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Acquired Resistance to Drugs Targeting Tyrosine Kinases

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Abstract

Resistance to chemotherapeutic drugs exemplifies the greatest hindrance to effective treatment of cancer patients. The molecular mechanisms responsible have been investigated for over fifty years and have revealed the lack of a single cause, but instead, multiple mechanisms including induced expression of membrane transporters that pump drugs out of cells (multidrug resistance (MDR) phenotype), changes in the glutathione system and altered metabolism. Treatment of cancer patients/cancer cells with chemotherapeutic agents and/or molecularly targeted drugs is accompanied by acquisition of resistance to the treatment administered. Chemotherapeutic agent resistance was initially assumed to be due by induction of mutations leading to a resistant phenotype. While this has occurred for molecularly targeted drugs, it is clear that drugs selectively targeting tyrosine kinases (TKs) cause the acquisition of mutational changes and resistance to inhibition. The first TK to be targeted, Bcr-Abl, led to the generation of several drugs including imatinib, dasatinib and sunitinib that provided a rich understanding of this phenomenon. It became clear that mutations alone were not the only cause of resistance. Additional mechanisms were involved, including alternative splicing, alternative/compensatory signaling pathways and epigenetic changes. This review will focus on resistance to tyrosine kinase inhibitors (TKIs), receptor TK (RTK)-directed antibodies and antibodies that inactivate specific RTK ligands. New approaches and concepts aimed at avoiding the generation of drug resistance will be examined. Many RTKs, including the IGF-1R, are dependence receptors that induce ligand-independent apoptosis. How this this signaling paradigm has implications on therapeutic strategies will also be considered.

Keywords

Receptor tyrosine kinases; Bcr-Abl; epidermal growth factor receptor; dependence receptors

Introduction

Cancer cells frequently exhibit resistance to the growth inhibitory and cytotoxic actions of chemotherapeutic drugs reflecting the potential to undergo a rapid form of molecular evolution as a means of developing a survival strategy. In this context, multiple

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mechanism(s) have been demonstrated as being responsible for the observed cancer cell chemoresistance/drug tolerance. These include acquiring mutations that enable survival, the “switching” between different receptor-driven signaling pathways, and the induction of transporter protein expression enabling efflux of drugs out of the cell. As more therapeutic strategies and molecularly targeted drugs that inhibit specific TKs are developed we have been provided the opportunity to probe deeper into the processes involved in drug resistance, it is clear that cancer cells have developed additional mechanisms of chemoresistance. In this review, the molecular basis for resistance to tyrosine kinase inhibitors (TKIs) will be discussed and how this compares to the resistance to receptor TKIs (RTKIs). Also included is a discussion of acquired resistance to monoclonal antibodies (mAbs) targeting RTKs and how the resistance mechanisms compare to RTKIs. These analyses will be considered in the context of tumor heterogeneity whereby cells populating any given tumor are heterogeneous and that natural selection by drug dosing is a key mechanism in this process.

Inhibition of Bcr-Abl and non-receptor tyrosine kinases

From a historic perspective, Gleevec (STI-571; imatinib) was the first therapeutically successful Abl tyrosine kinase inhibitor introduced for the treatment of chronic myeloid leukemia (CML). Accordingly, it has served as an instructional model for rational drug design of non-receptor, as well as receptor TKIs since its FDA approval in 2001. Given the fact that the structures of the tyrosine kinase family are highly homologous, particularly within the ATP-binding sites of their kinase domains (KDs), and these compounds competed with ATP for binding to the ATP-binding site, there was early concern that they would lack specificity for a single tyrosine kinase and fail to be effective drugs. The long succession of selective receptor and non-receptor tyrosine kinase inhibitors developed since imatinib proved this to be incorrect (). Based on elegant crystallographic studies of Abl kinase in the presence of imatinib (then referred to as STI-571 or CGP 57148) a mechanism of inhibition was determined, in which Imatinib binds to the ATP binding site of the kinase domain (KD), stabilizing the inactive non-ATP binding conformation of the Abl activation loop, thereby “locking” the kinase in the off position and preventing transphosphorylation (Schindler et al., 2000) (Marcucci, Perrotti, & Caligiuri, 2003).

In order to determine the rationale for imatinib's specificity and potency Lin and coworkers carried out a detailed analysis of the binding energies of imatinib for Abl and Src kinases (Lin, Meng, Jiang, & Roux, 2013). The premise for this comparison stemmed from the fact that these tyrosine kinases have 47% sequence identity, yet Src is not effectively inhibited by imatinib. Crystal studies of the Abl-imatinib complex revealed that the Asp-Phe-Gly (DFG) present near the N-terminal end of the KD activation loop adopts a “DFG-out” conformation corresponding to inactivation of the catalytic subunit (Schindler et al., 2000). It was initially assumed that Src and other TKs did not adopt the DFG-out conformation, precluding their abilities to bind imatinib. This view was eventually proven to be incorrect with the observation of c-Src bound to imatinib in the DFG-out state (Seeliger et al., 2007). The model then changed to one of induced-fit being responsible for imatinib preferentially interacting with Abl over Src kinase based on NMR analysis of enzyme-inhibitor complexes (Agafonov, Wilson, Otten, Buosi, & Kern, 2014). This was further confirmed based on analyses of common ancestral forms of these kinases and x-ray structural analysis. It was

postulated that affinity for imatinib increases as these kinases evolve toward Abl and lost as they evolve toward Src (Wilson et al., 2015).

More recent next generation TKIs targeting Abl include bosutinib (Bosulif®) and ponatinib (Iclusig®). Bosutinib was FDA approved in 2012 for use in treating adults with chronic-, accelerated- and blast-phase CML or Philadelphia chromosome-positive ALL. Bosutinib was developed to overcome resistance; that end result did not occur. However, ponatinib was FDA approved in November 2016 for the treatment of the above forms of AML as well as T315I-positive CML (all phases). Unfortunately, ponatinib has significant cardiovascular side effects and toxicities leading to life-threatening blood clots and severe vascular occlusive effects. These actions are consistent with the fact that in addition to Abl, ponatinib has inhibitory effects on upwards of 40 tyrosine kinases (T. O'Hare et al., 2009), including FGFR1-4 (Gozgit et al., 2012), thereby contributing to ponatinib's polypharmacology profile and toxicities.

