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Abstract

Recent advances in genetics and genomics have revealed new genes and pathways that are somatically altered in human malignancies. This wealth of knowledge has translated into molecularly defined targets for therapy over the past two decades serving as key examples that translation of laboratory findings can have great impact on the ability to treat patients with cancer. However, given the genetic instability and heterogeneity that are characteristic of all human cancers, drug resistance to virtually all therapies have emerged posing further and future challenges for clinical oncology. Here we review the history of targeted therapies, including early concepts with endocrine therapies for hormonally driven cancers and more contemporary examples of genetically defined cancer targets and their approved therapies. We will also discuss resistance mechanisms that have been uncovered with an emphasis on somatic genetic alterations that lead to these phenotypes.

Key words: Targeted therapies, drug resistance, somatic alterations, cancer

Endocrine Therapies: The first "targeted" therapies

Anti-Estrogens

The very first examples of target-specific cancer therapies were developed as endocrine or hormone therapies for certain cancers including breast and prostate cancers as well as for the M3 subtype of acute myeloid leukemia now known as acute promyelocytic leukemia. It is now well-established that estrogen receptors (ER) have been linked to breast cancer development and in particular ERα is now widely accepted to have a functional role in many breast cancers (1). ERα and ERβ are steroid hormone receptors belonging to the nuclear receptor superfamily (2). In normal breast epithelial cells, the estrogen receptors function as ligand-activated transcription factors to stimulate proliferation in response to estrogen. Additionally, the estrogen receptors can stimulate growth pathways through interaction with G-protein coupled receptors. Nearly 75% of all breast tumors express ERα, often at high levels compared to normal epithelium. These ER-positive cells are said to be estrogen dependent as they are known to regress if hormone is removed or blocked. This phenomenon was first discovered when it was noticed that the removal of the ovaries could lead to regression of many breast tumors. This observation led the drive to develop ER antagonists as a way to selectively target ER-positive breast cancers.

Tamoxifen, a compound synthesized in the late 1960s, was originally designed as a contraceptive and was one of the earliest anti-estrogens to be tested as a cancer therapeutic (3)(4). Following FDA approval in 1977, tamoxifen eventually became a widely accepted treatment for ER-positive breast cancers. In ER-positive breast tissue, tamoxifen binds to ER and forms a nuclear complex that competitively inhibits the action of estrogen. Additionally, it can recruit co-repressors in these tissues and downregulate transcriptional activity of proliferative genes whose expression is regulated by ER. Paradoxically, tamoxifen has been shown to act as an estrogen agonist in other cell tissues, such as bone and uterine tissue. In this case the tamoxifen-ER complex recruits co-activators increasing the gene expression of growth promoting ER regulated genes. Tamoxifen, and compounds of similar action, have been classified as SERMs (Selective Estrogen Receptor Modulators). It is this ability to selectively act as an estrogen receptor agonist or antagonist in differing tissue types that distinguishes this class of therapies.

Upon its approval for clinical use, tamoxifen displayed great promise as a targeted therapy against ER positive breast cancers. Unlike traditional cancer treatments that target all proliferating cells, tamoxifen inhibits the proliferation of estrogen dependent breast cells only. Moreover, because it antagonizes and/or mimics the action of a natural steroidal hormone, the side effects of tamoxifen are relatively well tolerated compared to traditional chemotherapies and radiation treatments. The success of tamoxifen brought about the development of many other SERM or SERM-like compounds. These include raloxifene, toremifene, lasofoxifene and others. However, despite its widespread use and significant success, tamoxifen-resistance (and resistance to the other SERMS) is a common occurrence. Many mechanisms of tamoxifen resistance have been described including change of tamoxifen's response from antagonism to agonism (5), and/or through activation of alternative growth pathways such as EGFR or HER mediated MAP Kinase and PI3 Kinase pathway signaling. Nevertheless, tamoxifen remains as a standard of care treatment for many patients.

