their survival. Oncogene addiction, that is, the acquired dependence of a cancer cell on the activity of a single oncogenic gene product, has been the basis for the targeted therapy paradigm, and operationally defines such signals. Additionally, cancer cells have altered metabolic requirements that create addictions to specific nutrients such as glucose and glutamine. In this review, I will discuss the therapeutic opportunities that these two types of molecular addictions offer, focusing on lessons learned from targeting members of the epidermal growth factor receptor family of kinases, and components of MAPK pathway. I will also discuss the challenges in simultaneously harnessing two types of molecular addictions for therapeutic benefit, and the importance of understanding not only the effects of oncogenic signal transduction on metabolism, but also the impact of metabolic states on signal transduction.

THE COMPLEXITIES OF IDENTIFYING AND TARGETING ONCOGENE ADDICTION

The notion that recurrent genetic lesions perturbing suspected (or *bona fide*) oncogenes mark potential therapeutic targets is based on the oncogene addiction paradigm, which dictates that a cancer cell's survival becomes unusually dependent on the activity of a single gene product (Weinstein, 2002). Accordingly, a number of therapeutic targets have been uncovered through focused or large-scale genomic profiling of human tumours. This approach has guided the successful development (or repositioning) of a handful of FDA-approved agents, such as imatinib for the treatment of mutant KIT (Hirota *et al*, 1998) and mutant PDGFRA (Heinrich *et al*, 2003b) gastrointestinal stromal tumours (Demetri *et al*, 2002; Heinrich *et al*, 2003a), vemurafenib for the treatment of mutant-BRAF melanoma (Davies *et al*, 2002; Bollag *et al*, 2010; Chapman *et al*, 2011), or crizotinib for the treatment of non-small cell lung carcinomas (NSCLCs) with anaplastic lymphoma kinase (ALK) translocations (Soda *et al*, 2007; Shaw *et al*, 2013). Although this genotype-to-phenotype strategy has provided clinical proof-of-concept for the oncogene addiction model, the approach has not always been successful.

In some instances, the clinical validation of addiction to a number of oncogenic drivers, for example, genomically amplified or mutated MYC proteins, mutationally activated ETS-family transcription factors, or mutant forms of RAS, has not been possible because pharmacological approaches to directly inhibit these types of targets have been challenging and/or insufficiently explored.

In the case of transcription factors, and with the exception of ligand-regulated nuclear receptors, no compounds until today have been described that directly bind to allosteric sites on this class of proteins to modulate their transcriptional activity. Instead, attempts have been made to indirectly inhibit their function by perturbing protein–protein or protein–DNA interactions.

In prostate cancer, the TMPRSS2-ERG fusion is found in ∼50% of all tumours, making it the single most common genetic lesion of this disease (Taylor *et al*, 2010; Yu *et al*, 2010). Unfortunately, only tool compounds such as DB1255 (Nhili *et al*, 2013), which modulates the ERG/DNA complex have so far been produced. Furthermore, the *in vitro* anti-tumour activity of DB1255 is yet to be demonstrated.

In the case of mutant RAS, a recent re-evaluation of RAS structure has finally identified a druggable pocket in one particular mutant form of RAS (Ostrem *et al*, 2013), namely RAS G12C. In this study, Ostrem *et al* described the synthesis of a series of compounds that specifically and covalently bind to the mutant cysteine in RAS G12C and prevent its activation. Furthermore, these compounds were shown to have *in vitro* anti-tumour activity in RAS G12C-dependent cell lines. However, the low potency of these inhibitors limits their *in vivo* utility, and further optimisation will be required before this class of compounds can be advanced into clinical testing.