Mechanisms of acquired vs. intrinsic resistance to TKIs

Over the years, a number of mechanisms have been identified as contributing to or causing resistance to receptor and non-receptor TKIs. Pre-existing, primary or intrinsic resistance refers to those mechanisms present in cells before they were ever exposed to an inhibitor (Jänne, Gray, & Settleman, 2009). Extrinsic or acquired mechanisms are represented by pharmacokinetic parameters that influence the efficacy of drugs as well as molecular changes in drug targets that alter drug efficacy. The tumor microenvironment (TME) has more recently been shown to contribute to both intrinsic and acquired drug resistance (Klemm & Joyce, 2015). The TME has been well accepted in contributing to tumorigenesis via angiogenesis/vascular re-modeling and the release of factors from various stromal cells, recruitment of cancer stem cells and the effects of cancer activated fibroblasts (Hanahan & Coussens, 2012). It is becoming increasingly evident that the TME also impacts therapeutic response including a role for the ECM in supporting primary and metastatic niches which can affect drug response (P. Lu, Weaver, & Werb, 2012).

Acquired resistance to Abl kinase TKIs

For patients treated with imatinib, the primary cause for relapse/resistance to imatinib is reactivation of the Bcr-Abl kinase as a result of the appearance of point mutation(s) within the KD; (T. O'Hare et al., 2005). These mutations alter imatinib action without significantly reducing ATP binding or kinase function (Deininger, Buchdunger, & Druker, 2005). Identification of the sites of point mutations in Bcr-Abl resulting from imatinib therapy, or the second-line Abl-kinase inhibitors dasatinib and nilotinib and their impact on kinase function have been well characterized by a number of investigative teams (Thomas O'Hare, Eide, & Deininger, 2007).

A number of kinase domain point mutations have been identified and characterized for their effects on Bcr-Abl function *in vitro* and sensitivity to dasatinib and nilotinib; these analyses have been reviewed elsewhere (Thomas O'Hare et al., 2007). The natural evolution of KD mutations in TKIs is typified by the T315I mutation in Abl, a key contact site for imatinib. T315I represents mutation of the "gatekeeper" residue in Abl and results in conferring

resistance to the Abl inhibitors, imatinib, dasatinib and nilotinib (Barouch-Bentov & Sauer, 2011). A key feature of gatekeeper mutations such as T315I in Abl is that they typically have no effect on kinase activity. Rather, they block TKI access to the hydrophobic pocket within the activation loop via steric hindrance which, in turn, blocks inhibitor binding via loss of the necessary hydrogen bonding required to form a stable enzyme-inhibitor complex (Zhang, Yang, & Gray, 2009). Additional point mutations located within the ATP binding loop prevent Abl from assuming a high affinity conformation capable of binding imatinib. Activation loop mutations are thought to stabilize the active conformation, which imatinib is unable to bind. Of note, a number of activation loop mutations were inhibitable with the second generation Bcr-Abl kinase inhibitors such as nilotinib (Weisberg et al., 2005) and dasatinib, a dual Src/Abl inhibitor (Shah et al., 2004), as a result of their increased affinity for Abl kinase compared to imatinib. Dasatinib has a 300-fold greater potency than imatinib and it binds to the catalytically active conformation of Abl, further enabling its ability to inhibit imatinib-resistant mutants (Shah et al., 2004). In differentiating between intrinsic and acquired resistance, Zhang et al., raise the issue that gatekeeper mutations may be pre-existing rather than acquired (Zhang et al., 2009).

The point mutations identified in the Bcr-Abl KD result in resistance to imatinib as a result of reduced KD flexibility, limiting its ability to form an inactive conformation necessary for imatinib binding and inhibition (Burgess, Skaggs, Shah, Lee, & Sawyers, 2005). On this basis, second generation inhibitors were developed with the goal of increased potency above that of imatinib. Indeed, mutations found to be resistant to dasatinib are present within contact sites (Burgess et al., 2005) while nilotinib-induced point mutations were also resistant to imatinib. (Ray, Cowan-Jacob, Manley, Mestan, & Griffin, 2007).

In contrast, *in vitro* induction of imatinib resistance is often associated with Bcr-Abl mRNA and protein overexpression, which is not always associated with gene amplification. Elevated P-glycoprotein expression and multidrug resistance-based drug efflux, as seen with many chemotherapeutics, has also been observed for imatinib (Mahon et al., 2000), and the activation of integrin and/or growth factor receptor signaling pathways have been described as mechanisms responsible for imatinib refractoriness (Deininger et al., 2005).

Receptor and non-receptor tyrosine kinases activate common pathways

Receptor and non-receptor tyrosine kinases utilize a variety of common effector proteins and pathways to mediate their downstream effects in normal cells and cancer cells. A key family of RTKs in tumorigenesis and therapeutic strategies in multiple cancer sites is the epidermal growth factor receptor (EGFR) also referred to as HER1 (human epidermal growth factor receptor1) or ErbB1 family (based on their relatedness to the avian viral erythroblastosis oncogene), is comprised of four members HER1-4 or ErbB1-4. Ligand binding leads to a conformational change in the 3D structure of the EGFR, its increased lateral mobility in the plasma membrane, homo- or heterodimerization and transphosphorylation of its partnering receptor's intracellular domain. The phosphorylated receptor dimer, through interactions of its phosphotyrosines, binds to effectors containing Src homology 2 (SH2) and phosphotyrosine binding (PTB) domains activating downstream pathways (Roskoski, 2014) including Ras-MAPK (Erk), PI3K/Akt and STAT activation downstream of the JAK non-

