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Molecular perspectives on the non-responder phenomenon

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With the advent of targeted therapies promising to revolutionise the nature and success of patient care, the field of clinical oncology is facing a highly exciting future. While much of this enthusiasm comes from the hope for improved patient outcomes, a review of clinical response/relapse rates for current therapies provides a more sobering perspective. Given that the majority of patients are intrinsically resistant to the therapeutic potential of these molecules, efforts are now directed at characterising such non-responsive system behaviour and causative molecular insults. Testament to this is an expanding catalogue of target and system-based aberrations, often defined through retrospective analyses of clinical tissue and associated outcome data. What has emerged is a complex picture, where numerous potential sources of cancer-specific aberration can contribute to refractory tumour behaviour. Clinicians, regulators and sponsors must now collaborate to determine how such knowledge should be used to enhance the clinical decision process and associated regulatory guidance.

Introduction

While there are many great scientific pursuits, perhaps the greatest of all derives from our attempts to decipher how genotype elicits phenotype. From a very basic perspective the phenomenon can be seen to result from a complex interplay between two disparate physiochemical milieus, the intracellular and extracellular environments. Understanding how extracellular-derived information – be it natural or exogenous (e.g. a drug) – is converted into a cellular and physiological response, is essential to defining the path from molecular insult to clinical presentation. In addition, such knowledge is essential to defining therapeutic strategies and to understanding why certain therapies fail under specific, commonly occurring, genetic/proteomic contexts. With the expanding characterisation of cancer genomes and a growing catalogue of resistance-associated aberrations, we are accumulating the knowledge required to design therapeutic modulators of the genotype to phenotype transition. While the enormity of the inherent challenge cannot be ignored, neither can the prize; a prize that promises a future of patient-tailored intervention strategies with vastly improved response rates.

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Molecular sources of the non-responder phenomenon

Deciphering the molecular bases of clinical response is fundamental to understanding the non-responder phenomenon. Despite the great complexity of this process, we can tackle it using a simple conceptual framework. Assuming that a diseased cell is in a non-quiescent state, at least three classes of component must interact to elaborate a therapeutic effect, namely (a) the drug(s), (b) the target(s), and (c) factors that affect, or are affected by, the drug or target (e.g. downstream proteins or regulatory microRNAs). Even the slightest change in structure or abundance of these components can have serious implications for how a patient responds to a drug. Responsiveness can therefore be mechanistically associated with a diversity of target-based and/or system-based aberrations. Target-based resistance typically occurs through structural or quantitative imbalances in the drug-to-target relationship. System-based sources, by contrast, arise from aberrations elsewhere in the molecular context of the drug and/or its target(s) (see Figure 1). We can further divide system-based resistance into two subclasses, the first affecting components of the disease-pathway – here termed 'pathway-dependent resistance' – and a second class that primarily affects the passage of drug to the diseased system (i.e. components of the absorption, distribution, metabolism, elimination (ADME) machinery)—here termed 'pathway-independent resistance'.