Over the past decade, fulvestrant, a non-SERM anti-estrogen drug, has been developed and approved for the treatment of metastatic ER-positive breast cancer. Fulvestrant is an ER antagonist that, similar to SERMs, competitively binds to ER and acts to antagonize estrogen signaling. Fulvestrant is unlike SERMs in that it does not have mixed agonistic properties. Moreover, once bound to ER, fulvestrant pharmacologically induces ER degradation. Likely due to its different mechanism of action, fulvestrant has been shown to be effective in a large percentage of women with tamoxifen-resistant breast cancer (6). However, resistance to fulvestrant is still prevalent, most likely due to activation of alternative growth pathways, including aberrant EGFR and HER2 signaling (7).

Another class of endocrine therapies, known as aromatase inhibitors was developed at the turn of the 21st century. These compounds work by blocking the action of aromatase, an enzyme that mediates the synthesis of estrogens from other steroid precursors. The main source of circulating estrogen in premenopausal women is production by the ovaries which is non-aromatase mediated. In contrast, aromatization of androgens is the primary source of estrogen in post-menopausal women. Aromatase inhibitors act to inhibit the final steps of the catalysis of androtestosterone into estrone, and testosterone into estradiol, thus decreasing the levels of circulating estrogen in patients. Clinically approved aromatase inhibitors include the steroidal inhibitor exemestane and the non-steroidal inhibitors anastrozole and letrozole. While these compounds can effectively block nearly 99% of all aromatase enzymatic activity and lead to marked decreases of estrogen levels in patents, between 30-70% of patients will develop clinical resistance to AI therapy (8)(9). Like SERM resistance, resistance to aromatase inhibitors is thought to be linked to changes in estrogen dependence and activation of alternative growth pathways (9). In fact, the similarities in resistance to both classes of endocrine therapies are exemplified by the cross-resistance often seen clinically in patients with progressive disease. Interestingly, ER mutations have rarely been seen as a mediator of resistance to endocrine therapies.

Anti-Androgens

Like ER and breast cancer, the link between androgens and prostate cancer has been appreciated for many years. In fact, this correlation was first described in 1941 when it was acknowledged that patients who underwent surgical or medical castration often displayed significant regression of their metastatic disease (10)(11). This discovery led to early crude attempts to block androgen production through the use of estrogen and estrogen agonists (12). An increase in estrogen inhibits LHRH (luteinizing hormone-releasing hormone), thereby decreasing testosterone production. The search for more refined anti-androgen therapies led to the development and approval of bicalutamide in the mid-1990's (13)(14).

Bicalutamide, and similar therapies such as flutamide and nilutamide, are non-steroidal anti-androgens. Similar to the mode of action of tamoxifen on ER, these drugs block the androgen receptor (AR) though competitive inhibition. Comparable to hormonal treatment for breast cancer, anti-androgens also can act to either inhibit or stimulate the AR depending on the cellular context of the cell. As has been described for endocrine therapy in breast cancer, it is well known that withdrawal of anti-androgen treatment in resistant tumors can lead to significant tumor regression (15). The genetic/genomic mechanisms of this are only now being elucidated.

Additional effective endocrine therapies for prostate cancer include the class of drugs known as LHRH agonists. These drugs include leuprolide and goserelin, and are thought to act as agonists on the pituitary-gonadal axis leading to suppression of LHRH through negative feedback, but may also act as antagonists to LHRH production directly (12). Newer iterations of anti-androgens with proven efficacy include enzalutamide, which blocks AR action by inhibiting nuclear re-localization and DNA-binding, and abiraterone which inhibits testosterone production (15)(16). However, similar to other hormonal therapies, the dual antagonistic and agonistic nature of these drugs, along with alternative growth signaling pathways likely contributes to a high incidence of resistance. Interestingly, unlike ER and endocrine therapy resistance, mutations that could lead to anti-androgen therapeutic resistance have been reported (17,18).