receptor tyrosine kinase. Of note, activation of the IGF-1R can result in “receptor cross-talk” as a result to protease activation and the shedding of membrane-tethered EGFR ligands. Alternatively, activation of the HIF-1 transcription factor resulting in VEGF expression and secretion can, in turn, activate the EGFR and/or VEGFR, respectively (Fig. 1; (Steven A. Rosenzweig, 2nd Edition; S. A. Rosenzweig & Atreya, 2010; Mark G. Slomiany et al., 2007; M. G. Slomiany & Rosenzweig, 2006)). Fig. 2 illustrates signaling pathways regulated by Bcr-Abl are common to those regulated by RTKs and other non-receptor tyrosine kinase leading to enhanced cell proliferation, tumorigenesis, invasion and metastasis (Steelman et al., 2004). The existence of overlapping or “redundant” pathways across receptor and non-receptor kinases provides insight as to how compensatory signaling pathways may take the place of those RTK pathways inhibited by a given molecularly targeted RTKI. These mechanisms, in addition to kinase mutations, represent important ways in which cancer cells become resistant to targeted therapeutics and will be reviewed below starting with Bcr-Abl TKIs and extending to a discussion of EGF and IGF-1 receptors. While this review is focused on receptor and non-receptor tyrosine kinase inhibitors and mechanisms of acquired resistance, it should be realized that there are currently inhibitors being evaluated or in clinical trials that target one or more kinase depicted in Figs 1 and 2 (Liu, Cheng, Roberts, & Zhao, 2009; S. A. Rosenzweig & Atreya, 2010). This further underscores the polypharmacology approach taken by some companies as an anti-cancer or anti-aging therapeutic strategy.

Receptor tyrosine kinase inhibitors and the Epidermal Growth Factor

Receptor (EGFR) family

As observed with chemotherapeutic agents that lack targeting specificity, rationally designed drugs (molecularly-targeted drugs; TKIs and mAbs) that selectively target receptor and non-receptor tyrosine kinases can also result in acquired resistance. As with Abl kinase TKIs, considerable experience has been gained in the study of drugs that target the EGFR family both in terms of acquired resistance and in defining sensitivity to drug. It was determined early on in the experience with sensitivity to the EGFR RTKIs gefitinib and erlotinib, that drug-sensitive patient populations could be selected for therapy based on the presence of an activating mutation in the EGFR (Lynch et al., 2004; Paez et al., 2004; Pao et al., 2004). For example, ~10% of all non-small cell lung cancer (NSCLC) patients in the United States - with a higher percentage in east Asia - exhibit gain of function mutations within the EGFR KD. These are attributable to a single amino acid substitution of arginine (R) for leucine (L) at position 858 (nucleotide 2573 TmG in exon 21) or an exon 19 in-frame deletion, removing the tetrapeptide Leu-Arg-Glu-Ala (Pao et al., 2005). Despite early positive responses to therapeutic intervention, most of these patients eventually developed resistance to erlotinib and gefitinib, as seen with acquired resistance to imatinib treatment in CML. The underlying cause for resistance was eventually determined to be caused by secondary mutations as observed in the Abl KD (Shah et al., 2002). These “loss of inhibition” mutations were found in over half of the patients exhibiting acquired resistance to imatinib, and were clustered within the ATP-binding and activation loops of the Abl KD, resulting in blocking imatinib binding to Abl (Shah et al., 2002). A single nucleotide change in the EGFR resulting in replacing a threonine with methionine at residue 790 (T790M) is

typically observed. It is notable that this represents a gatekeeper mutation analogous to other gatekeeper mutations such as, T670I in c-Kit, T674I in PDGFR- α and T315I in Abl described earlier, which in addition to causing resistance in CML, is responsible for acquired resistance to imatinib in gastrointestinal stromal tumors (GIST) (Heinrich et al., 2003; Pao et al., 2005).

Based on the previous experience with acquired resistance to imatinib, a number of investigators (Lynch et al., 2004; Paez et al., 2004; Pao et al., 2004) examined the EGFR kinase domain, spanning exons 18–24 in patients who were initially responsive to RTKI treatment, but whose tumors progressed over time. Pao and coworkers (Pao et al., 2005) examined the EGFR KD in 5 patients with acquired resistance to EGFR TKIs and found the presence of a second mutation in exon 20 at residue 790 (T790M). The net effect of replacing threonine with the bulkier and more hydrophobic methionine residue, is loss of the TKI binding cleft created by the threonine residue thereby eliminating this druggable site. This mechanism is common to multiple kinases including Abl, Src, FLT3 (FMS-like tyrosine kinase 3), platelet-derived growth factor β , (PDGFR β) and the fibroblast growth factor receptor (FGFR) (reviewed in (Jänne et al., 2009)). Moreover, this substitution, located within the ATP binding pocket, results in a greater affinity of the EGFR for ATP, reducing the potency of ATP-competitive drugs (Yun et al., 2008). Significantly, this mutation was not detected in tumor tissue from untreated patients, underscoring the selection for this somatic mutation by TKI treatment (Jänne et al., 2009). These findings underscore both the desire and need to carry out genomic studies on patients, which provides an advantage in screening patients for their drug-sensitivities as well as their potential and/or eventual drug resistance (Jänne et al., 2009).