1359-6446/06/$ - see front matter doi:10.1016/j.drudis.2009.01.005

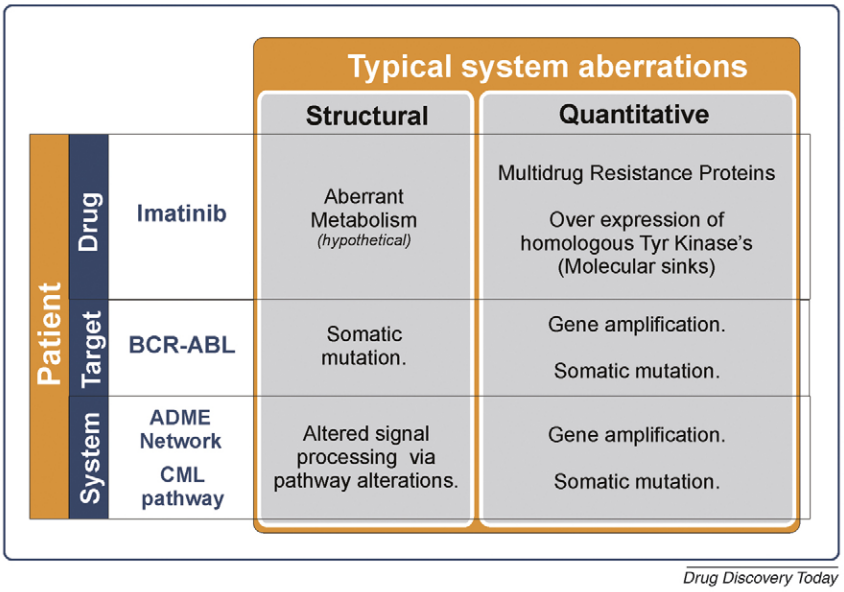

FIGURE 1

Using imatinib/BCR–ABL as an example, this figure highlights how genomic aberrations can lead to changes in the structure and abundance of factors (i.e. drug, target and pathway components) at all levels of the therapeutic system. Such effects can lead to refractory response to a drug in clinical settings.

Not surprisingly, each of these mechanisms poses a particular problem in oncology, where complex aberrations can evolve and propagate over time. A clinically pertinent example has emerged in the treatment of certain populations of chronic myeloid leukaemia (CML) patients with the tyrosine kinase inhibitor, imatinib mesylate [1,2]. While imatinib has rightfully secured a first-line status in the treatment of CML, the emergence of two classes of clinical resistance to the drug is proving particularly problematic. Primary resistance is observed in patients that fail to achieve sufficient clinical response after initial dosing, while secondary resistance is observed in patients who, despite continued imatinib treatment, relapse after achieving clinically complete remission. Viewed from totality of the diseased patient, many of the aforementioned mechanisms have been causatively associated with this behaviour.

The path to target

Once ingested, imatinib must pass a labyrinth of proteins as it transits to the disease system. At this stage resistance can occur in a disease-pathway independent manner, with effective drug concentration being influenced by perturbations in plasma binding proteins (primarily α-1-acid glycoprotein [3]), multi-drug resistance proteins (e.g. *P*-glycoprotein [4]), metabolising enzymes (e.g. CYP3A4 [5]), and possibly even homologues of the drug-binding domain within BCR–ABL, via 'molecular sink' effects (e.g. KIT [6]). By affecting the amount of drug reaching the disease system, inter-patient variation in components of ADME pathways (sometimes also mediated by co-medications) can potentially lead to non-responsive disease. This view is supported by studies that correlate low plasma levels of imatinib with poorer patient response. Picard *et al.* [7] have shown, for example, that median imatinib plasma concentration at steady state is higher in patients with a major molecular response (MMR) than in patients without. Clinical studies are presently underway to validate the benefit of higher dose imatinib in a larger patient setting. Related work by Blasdel *et al.* has further suggested that therapeutic drug monitoring may be useful in optimising targeted therapy in this context [8]. Here, worrying side effects such as erythropoietin-resistant anaemia led to the suspicion that toxic levels of imatinib may be accumulating in certain patients, a hypothesis they subsequently validated. While maybe not always leading to dose adjustment, these observations suggest that disease pathway-independent resistance may, at least under certain circumstances, be redressed through monitoring and maintenance of drug plasma levels within the correct therapeutic range.

When a drug meets its target

Having reached the disease pathway, the efficacy of a drug can be positively and negatively affected by variations in target protein structure or abundance, caused by single nucleotide polymorphisms (SNPs) or copy-number variations (CNVs), respectively. In the case of imatinib, Gorre and colleagues [9] were the first to detect such aberrations in a study comprising only nine non-responsive patients. Here, *bcr-abl* CNVs were found in three patients, with the remaining six harbouring a single mutation, corresponding to Thr315 (BCR–ABL^{T315I}), within the kinase domain. In the wild-type protein, this threonine commands a gatekeeper position at the periphery of the nucleotide-binding domain, and participates in a crucial H-bond interaction with imatinib [10–12]. Substitution with isoleucine abrogates this function and sterically hinders imatinib binding, rendering BCR–ABL^{T315I} refractory to inhibition. More than 90 additional mutations have since been identified, with mutations in Gly250, Tyr253, Glu255, Thr315, Met351 and Phe359, accounting for 60–70% of cases, many of which have been biologically characterised [13–18]. These mutations can act either directly or indirectly to effect imatinib binding and induce resistance. Substitutions such as T315I and F317L are clustered around

the imatinib binding site and act by directly abolishing important H-bond interactions or lipophilic contacts, or as a result of conformational changes that sterically oppose imatinib binding. Indirect mechanisms capitalise on the fact that imatinib binds to an inactive form of BCR–ABL, often referred to as the 'DFG-out' conformation in which an Asp–Phe–Gly triad is inverted out of its kinase-active position. Point mutations in this triad or *P*-loop residues (e.g. Gly250, Tyr253, Glu255) tend to destabilise the drug-induced inactive conformation shifting the free energy of the imatinib–BCR–ABL complex in favour of an active conformation [19].