Although currently 'undruggable' targets account for a fraction of potential molecular vulnerabilities identified through genomic profiling, not all oncogene addictions have failed clinical validation because of the unavailability of targeting agents. *De novo* resistance

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to inhibitors of the epidermal growth factor receptor (EGFR), for example, is well documented in glioblastoma, despite a 40% incidence of EGFR-activating lesions in this disease (Mellinghoff *et al*, 2005; Cancer Genome Atlas Research Network, 2008). This is in contrast to NSCLCs that carry activating EGFR mutations, where EGFR kinase inhibitors promote remarkable clinical responses (Rosell *et al*, 2009). These observations raise a number of interesting questions regarding oncogene addiction: (1) Do recurrent oncogenic lesions fail to create oncogene addiction in some contexts? (2) Can co-existing mutations create a pharmacological obstacle that mediates drug resistance without functionally relieving the cells from addiction? and (3) Is *de novo* drug resistance simply a reflection of inadequate pharmacological targeting?

TO BE OR NOT TO BE ONCOGENE ADDICTED

To answer the question of whether all recurrent lesions create oncogene addiction one must consider the following. Since it is not possible for functionally silent mutations to be positively selected during the natural history of a tumour, then, by definition, all recurrent mutations with high enough frequencies are functional. Given that oncogene addiction is defined operationally, it is of critical importance that a standardised set of criteria (akin to the definition of a RECIST clinical response) is considered when scoring addiction in experimental models. As originally described, targeting oncogene addiction should result in either the death or terminal differentiation of a cancer cell (Weinstein, 2002). Therefore, while inhibiting the activity of any recurrently mutated oncogene will likely have measurable functional effects (e.g., cytostasis), not every recurrent lesion will necessarily result in oncogene addiction as measured by death and differentiation following treatment with a targeted agent.

MUTATIONS, FRIENDS AND FOES OF TARGETED THERAPIES

Because cancer results from the accumulation of multiple mutations, there is a possibility that the mutational makeup of a cancer cell will be comprised of some elements that create a pharmacological vulnerability, and others that, while functionally synergistic, could mask this vulnerability. Therefore, it is possible that co-existing mutations could render a therapeutic ineffective against an otherwise valid target. Our current view of EGFR inhibitor resistance in glioblastoma provides an interesting example of this paradox.

Although EGFR mutations occur with high frequency in glioblastomas, loss of at least one copy of the tumour suppressor PTEN almost invariably accompanies these lesions (Cancer Genome Atlas Research Network, 2008; Vivanco *et al*, 2010) and is associated with the failure of EGFR inhibitors to elicit a clinical response in these tumours (Mellinghoff *et al*, 2005). A number of years ago, while trying to understand the molecular basis of this problem, we found through a series of biochemical experiments that loss of PTEN impaired the ubiquitination-induced degradation of activated EGFR leading to a net gain in the amount of cellular EGFR activity (Vivanco *et al*, 2010). This defect, in turn, resulted in a right shift in the biochemical and biological response to EGFR kinase inhibitors. Importantly, more complete inhibition of EGFR through exposure to higher levels of inhibitor or efficient knockdown of EGFR was able to overcome this type of resistance, suggesting that PTEN loss did not relieve EGFR-dependent cells from their addiction to EGFR. Although our study provided evidence that EGFR could still be a therapeutic target in PTEN-deficient tumours, it presented us with another pharmacological challenge: how to effectively suppress EGFR using clinically relevant drug doses.

DRUGS THAT DO NOT KILL *VS* CELLS THAT WOULD NOT DIE

Recognising the difference between a pharmacological inadequacy and the lack of oncogene addiction is one of the major challenges in deciding what might constitute a worthy therapeutic target. In the case of EGFR, our data suggested that while EGFR inhibitors have so far been unsuccessful in the treatment of glioblastomas, pharmacological refinement of currently available EGFR targeting agents could improve clinical outcomes.

Epidermal growth factor receptor mutations seem to almost exclusively target the kinase domain (KD) in lung cancer, but target the extracellular domain (ECD) in glioblastoma. We and others (Barkovich *et al*, 2012; Vivanco *et al*, 2012) have found that ECD and KD mutations cause distinct shifts in the conformational equilibrium of the KD, such that each type of mutation favours binding of one type of ATP competitive inhibitors, while limiting the binding and thereby the effectiveness of others. These differing conformations offer an explanation for why lung cancers that carry EGFR KD mutations respond so well to type I inhibitors (those that preferentially bind to the open conformation) such as erlotinib and gefitinib, while glioblastomas with ECD mutant EGFR fail to respond to this class of compounds, but respond (at least in experimental models) to type II inhibitors (those that bind the closed conformation), such as lapatinib and neratinib (Vivanco *et al*, 2012).