In addition to the acquired resistance in TKI-sensitive tumors stemming from the generation of secondary mutation(s) in the EGFR, additional mechanisms of acquired resistance have been described. Two such examples are overexpression of the Met receptor or of its ligand, hepatocyte growth factor (HGF), accounting for acquired resistance in a small percentage of tumors (Bean et al., 2007; Engelman et al., 2007). Additional studies using cell culture models of EGFR acquired resistance have confirmed that Met overexpression and phosphorylation can compensate for loss of EGFR (Mueller, Yang, Haddad, Ethier, & Boerner, 2010). In this case, it was shown that Met served as a co-receptor for the EGFR and that the physical link between these two proteins resulted in Met activation in the absence of HGF, but in the presence of c-Src kinase activity (Mueller et al., 2010). A study of gefitinib-resistant cell lines and human lung adenocarcinoma specimens showed that HGF overexpression (coupled with Met activation) leads to PI-3 kinase pathway restoration in the absence of Met amplification or T790M mutation of the EGFR (Yano et al., 2008). An important observation was that HGF expressed by tumor stromal cells affects gefitinib resistance in mutant EGFR expressing tumor cells (Yano et al., 2008). This underscores the role the TME (see above) plays in contributing to acquired resistance in what is referred to as non-cell autonomous drug resistance mechanisms *vs.* cell autonomous mechanisms; the latter occurring independently of cells in the TME, alterations in drug metabolism, angiogenesis, epigenetic changes or other considerations (Jänne et al., 2009; Ji, 2010).

Lapatinib, a dual kinase inhibitor of EGFR and HER2 and afatinib a covalent ErbB1 RTKI

The RTKI lapatinib (Tykerb®) is a dual action tyrosine kinase inhibitor that inhibits both the EGFR (ErbB1) and HER2 (ErbB2). The ErbB2/Neu receptor is overexpressed in 25% of all breast cancers where it typically serves as the primary driver of tumor cell growth in most of these cancers which exhibit addiction to ErbB2. Another aspect of ErbB2 is it lacks a known ligand. Owing to its stabilized conformation in the ligand-activated state, overexpression of the ErbB2 gene leads to ligand-independent hetero-dimerization, tonic stimulation of signaling pathways and cell proliferation (De Keulenaer, Doggen, & Lemmens, 2010). In ErbB2 amplified cells, the principal signaling unit is comprised of ErbB2/ErbB3/phosphoinositide 3-kinase (PI3K) complexes leading to Akt activation (Junttila et al., 2009). Lapatinib inhibits both ligand-dependent and independent signaling and as seen for the EGFR RTKIs erlotinib and gefitinib, can induce acquired resistance following its initial clinical benefit. As with the EGFR the gatekeeper residue T798M analogous to T790M in the EGFR (Roskoski, 2014). Rexer and coworkers demonstrated that stable expression of breast cancer cell lines with HER2 containing the gatekeeper mutation T798M resulted in resistance to lapatinib and trastuzumab (described below) and overexpression of the EGFR ligands EGF, TGF α , amphiregulin and HB-EGF. These cells were sensitized to trastuzumab treatment by co-addition of the anti-EGFR antibody cetuximab or lapatinib consistent with increased EGFR ligands as the cause for drug resistance (Rexer et al., 2013). Afatinib (Gilotrif®) is an irreversible EGFR TKI that covalently inserts into the active site at residue Cys797. Afatinib is capable of irreversibly inhibiting the gatekeeper mutation T790M in the EGFR and irreversibly inhibits ErbB2 (Cys805) and ErbB4 (Cys803) (Roskoski, 2014).

Lapatinib-induced kinome reprogramming and its role in resistance—As indicated, TKI or RTKI inhibition is beneficial following its initiation but that resistance to the drug usually develops. In addition to cell adaptation responses whereby gatekeeper and other residues in the ATP-binding site/activation loop are mutated to limit the drug efficacy. In addition to directly impacting the targeted kinase one often observes a compensation for this loss via the induction of alternative kinases or amplification of ligands for alternative growth factor receptors enabling a bypass of the inhibited pathway. The application of combination therapies has been proposed as a means around this phenomenon. As detailed above, ErbB2 is overexpressed in 25% of breast cancers where it heterodimerizes with ErbB3 leading to ErbB3 upregulation as a contributor to lapatinib resistance (Garrett et al., 2011). This is often accompanied by increases in multiple receptor and nonreceptor tyrosine kinases contributing to resistance ranging from IGF-1R, FGFR2, MET, FAK and Src family kinases (Rexer & Arteaga, 2012). Stuhlmiller and coworkers observed that tumors are capable of evading the long-term effects of kinase-targeting drugs by launching an adaptive kinome response, upregulating alternative kinases or by overcoming inhibition by reactivating the targeted pathway (Stuhlmiller et al., 2015). They refer to this as "adaptive kinome reprogramming" and in the case of ErbB2 signaling showed that lapatinib treatment of breast cancer cell line. Given the heterogeneity of the adaptive kinome response in different cell lines, multiple alternative inhibitors would have to be used during the course of intermittent therapy. To circumvent this issue, these investigators tested inhibitors of factors that modify or associate with chromatin (epigenetic enzymes) and determined that the BET

family of bromodomains (Delmore et al., 2011) were inhibited by JQ1 and caused the suppression of lapatinib-induced kinome reprogramming. This suppression of lapatinib-induced kinase expression blocked cell growth and caused lapatinib inhibition to be durable response (Stuhlmiller et al., 2015).

Related to kinome reprogramming, a recent study examined the proteome, kinome and phosphoproteome of ErbB2 overexpressing, lapatinib-resistant breast cancer cells employing mass spectrometric analysis. The results obtained both confirmed the occurrence of kinome alterations including overexpression of AXL kinase and reactivation of PI3K and further extended these findings with the demonstration that lapatinib resistance is associated with phosphorylation-based reprogramming of glycolysis (Ruprecht et al., 2017). Lapatinib-sensitive and resistant cells have been investigated by numerous laboratories and shown to undergo metabolic reprogramming of glycolysis during resistance (Komurov et al., 2012). However, the report by Ruprecht and coworkers, revealed a considerable amount of glycolytic enzyme posttranslational modification occurred following the inhibition of phosphorylation of the key regulator of the rate-limiting step in glycolysis, 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 2 (PFKFB2) on S-466 (Ruprecht et al., 2017). Of particular note is that the metabolic reprogramming observed was also supportive of the invasive/metastatic phenotype including the release of large amounts of glutamate, a known stimulator of growth and invasion (Li & Hanahan, 2013). This may have therapeutic ramifications in the future.