Retinoids

Acute promyelocytic leukemia (APL) is a distinct subtype of acute myeloid leukemia in that all cases are defined by abnormal translocations of retinoic acid receptor (RARα), generally occurring between the RARα and PML genes on chromosomes 15 and 17 [t(15;17)], respectively (19). RARs are ligand-dependent nuclear transcription factors that interact with several promoter response elements (RAREs) throughout the genome (20). When bound to retinoids, such as vitamin A, RARs dimerize with retinoid X receptors (RXRs) to transcriptionally regulate expression of genes involved in growth, vision, reproduction, embryonic development, differentiation of epithelial tissues, and immune responses (21). The abnormal expression patterns and unusual presence of RARα fusion proteins in APL sparked interest in targeting this pathway for therapy. In 1988, all-trans retinoic acid (ATRA) was proven to be a successful treatment for APL patients (22)(23). With further refinements in optimal dosing and combination with newer agents, the majority of APL patients undergoing ATRA treatment achieve clinical remission and have favorable long term outcomes (24). Unfortunately, acquired resistance to ATRA and remission can still occur and is thought to be caused by changes in retinoid clearance, increased retinoid sequestration or mutational changes in the PML-RARα fusion gene (20).

First Generation Targeted Therapies

The success of endocrine therapies hinted at the potential of having more refined compounds that selectively inhibited cancer cells. While hormonal therapies utilize natural pathways to inhibit receptor mediated growth responses, the nature of these systemic treatments and expression of steroid receptors on other normal cell types yielded predictable side effects. However, the benefit of endocrine therapies is derived from the fact that hormone receptors are only expressed in the tumor cells and a limited number of normal cells and tissues, resulting in tolerable side effects along with anti-cancer efficacy. Importantly, the number of cancers that are amenable to nuclear hormone receptor targeted therapies is limited, as only a few cancer types utilize these pathways for cell growth and proliferation. Therefore, the development of other drugs distinct from nuclear hormone receptors would herald the modern era of truly targeted therapies for human cancers, though not without significant challenges.

Multi-Kinase Inhibitors

The first and undoubtedly most famous drug that revolutionized the concept of small molecule kinase inhibitors was imatinib (STI571, Gleevec, Glivec). Imatinib was originally developed in the late 1990's and was the result of many peoples' work. Notably, Brian Druker demonstrated its value as a targeted therapy against the BCR-ABL protein expressed in the vast majority of chronic myelogenous leukemia (CML) patients (reviewed in (25)). Imatinib was discovered as part of a panel of small molecule inhibitors designed to target the BCR-ABL tyrosine kinase. CML develops from the reciprocal translocation between chromosomes 9 and 22, leading to the "Philadelphia chromosome" (Ph+) which is a genetic hallmark of this disease (26)(27). This translocation creates the *BCR-ABL* fusion gene product encoding for a hyper-activated ABL tyrosine kinase. This unique molecular prolife presented itself as an ideal target for therapy. Specifically, inhibition of ABL kinase activity would, in theory, specifically deter the proliferation of the malignant Ph+ clonal cancer cells, yet non-CML cells would not be affected. Imatinib was one of many small molecule inhibitors biochemically designed with this function in mind. High-throughput screening against protein kinase C (PKC) activity teased out imatinib as having high potential for pharmacologic inhibition (25). The success of imatinib for treating CML was widely viewed as a remarkable triumph for targeted cancer therapies. Prior to the use of imatinib, only 30% of CML patients survived 5 years after diagnosis. After FDA approval of imatinib in 2001, this percentage has steadily risen to nearly 90% (28).

Imatinib acts by blocking the kinase activity of the BCR-ABL protein. Specifically, imatinib occupies an area of the ATP-binding pocket of ABL, locking it in the inhibited or closed confirmation (29). In this way, the normally constitutively active BCR-ABL kinase is turned off and it can no longer phosphorylate downstream targets. This elegant mechanism of action leads to tumor cell senescence and apoptosis, through the down regulation of BCR-ABL mediated growth promoting proteins including RAS, SRC, PI3K and JAK/STAT. However, imatinib is not as specific to BCR-ABL as originally thought. It has proven to be an inhibitor to other kinases, most notably c-Kit and PDGFR(28). This promiscuity in hindsight, is in fact advantageous, as imatinib has proven to be an effective treatment for gastrointestinal stromal tumors (GIST) which exhibit mutated c-KIT or activated PDGFR (30).