A similar dichotomous pattern of response has been observed with the two most common ALK mutations found in neuroblastoma, Alk-F1174L and Alk-R1275Q. Each of these mutants accounts for ~40% of all ALK mutations in this disease, and shows a selective response to either type II inhibitors like crizotinib, as in the case of Alk-F1174L, or type I inhibitors like TAE684, as is the case for Alk-R1275Q (Bresler *et al*, 2011; Epstein *et al*, 2012).

Interestingly, our study (Vivanco *et al*, 2012) also found that even type II inhibitors were unable to induce cell death unless used in concentrations that can cause near-complete inactivation of EGFR. This requirement for potent inhibition of target is not unique to EGFR. Bollag *et al* (2010) has previously shown that in order for the BRAF inhibitor vemurafenib to elicit a proper clinical response in melanoma, a dose capable of inhibiting BRAF by >80% was needed. However, in the case of EGFR, we found that treatment of glioblastoma patients with the standard dose of lapatinib (750 mg p.o. b.i.d.) was insufficient to reach intratumoural drug concentrations >1 μM, which were necessary to induce a cytotoxic response in experimental models. Additionally, we found that in a preclinical model, a pulsatile high-dose schedule of lapatinib was superior to standard daily dosing. The clinical viability of such an alternative high-dose pulsatile schedule is currently being evaluated in a phase II lapatinib clinical trial (NCT01591577) for newly diagnosed glioblastoma patients.

Collectively, these data highlight the importance of (1) the use of quantitative tumour pharmacodynamic measurements to identify the level of target inhibition required for an optimal response, and (2) the implementation of various dosing schedules during early-phase clinical trials to find the proper dose that can achieve the desired level of target inhibition.

THE PROBLEM OF ACQUIRED RESISTANCE

Although the recognition of oncogene addiction as an effective platform for the identification of cancer-selective vulnerabilities has been the main force behind most of the recent advances in precision medicine, the problem of acquired and *de novo* resistance to targeted therapies remains a major obstacle in achieving

long-term responses. Every initial success has been inevitably followed by the emergence of drug-resistant disease. Interestingly, in almost all cases of acquired drug resistance, the molecular mechanism responsible for the relapse has involved the reactivation of either the target itself, or the signalling pathway that it regulates, thus reinforcing the notion of true addiction.

The current strategy to overcome acquired resistance involves molecular profiling of the resistant tissue, either from a relapsed patient or from *in vitro* or *in vivo* models that have been made resistant through chronic exposure to drug, in hopes that resistance factors can be identified and pharmacologically targeted. Although this approach has yielded effective secondary therapies in a number of cases, resistance to these compounds also invariably ensues, leading to a new iteration of the approach to identify subsequent lines of therapy. This success/failure cycle raises an important question: Do cancer cells have additional molecular dependencies that can be targeted concurrently to minimise the potential for the emergence of resistance?

ONCOGENIC SIGNALLING AND METABOLIC ADDICTION

The molecular routes used by cancer cells to generate energy and support biosynthesis differ from those employed by normal cells. Warburg (1956) noted that cancer cells prefer to break down glucose through glycolysis, even in the presence of sufficiently high concentrations of oxygen to support oxidative phosphorylation. This metabolic behaviour, now known as the Warburg effect, renders most cancer cells addicted to glucose. Furthermore, cancer cells are also more dependent on fatty acid synthesis and glutaminolysis. These metabolic requirements represent an additional pharmacological liability for cancer cells, but more importantly, the co-existence of metabolic and oncogene dependencies could offer a unique opportunity for therapeutic strategies that simultaneously take advantage of two distinct types of molecular addictions.