Epigenetic mechanisms of resistance

Epigenetic alterations have also been shown to affect resistance mechanisms in addition to their well known effects on tumor induction and development. Histone acetyltransferases acetylate histone N-terminal lysine residues promoting chromatin expansion and transcription factor access to promoter regions. Histone deacetylases (HDACs) catalyze the removal of acetyl groups from histone lysines resulting in DNA/histone complex compaction that blocks transcription factor access to binding sites decreasing gene transcription. Blockade of this modification with HDAC inhibitors favors growth arrest, differentiation, and apoptosis (Bolden, Peart, & Johnstone, 2006). Consequently, HDAC inhibitors such as vorinostat have anti-tumor activity and are effective as cancer therapeutic drugs (Lane & Chabner, 2009).

Epigenetic mechanisms may further participate in RTKI resistance mechanisms. One example is the EGFR, which along with many other RTKs requires the chaperone protein heat shock protein 90 (Hsp90) for its proper folding and function. The HDAC inhibitor LBH589 (panobinostat) increases Hsp90 acetylation thereby decreasing its association with EGFRs, causing down-regulation of survival signaling proteins and inducing cell death (Edwards, Li, Atadja, Bhalla, & Haura, 2007). Accordingly, the EGFR is sensitive to the actions of HDAC inhibitors. Consistent with this, in cells lacking EGFR-dependence, LBH589 has a negligible effect on apoptosis causing cell cycle arrest instead. A 10-fold increase in LBH589 dose was required to deplete EGFR and Akt in cells lacking EGFR mutations. Co-treatment of cells with the EGFR TKI erlotinib and LBH589 resulted in synergistic actions on lung cancer cells dependent on EGFRs for growth and/or survival.

This suggests that EGFR mutation status may be predictive of a positive response to LBH589 and other HDAC inhibitors (Edwards et al., 2007).

Taken together, these observations underscore the notion that drug resistant cell populations may be selected via multiple mechanisms ranging from drug efflux, modulation of drug metabolism, secondary mutation of the target protein, induction of alternate signaling pathways and the induction of epigenetic mechanisms (Trumpp & Wiestler, 2008). An additional mechanism to consider is the selection of drug refractory cancer stem cell populations or cancer-initiating cells; their existence also underscores the well known cellular heterogeneity present within a tumor that enhances a tumor's ability to adapt to a changing environment (Dannenberg & Berns, 2010). Consistent with the idea that cancer cell populations within a tumor are heterogeneous, Sharma et al., (Sharma et al., 2010) recently described a subpopulation of PC9 cells (an EGFR mutant NSCLC cell line) that were reversibly drug-tolerant and labeled as "drug-tolerant persisters" (DTPs). These cells remained viable under conditions that killed-off the majority cell populations. DTPs were detected following expansion of single drug-sensitive cells and their phenotype remained reversible. Because DTPs occur at frequencies higher than expected as a result of mutation, it was reasoned that epigenetic regulatory mechanisms may be responsible (Sharma et al., 2010). While DTPs are quiescent cells, owing to their heterogeneity, a small percentage (~20%) of these cells exhibit normal proliferation responses when grown in the presence of drug and were thereby termed "drug-tolerant expanded persisters": (DTEPs).

In an effort to define the underlying mechanisms of the drug tolerant state, Sharma et al., determined that the resistant cells retained the sensitizing EGFR mutation and did not acquire the T790M "gatekeeper" mutation or MET gene amplification, suggesting an alternative modification may be occurring (Sharma et al., 2010). Using genome-wide gene expression analysis of parental PC9, DTP and DTEP cells, significant expression differences were identified among the three cell lines. The DTPs and DTEPs exhibited a single gene elevation, KDM5A/RBP2/Jarid1A (KDM5A), a histone H3KA-demethylase (Fattaey et al., 1993; Klose et al., 2007). Importantly, silencing KDM5A in PC9 cells reduced the number of DTEPs generated in response to cisplatin challenge without affecting PC9 cell proliferation. It was thus concluded that KDM5A expression was a necessary requirement for induction of reversible drug tolerance (Sharma et al., 2010). Because KDM5A is known to interact with HDACs (Klose et al., 2007), HDAC inhibition was tested for its ability to phenocopy KDM5A knockdown in PC9 cells. Addition of the HDACI/II inhibitor, trichostatin A caused the rapid death of DTPs and DTEPs without having an effect on parental PC9 cells and this was verified by demonstrating HDAC inhibitor co-treatment of PC-9 cells in the presence of an EGFR TKI eliminated the emergence of DTEPs, suggesting that drug-tolerant cell populations are susceptible to HDAC inhibition. More recently, several groups have reported the successful identification of selective inhibitor of the KDM5 family of histone demethylases (Gale et al., 2016; Vinogradova et al., 2016). One of these compounds, CPI-455, behaved as predicted; it inhibited KDM5, elevated levels of H3K4 trimethylation and decreased the number of DTPs in multiple cancer cell lines (Vinogradova et al., 2016). The development of this inhibitor has confirmed that removal of the DTP subpopulation of cancer cells may further reduce relapse/acquired resistance in these cancer sites.

It is noteworthy that as an alternative to the use of HDAC inhibitors, treatment of cells with the IGF-1R TKI, NVP-AEW541 (S. A. Rosenzweig & Atreya, 2010) was capable of inhibiting the emergence of DTEPs, suggesting that IGF-1R signaling can lead to chromatin modifications resulting from altered KDM5A activity or expression. A small percentage of DTEPs harboring the T790M EGFR mutation arose during treatment of PC9 cells with NVP-AEW541 and erlotinib, consistent with mutational mechanisms being responsible for mediating the pathway to drug resistance.