Many of these target-based aberrations also represent a major source of relapse due to emergent secondary resistance. Interestingly, evidence suggests that imatinib may provide a selective growth advantage to clonal subpopulations of leukaemic cells harbouring such mutations [20]. These cells, which appear to function independently of BCR–ABL activity, are causative suspects for the re-emergence of disease in patients who have terminated imatinib treatment. Of particular relevance is the fact that BCR–ABL has been linked to the production of reactive oxygen species, causing increased DNA damage, a problem confounded by additional kinase-dependent effects on the DNA repair machinery [21,22]. It has thus been suggested that BCR–ABL directly powers the accumulation of DNA damage in leukaemic cells and that this manifests in the form of imatinib-resistant progressive disease. These observations stress the importance of depleting leukaemic stem cell populations as soon as possible after initial diagnosis, a strategy that should be aided by the recent development of second generation BCR–ABL inhibitors such as dasatinib (BMS-354825; Bristol-Myers Squibb), nilotinib (AMN107; Novartis), AZD0530 (AstraZeneca), Bosutinib (SKI-606; Wyeth) or INNO-404 (Innovive).

The epidermal growth factor receptor (EGFR) family (for review see Ref. [23]) has also been particularly instructive of the molecular mechanisms leading to non-response. Today, after more than two