Although an undeniable success story, at least 10% of patients will not demonstrate prolonged benefit from imatinib therapy (31). This can be due to initial resistance upon treatment or an acquired resistance after an initial response to the drug. Several mechanisms for innate and acquired resistance to imatinib have been proposed. Amplification or duplication of the BCR-ABL kinase, c-KIT or PDGFR may be one such mode of resistance, in which the increased tyrosine kinase production outcompetes the action of the drug (31)(32). Importantly, point mutations in the *ABL* gene have also been linked to imatinib resistance, most frequently a threonine to isoleucine substitution (T315I) in the kinase domain that prevents imatinib association with the ATP-binding pocket (31–33). Not only do these mutations confer resistance to imatinib treatment, but they also exhibit increases in cancerous phenotypes (34). Many other mutations of the *ABL* gene have been identified as contributing to resistant phenotypes including mutations within the P-loop, SH2 domain, A-loop, C-helix and substrate binding domain (31). Mutations in c-KIT and PGDFR are also associated with imatinib resistance in GIST patients (32). Alternative mechanisms of imatinib resistance have also been described and include changes in drug influx/efflux, epigenetic modification and activation of alternative growth pathways independent of tyrosine kinase receptors (31,32).

Following the success of imatinib in treating CML and GIST, the race to replicate these results with similar kinase inhibitors to benefit other forms of cancers began in earnest. There were many compounds that

were developed and approved as a result of this new areas of focus, including several compounds that similar to imatinib, target several different kinases. Sorafenib and sunitinib are two such compounds. These drugs have shown to inhibit various growth signaling pathways involved with a number of receptor tyrosine kinases, as well as VEGFR-dependent angiogenesis (35). In a manner similar to but distinct from imatinib and ABL kinase, sorafenib and sunitinib interact with the ATP-binding pocket within the kinase domain of their respective targets, competitively deactivating the growth signaling of the various kinases (36,37). Originally approved for clinical use in 2006, sorafenib is a small molecule inhibitor of VEGFR, PDGFR and the RAF kinases CRAF and BRAF. Originally, designed as a RAF kinase inhibitor, sorafenib has been utilized as a successful treatment for renal and liver cancers (36,38). Sunitinib was approved in 2006 for renal cell carcinoma and GIST, and has been shown to target VEGFR, PDGFR, KIT, RET, FLT3 and other receptor tyrosine kinases (35,39–43). Sunitinib inhibits c-KIT and PDGF in a manner distinctly different than imatinib, and therefore it is clinically used with some success for the treatment of imatinib resistant GIST (44). Although in theory, the multi-kinase inhibition of these molecules might reduce the emergence of drug resistance, in practice resistance to sorafenib or sunitinib is common. In addition, the multi-kinase inhibitory properties also make these drugs less selective for cancer cells resulting in numerous side effects for most patients. Resistance can arise to either or both the anti-proliferative and anti-angiogenic properties of these kinase inhibitors. Activation of alternative signaling pathways (AKT, PI3K, etc.), drug sequestration, upregulation of non-VEGF dependent angiogenesis factors (FGF, IL8, etc.), and pro-angiogenic pericyte and monocyte recruitment are some of the documented mechanisms of resistance to PDGFR and VEGFR inhibitors including sorafenib and sunitinib (32,45,46). Although these drugs are effective for certain indications, their lack of specificity and toxicity profiles dictated further refinements in targeted therapies for cancer.

mTOR Inhibtors

The mammalian target of rapamycin (mTOR) has proven to be a much sought after target in drug development. It is a regulator of cell signaling found downstream of several growth pathways that are mutated in cancers including the PI3K/AKT signaling cascade. As the name suggests, mTOR was discovered as a target for the anti-fungal rapamycin in yeast cells (47–49). Rapamycin interacts with the protein FKBP12 (Rbp1) forming a complex that then binds the FRB (FKB) domain of mTOR, thereby inhibiting mTOR kinase activity and mTOR substrate interaction (50)(51). mTOR associates with two proteins; RAPTOR and RICTOR, forming two complexes mTORC1 and mTORC2, respectively(48)(51). Rapamycin predominantly interacts with mTORC1-RAPTOR and inhibits the phosphorylation of the targets S6 kinase 1 (S6K1, p70) and eukaryotic initiation factor 4E binding protein (4EBP1) (52,53).