Resistance to receptor tyrosine kinase inhibitors vs. receptor targeted antibodies: IGF-1R

As detailed above, Abl kinase and the EGFR provide clear examples of how KD and gatekeeper mutations affect the sensitivity to drugs and the resistance to small molecule TKIs, additional mechanisms may also be in place for these and other RTKs and non-receptor TKs. An example highlighting this is the insulin-like growth factor-1 receptor (IGF-1R), which was at one point, a major focus of targeted therapeutic strategies, and a large number of TKIs and antibodies were developed to target this receptor in various cancer sites (reviewed in (S. A. Rosenzweig & Atreya, 2010)). The IGF-1R is a prosurvival, anti-apoptotic signaling growth factor receptor tyrosine kinase that is frequently overexpressed in cancer, with little evidence available demonstrating that it has a propensity for undergoing mutational change, SNPs or true gene amplification. The small molecule, dual-kinase IGF-1R/insulin receptor (IR) TKI, BMS-754807 was reported to inhibit IGF-1R signaling *in vitro* and in *in vivo* animal models (Huang et al., 2010). Huang et al., (Huang et al., 2010) generated two drug resistant rhabdomyosarcoma cell lines from parental Rh41 cells: Rh41-807R with acquired resistance to BMS-754807 and Rh41-MAB391R cells with acquired resistance to an IGF-1R blocking antibody, MAB391, in an effort to determine the mechanisms responsible for the acquired resistance to TKIs and mAbs targeting the IGF-1R. By applying gene expression profiling and DNA copy number analyses both unique and common mechanisms of resistance were identified. In common, both resistant cell lines upregulated alternate signaling pathways, but the pathways induced differed in each case. PDGFR α was amplified, overexpressed and constitutively activated in Rh41-807R cells; knockdown of PDGFR α resulted in re-sensitization of the cells to BMS-754807. Interestingly, Axl expression levels were upregulated in Rh41-MAB391R cells, in contrast, this pathway was downregulated in Rh41-807R cells. Although both inhibitors target the IGF-1R, their mechanisms of action significantly differ, presumably contributing to the observed distinctions in the mechanisms of acquired resistance described. Whether the mechanisms involved depend upon mutational or epigenetic pathways has not been determined. A main difference in the actions of these agents is that small molecule TKIs are able to access all intracellular compartments, unlike mAbs, enabling them to bind to and potentially influence multiple proteins (protein kinases in particular) besides the RTK to which they are targeted. Specific to the IGF-1R, which, as mentioned, typically lacks mutations or amplification in cancer, induction of alternate compensatory pathways over mutational changes may be the more expected outcome. Acquired resistance to trastuzumab (see below) occurs whether it is administered as monotherapy or as the more common combination therapy with a standard of care chemotherapeutic (Slamon et al., 2001).

Other mAbs and acquired resistance: trastuzumab

The Human EGF Receptor-2 (HER-2, erbB2/neu) is overexpressed in 20–25% of metastatic breast cancers (Slamon et al., 1987). Trastuzumab (herceptin), is a humanized mAb directed against subdomain IV of the ErbB2 extracellular domain (ECD), that is in current use as a targeted therapy in cases where HER2 is shown to be overexpressed (Nahta & Esteva, 2006). The mechanism by which trastuzumab action leads to tumor regression is not completely known, it is known that treatment of tumor cells with trastuzumab results in reduced ErbB2 signaling, cell cycle arrest, reduced proliferation, ErbB2 endocytosis and downregulation (Nahta & Esteva, 2006). Mechanistically, trastuzumab binds to subdomain IV of ErbB2 to block the ligand-independent signaling mediated by ErbB2–ErbB3 heterodimers - the principal signaling unit in ErbB2 overexpressing cells (Junttila et al., 2009). Pertuzumab (perjeta®) is a humanized monoclonal antibody that binds to subdomain II of ErbB2 and sterically hinders the binding pocket required for ligand-dependent dimerization and downstream signaling (Badache & Hynes, 2004; Franklin et al., 2004).

Whether used as monotherapy or in combination therapy, patients who initially exhibited a positive response to trastuzumab eventually exhibit acquired resistance (Slamon et al., 2001). A number of underlying mechanisms may be responsible for acquired trastuzumab resistance. One clear possibility is mutation of the ErbB2 ECD, precluding trastuzumab binding to the HER2 ECD, similar to mutational events seen in response to EGFR TKIs (see above). Alternatively, elevated EGFR:ErbB3 heterodimers, EGFR homodimers or loss of ErbB2 could be responsible for a loss of trastuzumab sensitivity. Akt and/or PI3K activation (Yakes et al., 2002) or loss of PTEN activity (Nagata et al., 2004) can also lead to trastuzumab resistance. Induction of alternate signaling pathways has been observed in trastuzumab resistance, in particular, elevation of IGF-1R signaling (Y. Lu, Zi, Zhao, Mascarenhas, & Pollak, 2001). This is similar to the induction of the redundant Met pathway (Bean et al., 2007; Engelman et al., 2007; Jänne et al., 2009) in EGFR TKI resistance. Indeed, IGF-1R levels were found to be increased in herceptin-resistant breast cancer cell lines; treatment with the IGF-1R TKI, NVP-AEW541 restored sensitivity to trastuzumab (Browne et al., 2011). It has also been reported that trastuzumab treatment of trastuzumab-sensitive SKBR3 breast cancer cells induces insulin-like growth factor binding protein-3 (IGFBP-3) secretion which blocks autocrine and paracrine expressed IGF-1/2 access to the IGF-1R causing growth inhibition (Dokmanovic, Shen, Bonacci, Hirsch, & Wu, 2011).

Induction of IGF-1R signaling has also been implicated in acquired resistance to EGFR TKIs. Generation of gefitinib-resistant A431 squamous cancer cells was associated with the loss of IGFBP-3 and IGFBP-4 expression leading to increased IGF access to the IGF-1R (Guix et al., 2008). Treatment of cells with recombinant IGFBP-3 restored gefitinib sensitivity and co-treatment of mice bearing A431 xenografts with gefitinib and an IGF-1R targeting mAb blocked tumor growth, whereas either treatment alone had no effect on tumor growth (Guix et al., 2008).