Although conceptually appealing, whether such therapeutic approach will perform better than individual targeting of each separate addiction might depend on whether these two seemingly independent phenotypes (i.e., oncogene and metabolic addictions) are in fact channelled through non-overlapping molecular pathways. However, a number of studies suggest that cancer-associated changes in cellular metabolism are a direct consequence of oncogenic signal transduction (Table 1) rather than an adaptive response to the increased metabolic demands of oncogenic growth. For example, 10 years ago, Elstrom *et al* (2004) showed that constitutive activation of the oncogenic serine/threonine kinase AKT can induce the Warburg effect and render cells addicted to glucose. Similarly, activating BRAF mutations increase glycolytic output in melanoma cells (Haq *et al*, 2013). Subsequent work by Wise *et al* (2008) showed that activation of the MYC oncogene promotes mitochondrial glutaminolysis and glutamine addiction, and a more recent study by Son *et al* (2013) has found that a

non-canonical glutaminolysis pathway that uses aspartate transaminase instead of glutamate dehydrogenase may be responsible for glutamine addiction in mutant-KRAS pancreatic ductal adenocarcinoma cells. These findings provide evidence of oncogene-initiated metabolic addictions and raise the question of whether targeting these dependencies could simply provide an alternative way of inhibiting the addicting oncogenic lesion.

More recent evidence of oncogene-mediated metabolic rewiring comes from an elegant study by Ying *et al*. Using an inducible mouse model of pancreatic cancer, this group showed that oncogenic RAS caused the metabolic reprogramming of tumour cells through a number of specific transcriptional changes that affected the expression of glucose transporter and various rate-limiting enzymes (Ying *et al*, 2012). These metabolic changes not only involved an increase in glycolytic flux and ribose biosynthesis, but also a rechannelling of glycolytic intermediates into the hexosamine biosynthesis and pentose phosphate pathways. Importantly, interruption of transgene expression in this model caused complete tumour regression, consistent with RAS addiction.

A later study by Palorini *et al* (2013) identified a functional link between some of the same RAS-induced metabolic changes described in the Ying manuscript and glucose addiction. Their study found that glucose deprivation caused cell death in RAS-transformed 3T3 mouse fibroblasts and RAS-mutant breast cancer MDA-MB-231 cells by inhibiting the hexosamine biosynthetic pathway. These findings suggest that glucose addiction, rather than existing as a parallel and independent phenotypic entity, might in fact, be a 'downstream' effect of RAS addiction. However, the linear nature of this relationship may not necessarily undermine the utility of concurrently targeting two linearly related addictions.

Consistent with this notion, the US FDA recently granted accelerated approval for the combined use of the BRAF inhibitor dabrafenib and the MEK inhibitor trametinib in the treatment of mutant-BRAF melanoma, a decision based on the results of an open-label phase I/II clinical trial in which combination treatment nearly doubled the duration of response and significantly improved overall response rates when compared to dabrafenib alone (Flaherty *et al*, 2012). These drugs target two concurrent oncogene addictions in these tumours, both of which are a direct functional consequence of mutational BRAF activation. Such an approach can not only achieve a greater degree of MAPK signal output inhibition, but may also lessen the degree of negative feedback relief caused by acute BRAF suppression (Lito *et al*, 2012). Of note, despite the improvement on the durability of the response, patients on combination therapy eventually relapsed with drug refractory disease.

Although the dabrafenib/trametinib trial provides good evidence that stronger perturbation of a molecular addiction and a higher therapeutic index can be both achieved through drug combinations that target multiple nodes within a single addicting pathway, it also demonstrates that this approach alone may not be sufficient to prevent the emergence of acquired drug resistance. However, it remains possible that co-targeting the metabolic component of the addiction could deliver a harder blow to the cancer cell because energy production and biosynthesis are distal to the oncogenic lesion, and more proximal to direct regulators of cell survival and apoptosis.