The pursuit of mTOR inhibitors as an anti-cancer therapy first gained traction with the discovery of the rapamycin analogs (rapalogs) temsirolimus, everolimus and ridaforolimus (deforolimus). Like rapamycin, the rapalogs target FKBP-12 rapamycin binding domain (FRB, FKB) of the mTORC1 complex (52,53). Unlike rapamycin these rapalogs do not have the same degree of immunosuppressive properties, and as a result exhibit lower infection related toxicity. Temsirolimus and everolimus are prodrugs, such that their metabolites are the active rapamycin-like forms. These compounds inhibit several downstream targets of mTOR such as HIF1α, cyclin D and VEGF thereby affecting angiogenesis, cell proliferation, cellular metabolism, and survival (54). Temsirolimus, the first rapalog to be approved for clinical use, is primarily administered for the treatment of renal cancer. In the past few years, everolimus has been approved for the treatment of kidney, pancreatic and ER positive, HER2-negative breast cancers.

mTOR remains a promising target for cancer treatments, as it can control many tumorigenic factors such as angiogenesis, proliferation, survival etc. However, it is this role as a regulator of such a diverse range of functions that allows rapalog resistance to arise by many different mechanisms. One source of resistance is due to the fact that the rapalogs depend on the interaction with FKBP-12 for activity. Mutations in FKBP12 or the FKBP12 binding domain of mTORC1 can result in rapalog resistance due to a drop in binding affinity (55–57). Additionally, alternative proliferative signaling cascades can be activated in response to negative feedback from mTOR targets. Upregulation of PI3K, AKT, MAPK, PIM kinase and PDK activity has been observed in response to rapamycin and rapalog treatment (58–60). This mechanism of resistance is common among targeted therapies, that is, when one signaling pathway of proliferation or survival is disrupted, alternative pathways become activated by negative feedback loops. This mechanism of resistance is especially vexing with the rapalogs because mTORC2 will phosphorylate AKT at serine 473 in response to negative feedback from mTORC1 inhibition (61). The expression patterns of the targets of mTOR can also become altered and thereby cause rapalog resistance. Decreases in 4E-BP1 protein levels can confer resistance to rapalogs, and increased expression of eIF4E can also lead to resistance (62,63). Rapalogs are believed to cause apoptosis by stimulating the release of cytochrome c thereby initiating the mitochondrial caspase9/caspase3 apoptotic pathway. Thus, increases in anti-apoptotic signals, through upregulation of Bcl-2 or the IAPs, has been indicated to contribute to rapalog resistance (64,65). Inhibition of mTOR also decreases HIF-1α expression, a protein that controls VEGF transcription, and therefore it is proposed that inhibition of HIF-1α by rapalogs can trigger decreases in angiogenesis and limit tumor growth (65,66). Accordingly, activation of alternative angiogenesis signals can bypass this component of the anti-tumor effect of rapalogs and contribute to decreased effectiveness of these compounds.

Several strategies are being pursued to overcome rapalog resistance in cancers. Second generation mTOR inhibitors (mTOR Kinase inhibitors) are being developed that competitively inhibit the serine/threonine kinase activity of mTOR (67). The advantage of this mode of mTOR inhibition is that it is independent of FKBP12 and targets both mTORC1 and mTORC2. Another potential method to overcome the common causes of rapalog resistance is treatment with rapalogs together with compounds that target alternative proliferative pathways such as the PI3 Kinase and/or MAP Kinase pathways. Furthermore, single compounds that block both PI3K and mTOR are being explored for improved efficacy (67)(68). Thus, mTOR remains a desirable target for many cancer therapies.

Current and Future Cancer Targeted therapies

CD20 and rituximab

Antibodies have always held high promise as targeted therapies due to their potential specificity and presumed lack of toxicity. They are naturally designed to only interact with their target proteins and therefore present a low likelihood for serious side-effects (depending on the target protein). One of the first and most successful antibodies in cancer therapy is rituximab, a monoclonal antibody (mAb) against the B-cell antigen CD20. CD20, initially described in 1980, is a member of the membrane-spanning 4-A (MS4A) family of proteins (69–72). CD20 plays an important role in B-cell-to-plasma differentiation; it is expressed exclusively in pre-B cells and developing B-cells and its expression is lost in plasma cells. The clinical significance of this protein was quickly realized upon the observation that CD20 is highly expressed in B-cell lymphomas, hairy cell leukemias and B-cell chronic lymphocytic leukemias (70,71).