The scaffold protein IQGAP1 has been reported to interact with ErbB2 to mediate trastuzumab resistance (White, Li, Dillon, & Sacks, 2011). Herceptin resistant human breast epithelial cells were shown to overexpress IQGAP1, with reduction of IQGAP1 levels resulting in restoration of trastuzumab sensitivity (White et al., 2011). The tumor suppressor

DACH1 can downregulate EGFRs and cyclin D1, exhibited loss of its suppressor activity in response to IGF-1 stimulation which suggests that IGF-dependent cancer cells are capable of escaping the tumor suppressive effects of DACH1 (DeAngelis, Wu, Pestell, & Baserga, 2011).

IGF-1R and dependence receptors in drug resistance

For a while the IGF-1R was the focus of a number of therapeutic strategies aimed at targeting a number of solid tumors (S. A. Rosenzweig & Atreya, 2010). The IGF-1R is an important regulator of prosurvival, anti-apoptotic signaling that has surfaced as a significant target in multiple cancers. A key reason for this relates to the fact that the IGF-1R is a potent activator of Akt which consistent with the findings that inhibition of mTOR signaling by rapamycin frequently results in the loss of feedback inhibition of IGF-1R signaling, in turn, leading to Akt activation (Wan, Harkavy, Shen, Grohar, & Helman, 2007). Similar findings to these findings have been reported by a number of laboratories and support the strategy of combination therapy with rapamycin analogs plus an IGF-1R targeting TKI or mAb (Kolb et al., 2011). In addition to its involvement in the acquired resistance to EGFR TKIs and herceptin ((Jameson et al., 2011) and described above), IGF-1R signaling was reported to regulate RON receptor activation by direct physical interaction in pancreatic cancer cells, suggesting that RON activation may be involved in acquired resistance to IGF-1R therapies (Jaquish et al., 2011). IGF-1Rs have been found to be downstream of RTKs (Ahmad, Farnie, Bundred, & Anderson, 2004) and G-protein coupled receptors with crosstalk occurring at the receptor level, as well as via downstream effectors (Rozengurt, Sinnott-Smith, & Kisfalvi, 2010). For example, IGF-1R crosstalk between with neurotensin receptors was shown to be Src-dependent, providing evidence for IGF-1R dependent regulation of inflammatory signaling in human colonic epithelial cells (Zhao et al., 2011).

As indicated, the IGF-1R is well known for its prosurvival anti-apoptotic signaling paradigm mediated by PI3K/Akt signaling. The IGF-1R, and insulin receptor (IR) were both shown to be “dependence receptors” (Boucher et al., 2010). Dependence receptors are so named based on the fact that when they are unliganded, they promote apoptosis; therefore cells expressing them are dependent upon ligands for survival (Goldschneider & Mehlen, 2010). There are over 12 members of this family, which includes a wide variety of membrane receptor proteins including the p75 neurotrophin receptor, MET, RET, ALK, EphA4, integrin $\alpha 5\beta 1$ and the androgen receptor (Goldschneider & Mehlen, 2010). They lack specific homology domains, however many dependence receptors possess caspase cleavage sites enabling them to recruit and bind to caspases, which may reflect their mechanism of action (Mehlen, 2010).

Double knockout (DKO) cells lacking both IGF-1Rs and IRs were resistant to apoptosis via intrinsic or extrinsic pathway stimulation (Boucher et al., 2010). The dependence-pathway is receptor-dependent, ligand-independent and required for cells to undergo apoptosis. Therefore, re-expression of one of these receptors cells enables cells to undergo apoptosis in the absence of ligand; re-expression of a kinase-dead mutant also results in the ability of cells to undergo apoptosis (Boucher et al., 2010). The impact of this pathway with respect to cancer therapeutics is likely of significance. If the IGF-1R is required/permissive to cell death, then mechanisms that down regulate IGF-1Rs or remove them from the cell surface

may have the unwanted effect of promoting cancer cell survival. IGF-1R TKIs, on the other hand, have no effect on receptor expression, but may promote a TK-independent cell death signaling paradigm. There have been a number of reports describing the TK-independent activation of IGF-1R signaling pathways. IGF-1 treatment of smooth muscle cells was reported to result in extracellular-regulated kinase 1/2 phosphorylation/activity in the presence of IGF-1R TKIs (Perrault, Wright, Storie, Hatherell, & Zahradka, 2011). This observation further supports the concept that this activity was independent of IGF-1R tyrosine kinase signaling.

In a study of the IGF-1R, we tested the effects of IGF-F1-1, a cyclic hexadecapeptide identified by phage display screening technology and developed to be an IGFBP-mimetic (Robinson & Rosenzweig, 2006) based on its ability to block IGF-1 action. We found that IGF-F1-1, which interacts with the IGFBP-binding domain on IGF-1, inhibited IGF-1 binding to MCF-7 cells, but also increased Akt activation, S-phase transition and thymidine incorporation into DNA without stimulating IGF-1R tyrosine phosphorylation/tyrosine kinase activation. Paradoxically, these activities could be blocked by the IGF-1R/IR TKI NVP-AEW541 (Robinson & Rosenzweig, 2006). The signaling mechanisms responsible for these actions along with the TK-independent apoptotic signaling of the IGF-1R, although not well understood, could provide insight into future cancer therapeutic strategies. Based on the IGF-1R being a dependence receptor, novel approaches to cancer therapeutics that promote apoptosis via the unliganded IGF-1R may be developed. Using this paradigm, combination therapy comprised of inactivating RTKs in conjunction with either an antagonist that blocks endogenous ligand binding, the use of a decoy receptor (Pavet, Portal, Moulin, Herbrecht, & Gronemeyer, 2011) or an alternative method for ligand inactivation/removal may have merit as a future therapeutic strategy. These observations provide a rationale for targeting RTKs in a way that does not induce their endocytosis and down-regulation in future therapeutic strategies.