With the objective of effectively inhibiting glycolysis while exploiting oncogene-induced metabolic programs to maximise the therapeutic window, efforts have been made to develop pharmacological agents that target rate-limiting enzymes. One in particular, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3 (PFKFB3), has been an attractive target because its expression seems to be differentially induced during oncogenic transformation. The enzyme PFKFB3 is overexpressed in a number of human cancers (Atsumi *et al*, 2002) and produces fructose-2,6-bisphosphate, a potent stimulator of glycolysis. Advanced Cancer

Table 1. Oncogenes that rewire cellular metabolism and generate metabolic addictions

Activated oncogene	Associated metabolite addiction	Reference
BRAF	Glucose	Haq *et al*, 2013
KRAS	Glucose/glutamine	Ying *et al*, 2012/Son *et al*, 2013
AKT	Glucose	Elstrom *et al*, 2004
MYC	Glutamine	Wise *et al*, 2008

Therapeutics has recently gained FDA approval to commence a phase I clinical trial (NCT02044861) to test the safety of a small-molecule PFKFB3 inhibitor called PFK158 in patients with advanced solid tumours. Furthermore, they have reported that PFK158 can synergise with vemurafenib to induce mutant-BRAF melanoma cell death (O'Neal *et al*, 2014) in experimental models. Whether this synergy will translate into improved clinical outcomes, including the delay or prevention of acquired drug resistance, remains to be seen.

THE INFLUENCE OF NUTRIENTS ON ONCOGENIC SIGNAL TRANSDUCTION; WHEN METABOLISM TALKS BACK

The influence of oncogenic signal transduction on cellular metabolism has been and continues to be of great interest to basic and translational cancer biologists. One related area that has been relatively neglected in this regard is the study of how metabolic states might influence the nature of the oncogenic signal and its biological manifestation. If we are to understand the molecular basis of a particular response to a targeted therapy, or the potential routes to acquired resistance, it is critical that we understand the exact biochemical consequences of perturbing a particular addiction.

A few studies published within the last 3 years highlight the importance of this understanding. First, using a network-based approach, Komurov *et al* (2012) analysed global gene expression data from a HER2-dependent breast cancer cell line called SKBR3, and an isogenic derivative that was made resistant to the HER2 inhibitor lapatinib by chronic exposure to gradually increasing doses of the drug. They found that lapatinib resistance in this model was associated with an increase in the expression of glucose deprivation response network genes, including those that regulate glucagon signalling, glucose uptake, gluconeogenesis and the unfolded protein response. In parental cells, lapatinib treatment led to a decrease in glycolysis suggesting that this metabolic process was HER2-dependent. Interestingly, inhibition of glycolysis through glucose depletion conferred protection against the cytotoxic effects of lapatinib in two independent HER2-dependent cell lines. This observation is at odds with the notion that concurrent targeting of oncogene and metabolic addictions might provide a response that is superior to individual treatment, and raises the question of how the metabolic state of the cell might alter the outcome of perturbing an oncogene addiction. In fact, this anti-correlation seems not to be restricted to oncogenic kinases or glycolytic addiction. Work by Qing *et al* (2012) has demonstrated that in N-MYC-addicted neuroblastoma cells, which are concomitantly dependent on glutaminolysis for survival, glutamine-withdrawal-induced cell death can be significantly rescued by N-MYC inactivation.

To understand the signal transduction changes that accompany the cell-death-inducing perturbation of glucose addiction, Graham *et al* (2012) used a mass spectrometry-based approach to interrogate the changes in the phosphotyrosine proteome that follow glucose deprivation. The study found that in glucose-dependent cell lines derived from glioblastoma, sarcoma and melanoma, depletion of glucose caused a supraphysiological increase in phosphotyrosine signalling promoted by a positive feedback that involves ROS-mediated inactivation of tyrosine phosphatases. These findings provide a potential explanation for the observed antagonistic effects of lapatinib treatment and glucose deprivation, since HER2 inhibition might provide a means to buffer or normalise the death-inducing increase in phosphotyrosine signalling that results from the inhibition of glycolysis.

CONCLUSIONS

Although in the last 10 years significant progress has been made in the identification and classification of oncogenic lesions that mark potential therapeutic targets, validation of these targets using pharmacological tools has suffered from an incomplete understanding of what represents meaningful target inactivation. The systematic evaluation and comparison of compounds with distinct modes of inhibition and/or different conformation specificities should provide a very powerful approach to evaluate the best targeting strategy and interrogate the existence of a molecular addiction.