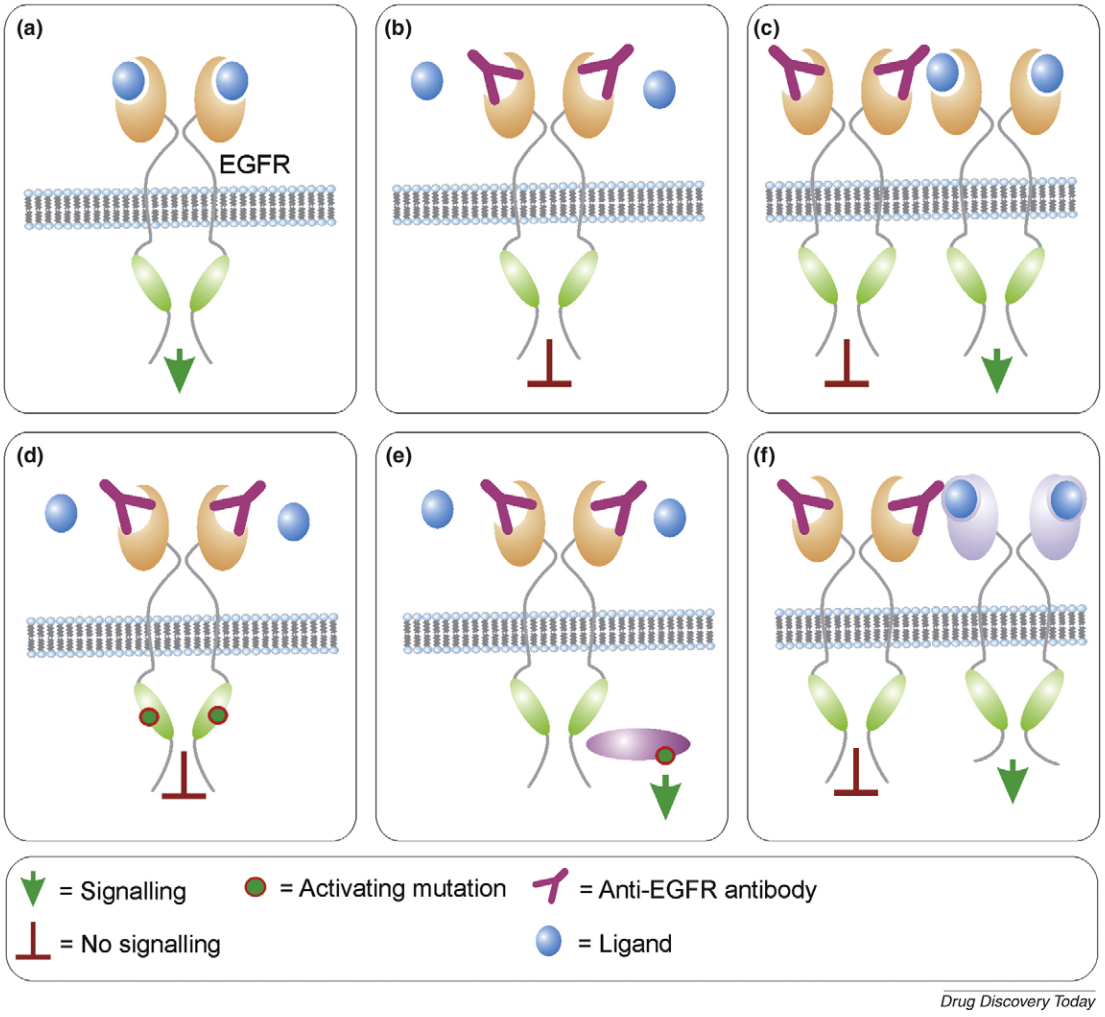

FIGURE 2

Schematic overview of **(a)** wild-type EGFR, (b) the effect of anti-EGFR antibodies on its function and **(c–f)** the effects system components on this activity. As depicted in (c), heavy tumour-load can lead to imbalance in the quantitative relationship between drug and target. Higher concentrations of drug may address this problem. Also noteworthy are the effects of cetuximab on kinase-mutated EGFR receptors (d), with preliminary results suggesting clinical efficacy in this context. By contrast, the effects of downstream mutations on proteins such as KRAS or BRAF (panel e) can render tumour cells insensitive to therapy. Such resistance has also been attributed to the presence of homologous proteins such as ERBB3 [42] (panel f).

decades of development, five EGFR antagonists have been approved for the treatment of five metastatic epithelial cancers: non-small-cell lung cancer (NSCLC), pancreatic cancer, squamous-cell carcinoma of the head and neck, breast cancer and colorectal cancer. EGFR is an interesting target in that small molecule and antibody-based inhibitors have been approved, the former targeting the intracellular kinase domain (e.g. erlotinib, gefitinib), while the latter target the extracellular domain (e.g. cetuximab, panitumumab). A variety of perturbations have been described that confer either sensitivity or resistance to these drugs (depicted in Figure 2; reviewed in Ref. [24]). In NSCLC, for example, 'activating mutations' in the kinase domain of EGFR (Figure 2d) have been shown to sensitise tumours to EGFR tyrosine kinase inhibitors (EGFR-TKIs). Such mutations tend to cluster around the ATP-binding pocket, with about 80% consisting of either a single missense mutation (L858R) or nested in-frame deletions (delE746-A750 and variants thereof) [25–29]. Numerous prospective Phase II studies using erlotinib or gefitinib in NSCLC patients harbouring *egfr* mutations have revealed astounding response and disease control rates of about 85%. Impressive progression free survival (PFS) rates of 7.7–14.0 months are also reported, significantly exceeding the PFS rates of 4–6 months reported for unselected patients treated with other chemo-/targeted-therapies [30–32]. Comparative studies are now required to validate EGFR-TKIs prospectively as the treatment choice for patients with kinase-activated EGFR mutants. Notwithstanding, many patients do still relapse owing to the emergence of secondary somatic mutations (e.g. T790 M), which reduce EGFR-TKI binding [33]. As we have just seen in the case of CML, this phenomenon exemplifies the enormous challenge in treating what is essentially a molecularly heterogeneous and ever evolving disease.

The efficacy of EGFR-TKIs in treating tumours with kinase-activated EGFR mutants suggests that these tumours may be somehow 'addicted' to the enhanced signalling capacity of the receptor; however, only few cancers have been shown to harbour such mutations to any significant degree (see Table 1). In a study of 98 patients with colorectal cancer, for example, Lee *et al.* failed to identify any kinase mutations at all, and this despite the fact that cetuximab targets EGFR in this indication. Recent studies on a subgroup of cetuximab responders have however identified a potential alternative mode of action, this time dependent on attenuated receptor signalling. In a study of 32 patients, Gonçalves *et al.* described 11 responders, all of whom possessed a variant of exon 13, causing a conservative R521K substitution within the extracellular domain [34]. Previously identified in glioma and lung cancer, this mutation has been shown to reduce TGF-α binding and EGFR signalling. These findings have led to the speculation that altered ligand-binding specificity and/or attenuated signalling capacity may increase the sensitivity of cells to targeted receptor inhibition. Given that the R521K mutation may also positively influence drug binding and/or effect, such hypotheses await further clinico-molecular insight. It also remains to be seen why certain cancers appear susceptible to *egfr* mutations (e.g. NSCLC), while others remain apparently unaffected (e.g. gastric cancer).