Conclusions and Future Perspective

The experience gained through administering receptor and nonreceptor TKI therapeutics has led to the realization that selecting patient populations sensitive to a particular inhibitor - based on the presence of a specific mutation or the existence of oncogene addiction - provides a key therapeutic advantage toward treatment success. There have also been attempts to predict patient populations that may become resistant to targeted therapeutics such as erlotinib (Goodin, 2006; Van Schaeybroeck et al., 2006), with women, Asian patients having adenocarcinoma and never-smokers, all being more likely to positively respond to erlotinib and gefitinib treatment due to select mutations within the EGFR TK domain or exhibiting EGFR amplification (Tsao et al., 2005). While erlotinib and gefitinib sensitivity may predict responsiveness, this does not, in turn, translate to survival. The same unpredictability has been seen with IGF-1R TKIs. Here, acquired resistance to NVP-AEW541 in a mouse model of metastatic alveolar rhabdomyosarcoma was caused by ERK reactivation and HER2 overexpression instead of the predicted induction of PDGFR α (Abraham et al., 2011). There is a possibility that this is caused by HER2:IGF-1R heterodimerization and receptor cross-phosphorylation by alternate ligands. In this particular instance, combination therapy with lapatinib and an IGF-1R TKI was more effective than

either drug alone. The physical association of heterologous receptors adds a new dimension to current and future therapeutic strategies. In addition to the identification of RTK heterodimerization the future clearly holds promise for the development of new RTKIs and mAbs, as well as the identification of new cancer-related receptors belonging to the dependence receptor family. While autocrine/paracrine signaling by these receptors is maintains normal cell and tissue growth and physiology, ligand overexpression will lead to tumor survival. Thus, future therapies may focus on targeting RTK ligands in order to enhance apoptotic signaling. It is clear that each tumor, owing to its heterogeneity and the contributions of the tumor microenvironment to tumor progression, will require personalized therapeutic strategies for each patient.

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Nomenclature and Abbreviations

Akt	Ak (mouse strain) - thymoma
Axl	a receptor tyrosine kinase
Bcr-Abl	breakpoint cluster-Abelson tyrosine kinase
CML	chronic myelogenous Leukemia
CrkL	v-crk sarcoma virus CT10 oncogene homolog (avian)-like
DACH1	dachshund homolog 1 (Drosophila)
DFG	Asp-Phe-Gly
Dok	docking protein (downstream of tyrosine kinase 1)
DTP	drug-tolerant persisters
DTEP	drug-tolerant expanded persisters
ECD	extracellular domain
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
Erk	extracellular-regulated kinase
FGFR	fibroblast growth factor receptor
FIT3	FMS-like tyrosine kinase 3
GIST	gastrointestinal stromal tumor
Grb2	growth factor receptor bound-2

HB-EGF	heparin binding-epidermal growth factor
HDAC	histone deacetylase
HER2	human epidermal growth factor receptor 2
HGF	hepatocyte growth factor
IGF	insulin-like growth factor
IGF-F1-1	cyclic hexadecapeptide, IGF antagonist
IGF-1R	insulin-like growth factor-1 receptor
IGFBP	insulin-like growth factor binding protein
IQGAP1	Ras GTPase-activating-like protein
Jak	Janus kinase
KD	kinase domain
mAb	monoclonal antibody
MAPK	mitogen-activated protein kinase
MDR	multidrug resistance
MEK	map kinase kinase
Met	MNNG HOS Transforming gene
mTOR	mammalian target of rapamycin
NMR	nuclear magnetic resonance
NSCLC	non-small cell lung cancer
OCT1	organic cation transporter 1
PDGFR	platelet-derived growth factor
PDK1	phosphoinositide-dependent kinase 1
PFKFB2	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 2
PH	pleckstrin homology
PI3K	phosphoinositide 3-kinase
PTB	phosphotyrosine binding domain
PTEN	phosphatase and tensin homolog
Raf	Ras family member
Ras	rat sarcoma

RTK	receptor tyrosine kinase
SH2	src homology 2 domain
Shc	SH2 domain containing
SHIP	SH2 domain-containing inositol phosphatase
Sos	son of sevenless
c-Src	cellular-sarcoma
Stat	signal transducer and activator of transcription
TGFα	transforming growth factor α
TKI	tyrosine kinase inhibitor
Vav	guanine nucleotide exchange factor; vertical line; pillar (Hebrew)
VEGFR	vascular endothelial growth factor receptor

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Figure 1. Receptor tyrosine kinase signaling pathway crosstalk

Following ligand-induced receptor transphosphorylation, growth factor receptor tyrosine kinases such as the ErbB1 and IGF-1R recruit effector molecules containing SH2 or PTB domains to initiate a downstream cascade activating the Ras-Erk or PI3-K/Akt pathways, which impinge upon a number of additional pathways and activities including mTOR regulation.

Figure 2. Bcr-Abl signaling pathways

Formation of the Bcr-Abl fusion protein results in its mis-localization within the cell. This, in turn, leads to the phosphorylation and activation of a number of pathways common to receptor tyrosine kinases. Also shown is signaling by ErbB1 (EGFR).

Figure 3. Mechanism of action of ErbB2 inhibitors

A. Trastuzumab is a humanized mAb that binds to subdomain IV of ErbB2 blocking ErbB2 dimerization and ErbB2–ErbB3 complex formation which represents the major signaling unit in ErbB2 overexpressing cancer cells. This represents inhibition of ligand-independent heterodimers and signaling through PI3K and Akt. **B.** Pertuzumab is a humanized mAb selective for subdomain II of ErbB2 through which dimerization occurs with other ErbB family members. Pertuzumab treatment blocks ligand-induced heterodimerization and signaling. **C.** Lapatinib is a RTKI selective for ErbB1 and ErbB2. Lapatinib is an ATP competitive inhibitor that binds to the kinase domain of Erb1 and ErbB2 to inhibit kinase activity resulting in blockade of ligand-dependent and independent signaling (after (De Keulenaer et al., 2010)).