The example of *de novo* EGFR inhibitor resistance in glioblastoma illustrates the importance of pharmacodynamics measurements in the assessment of oncogene addiction and in choosing the appropriate dosing scheme to achieve a relevant level of target inactivation that can elicit a meaningful biological response.

Although targeting oncogene addiction has proven to be a successful way to treat certain tumours, acquired drug resistance remains a major challenge. The altered metabolic state of cancer
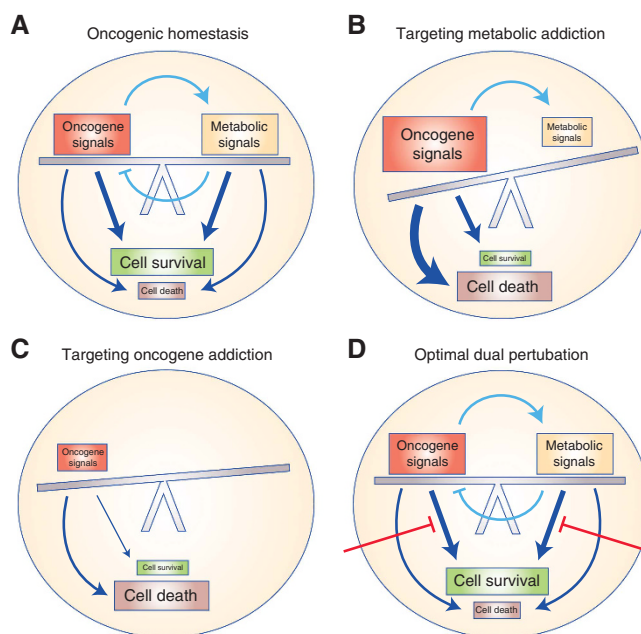
Figure 1. A cancer cell's circle of life. (A) In cancer cells, homoeostasis is maintained by the effective balance between oncogenic signals that promote accelerated growth and survival and prevent cell death, and metabolic signals that provide the necessary energy and biosynthetic power to enable proliferation and survival. An additional set of functional interactions between oncogenic and metabolic signals fine tunes the homoeostatic balance and allows the system to maintain an appropriate output. Inhibition of either set of signals, has the potential to throw the system out of balance and cause death. (B) By targeting metabolic addiction, available experimental data suggest that the oncogenic signalling output is overstimulated and becomes toxic, perhaps by a disproportional increase in pro-apoptotic signals. (C) By targeting oncogene addiction, the cells lose important survival signals from both the oncogenic lesion itself and the metabolic programs it regulates. Resistance could emerge if oncogenic signals are restored through mutation or pathway reactivation. (D) The most efficient way to kill a cancer cell is to inhibit the specific survival signals generated by both the oncogene and the metabolic process associated with oncogene activation. This can only be achieved if the molecular identity of these signals is well characterised.

cells and the acquired dependence on specific metabolic processes such as glycolysis provide an additional level of targetable molecular addictions. However, in order to effectively prevent or delay the emergence of resistance through concurrent targeting of metabolic and oncogene dependencies, it is important to first understand the consequences of metabolic perturbations on oncogenic signal transduction (Figure 1A–C). The studies discussed in this review suggest that perturbation of a metabolic addiction could negatively impact the therapeutic benefit of targeting oncogene addiction (Figure 1B). To circumvent this problem, one could potentially use time-staggered treatments to temporally segregate the cytotoxic effects of individual agents, but precise pharmacokinetic and pharmacodynamic measurements will be of paramount importance for the proper implementation of this approach. Lee *et al* (2012) has demonstrated, for example, that sequential (but not concurrent) treatment of triple-negative breast cancer cell lines with EGFR kinase inhibitors and DNA-damaging agents is cytotoxic. Ultimately, the molecular characterisation and comparison of the pathways that mediate cell death following the perturbation of either oncogene addiction or metabolic addiction should provide a clearer picture of the therapeutic opportunities afforded by a combination approach (Figure 1D).

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