TABLE 1

Synopsis of EGFR mutation rates found across diverse cancers types, determined in numerous independent studies. It is noteworthy that certain cancer types appear more susceptible to acquiring such mutations than others.

Cancer type	EGFR mutation rate in patient population	Reference
<5% Mutation rate detected		
Acute adult leukaemia	0/88	[58]
Breast	0/42	[58–60]
	0/11	
	1/93 (1.1%)	
Colon	0/98	[58]
	1/293 (0.34%)	[61]
Gastric	0/185	[58]
Glioblastoma	0/59	[61]
Ovarian	2/57 (3.5%)	[62]
Other	9/566 (2%)	[63]
Pancreatic	2/55 (4%)	[64]
≥5% Mutation rate detected		
Barrett's oesophagus	3/21 (14%)	[64]
Cholangeocarcinoma	3/22 (14%)	[65]
Oesophageal adenocarcinoma	2/17 (12%)	[64]
Head and neck	17/108 (16%)	[66]
	1/100 (1%)	[67]
	3/41 (7%)	[58]
NSCLC (adenocarcinoma)	2/40 (5%)	[63]
NSCLC (bronchioadenocarcinoma)	6/33 (18%)	[63]

From target function to disease pathway context

Recent work has further expanded our appreciation of the non-responder problem beyond the level of drug target, to include its functionally associated partners (i.e. pathway-dependent resistance). Our first insights have come from point mutations in genes encoding pathway components such as KRAS and p53. Lièvre *et al.* were the first to describe the negative impact of *K-ras* mutations on patient response/prognosis to cetuximab [35]. No *K-ras* mutations were detected in tumours from 11 cetuximab-responding patients, whereas 13 of the 19 non-responders ($p = 0.0003$) had a *K-ras* mutated tumour. Similar effects are also seen in panitumumab treated patients, meaning *K-ras* mutational status can be used to define those patients most likely to achieve clinical benefit from approved anti-EGFR antibodies. The European Medicines Agency has welcomed these findings by approving panitumumab for use in mCRC patients whose tumours harbour wild-type KRAS. Still, this effectively defines around 85% of patients for whom the drug will probably fail.

K-ras mutations account for 30–40% of non-responsive patients, raising questions about other causative insults. Hypothesising that other members of the RAS-RAF-MAPK pathway may be involved, Di Nicolantonio *et al.* identified a *BRAF V600E* mutation in 11 of 79 patients who had a wild-type *K-ras* gene [36]. None of these patients responded to cetuximab/panitumumab treatment, while none of the responders carried this mutation. Not only did this reveal a strong clinically important epistatic relationship between EGFR and BRAF, but it also supports the development of rectifying combination therapies. Combined mutational analysis of *K-ras* and *BRAF* might therefore be used to define prospectively those mCRC patients eligible for such treatment. While still leaving about 40% of non-responders for which no causative aberration is known, the number and nature of genes with suspected involve-

ment in the phenomenon is increasing. *PTEN*, a gene involved in controlling AKT activation status is a prime candidate. Failure to downregulate AKT activity in response to EGFR-TKI treatment is typical of resistant tumour cells [37]. Although less than 10% of NSCLC patients possess mutations of the *PTEN* gene, about 70% bear no detectable expression, probably due to epigenetic promoter methylation [38]. Receptor proteins such as the insulin-like growth factor receptor (IGFR-1) [39], MET [40,41] and even the EGFR homologues ERBB2 and ERBB3 [42], have also been proposed to mediate resistance to anti-EGFR therapies.

Interestingly, despite the fact that EGFR is the primary target for five approved therapies, the comparative clinical effects of these molecules range from overlapping to distinct. This begs the question as to how, given their common target, might we explain this diversity of clinical effect among the different combinations of EGFR-antagonist:cancer-type treatment regimens? Why is response achieved in one EGFR-positive cancer type but apparently not so in others? To the best of my knowledge, no large-scale study has yet been pursued in an attempt to address these issues, so let us examine some of the factors that need to be considered, all of which could have an effect on how we interpret the sources of resistance for small molecule drugs. The first relates to the promiscuous nature of most small molecule kinase inhibitors. Recent chemoproteomic studies have elegantly emphasised the system-wide affinity of small molecule drugs for diverse components of complete proteomes. While chemical promiscuity is clearly a fact of life, many small molecule compounds were until recently belittled as 'dirty drugs'. But as more information emerges it appears that such system-wide promiscuity is an important characteristic of drug mode of action. Caffeine, the favourite small molecule of many scientists, is particularly instructive in this regard. Performing the following PubMed search, 'caffeine AND (bind* OR antagoni* OR agoni* OR inhibit*)' reveals just how systemically acting this molecule is. While many of the 7512 abstracts returned refer to caffeine's antagonism of the adenosine A(2A) receptor [43], many other structurally distinct binding partners are reported, including ryanodine receptors [44], calcium channels [45], and the ATM and PKC kinases (an effect that inhibits UV-induced NF-κB activation) [46].

Until recently, our appreciation of drug promiscuity remained obscured by reductionist approaches to single target assessment, but recent advances in proteomics technologies have thankfully provided more system-wide perspectives. Bantscheff *et al.*, for example, have recently developed a novel proteomics method based on 'kinobeads' [47]. This approach has been used for quantifying the system-wide promiscuity of kinase inhibitors *in vivo*. Using quantitative mass spectrometry to monitor binding of specific proteins to the beads as concentrations of a free kinase inhibitor are increased, the authors established binding constants for hundreds of kinases from multiple cell lines and tissues. Applying the method to imatinib, dasatinib and bosutinib highlighted not only the large degree of kinase binding promiscuity of these molecules but also a subset of potentially novel targets. Related efforts by the Zarrinkar laboratory have extended the coverage of kinase inhibitors assessed and revealed striking differences between the affinity profiles of established EGFR-TKI inhibitors including erlotinib and gefitinib [48]. These important observations tell us that marketed EGFR-TKIs are inherently multi-targeted, and also suggest that, for many indications, inhibition of a single kinase may not be sufficient to affect the disease process significantly. Indeed, there exists an ever-growing sentiment that interfering simultaneously with multiple protein activities (e.g. Sorafenib and Sunitinib—targeting VEGFR, PDGFR, FLT-3 and c-Kit) may be key to achieving higher rates of efficacy and response. We still have little understanding, however, as to how such multi-targeting might affect the likelihood of resistance.

A key question from these observations is whether the kinase complement of specific disease systems, when matched to the affinity profile of specific EGFR-TKIs, provides an additional rationale for the observed dichotomy of clinical response to these molecules. In other words, does the 'kinase content' of a cancer cell influence the clinical outcome, given that a kinase inhibitor will target a subset of the cancer-specific kinome? This is clearly an important question, as cancer is a molecularly diverse disease. Heterogeneity is found not only between the proteomes of different cancer types, single tumours can also harbour several different clonal populations, each with distinct proteomes, mutations and phenotype. Understanding to what degree the complementarity between compound affinity profiles and disease system proteomes plays a role in clinical response will be important to prioritise rationally specific cancers for clinical testing. Those with the highest degree of complementarity might be assumed to be inherently more responsive, although this may depend on the functionality of the individual kinases and their contribution to tumour progression. Several hypotheses have been proposed, for example, as to why the efficacy/response seems different for erlotinib and gefitinib in the similar BR.21 [49] and ISEL [50] Phase III NSCLC studies. While one explanation is differences in dosage/biovailability (erlotinib was used at the maximum tolerated dose, whereas gefitinib was provided at a lower dose), the importance of system-specific multi-kinase inhibition remains a compelling possibility.

Recent evidence has also allowed us to step beyond such 'kinase-centric' interpretation of kinase inhibitor activity to uncover an important new aspect of non-response; this time driven by enhanced understanding of EGFR biology. That EGFR expression levels are correlated with patient prognosis but not with anti-EGFR responsiveness suggests an EGFR kinase-independent effect on disease progression [51]. Supporting this premise is the observation that loss of EGFR kinase activity does not phenocopy loss of EGFR protein *in vivo* [52,53]. Moreover, while EGFR-TKI-based inhibition of kinase activity often leads to decreased cell proliferation [54], knockdown of the receptor causes cell death [55]. Building on these findings, Weihua *et al.* at the M.D. Anderson Cancer Center, Texas, found that EGFR functions to prevent autophagic cell death in a kinase-independent manner [56]. Strikingly, EGFR was found to interact with and stabilise the sodium/glucose co-transporter 1 (SGLT1), thereby enabling cells to extract the energy substrate, glucose, from their environment. Such function is purported to endow tumour cells with increased survival capacity, further suggesting that combined inhibition of kinase-dependent and kinase-independent receptor activities are necessary to derive full therapeutic potential. Since inhibition of SGLT1 might prove rather toxic, the dual inhibition of downstream receptor specific kinases may prove feasible in achieving this goal.

Clinical and regulatory implications

A key question for the community today is how to best translate such knowledge into actionable clinical and regulatory directives. One complication is that most data regarding the molecular sources of non-response have been obtained from retrospective analyses of previously completed clinical studies. Under such conditions an important question facing clinicians, regulators and trial sponsors is the level of evidence required to validate the clinical utility of a predictive molecular aberration (i.e. biomarker). Numerous variables need to be considered, such as the overall risk:benefit profile, safety issues and the availability of alternative therapies. While clinical oncologists have quickly embraced the significance of *K-ras* mutation as a theranostic biomarker for cetuximab and panitumumab treatment, regulatory directives from the FDA are still awaited. Notwithstanding, pharmacogenomics has been recognised as a key element of the FDA's Critical Path Initiative and, since then, a number of agency guidance documents have been issued to encourage and assist sponsors in evaluating the influence of molecular aberrations on treatment choice [57]. An important component of these guidelines is the encouragement to make Voluntary Genomics Data Submissions (VGCS) when provision of such data is not otherwise required.

The *K-ras*:cetuximab/panitumumab case has been instructive as to the regulatory challenges faced. The FDA has provided some of the following requirements regarding the retrospective analysis of such putative biomarkers:

- Associated trials must be adequately sized, well conducted and well controlled.
- The trial must be of sufficient sample size to ensure random allocation to each of the study arms of factors that were not used as stratification variables for randomisation.
- Tumour tissue obtained in ≥95% of the registered and randomised study subjects. Results available for assessment from ≥90% of these.
- Before analysis, the FDA must review the assay methodology and determine that it has acceptable analytical performance.
- Genetic analysis must be performed according to the qualified assay method by individuals who are masked to treatment assignment and clinical outcome results.
- Before analysis, the FDA must agree on analysis plan for hypothesis testing.

Thus, despite the fact that substantial clinical and biological evidence exists linking the *K-ras* mutation to negative pharmacologic response to anti-EGFR antibodies, many of these study controls are missing. For example, while retention of tumour samples is required for these studies, compliance has not been determined. Moreover, details regarding tumour acquisition and sample handling were not pre-specified and thus there is no way to ensure that accurate test results could be obtained. The ideal scenario from a regulatory perspective is one where development of the resistance-associated biomarker is an integral part of the drug development programme. Here, the clinical studies required to prove drug efficacy and those needed to establish the prognostic/predictive value of the aberration should optimally occur in tandem. Still, in a case such as *K-ras* where the data supporting a theranostic function are compelling, clinicians can still utilise the information as part of their clinical decision process.

Concluding remarks

Together, these insights demonstrate how many combinations of system variant can contribute to the existence and emergence of non-responsive disease. Such differences can range from factors affecting the pharmacokinetics of the therapy (e.g. mutations affecting specific cytochrome P450 s) to aberrations within primary or secondary targets and/or downstream factors mediating drug response. As a consequence, we now know that many diseases that phenotypically conform to a specific diagnosis can be distinct at the system level. Such a systemic vantage point provides a better understanding of why large percentages of treatment naïve patients are refractory to targeted therapies. The implications for clinical trial design are thus significant, with molecular diversity rendering many primary endpoints inevitable statistical failures. Hopefully, however, as we continue to catalogue the array of aberrations responsible for this behaviour, we will become less reliant on the retrospective identification of resistance factors. Future clinical trials, driven by advances in molecular profiling and *in silico* discovery technologies, may include prospective, hypothesis-driven designs that help us obviate the problems of such retrospective findings. Key to this goal will be the generation of cancer-specific disease models, where the functional importance of individual components can be modelled and assessed with respect to the drugs mode of action. System-based discovery is thus essential to reaching new levels of innovation in redressing the non-responder problem.

Acknowledgements

My sincere thanks to Hartmut Voss, Guillaume Taglang and Julien Gagneur for their helpful comment and critique.

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