Rituximab is a chimeric mouse/human antibody against CD20 developed in 1991 at IDEC pharmaceuticals (73). It was approved to treat non-Hodgkin’s lymphoma in 1997 becoming the first monoclonal

antibody approved for the treatment of cancer. It has since gone on to be approved for use against other lymphomas and leukemias, as well as autoimmune diseases such as rheumatoid arthritis. Rituximab is quite successful as an anti-cancer agent, showing promise as a single agent but true clinical value in combination with chemotherapy (74). Rituximab marks CD20 positive cells for destruction by natural killer cells (NK), neutrophils and macrophages, thereby selectively targeting malignant B-Cells for antibody-dependent cellular cytotoxicity (ADCC) (74,75). Rituximab triggers ADCC by permanently attaching to CD20 on the surface of B-cells. The CD20 bound rituximab recruits the innate effector cells responsible for ADCC through an interaction of the Fc region of rituximab and the Fc gamma receptor (FcγR, CD16) on NK cells (76,77). Additionally, rituximab can deplete B-cells via apoptotic upregulation and complement-mediated cytotoxicity (CMC, CDC). Apoptosis is triggered by rituximab by the reorganization of CD20 into lipid rafts and induces changes to p38, NF-κB/bcl-2, ERK, and AKT (78). CMC in response to rituximab is linked to binding of the serum protein C1q to the Fc portion of the antibody thereby leading to cell lysis(78).

Rituximab, while highly effective in many patients, is certainly subject to therapeutic resistance. It is estimated that 30-60% of patients may show resistance to rituximab (79). As there are several mechanisms of rituximab cytotoxicity, there are likewise many means by which resistance may develop. For example, CMC in tumor cells may be blocked through the differential expression of membrane complement-regulatory proteins (mCRP). These proteins, such as CD46, CD55 and CD59, inhibit the complement cascade or membrane attack complex that is responsible for cell lysis in response to CMC (79,80). Also, as CMC is dependent on the interaction of rituximab with C1q, a depletion of this effector may lead to resistance to rituximab mediated cytotoxicity (79). The destruction of rituximab bound B-cells by immune effector cells is dependent on two variables: the binding of rituximab to FcγR, and the congregation of the bound CD20 into lipid rafts (77–79). Thus, decreases in the affinity of FcγR to rituximab or low numbers of NK cells with FcγR can diminish the efficacy of the treatment (78,79). Similarly, a lack of rituximab-CD20 polarization can inhibit ADCC (75). Additionally, resistance to the apoptotic effects of rituximab may be linked to decreases in pro-apoptotic signaling and upregulation of anti-apoptotic signaling. Overexpression of the MAP Kinase or PI3 Kinase pathways, as well as NF-κB hyperphosphorylation can all contribute to an attenuation of the pro-apoptotic nature of rituximab (78,81). While it would seem that the most likely mechanism for resistance would be changes to CD20 expression or affinity to rituximab, evidence for this is lacking (79).

CD20 remains a promising target for therapy in leukemias and lymphomas and as such there are several anti-CD20 monoclonal antibodies approved or in various stages of development. These include ocrelizumab, ofatumumab and others. These compounds are mechanistically similar to rituximab, but also likely share resistance mechanisms akin to rituximab.

BRCA and PARP Inhibitors

The tumor suppressor genes BRCA1and BRCA2 are well-known for their association with hereditary breast and ovarian cancers. Carriers of BRCA1 and BRCA2 mutations have up to an 80% lifetime risk of developing breast cancer (82). These patients also have up to a 55% chance of developing ovarian cancer (82). The BRCA1 and BRCA2 genes are expressed in breast, ovarian and other tissues where they function to repair damaged DNA. Specifically, BRCA1 and BRCA2 act as part of a complex of proteins, most notably interacting with the recombinase RAD1, to repair double-strand breaks through homologous recombination (HR) and stabilization of stalled DNA synthesis (83–86). Cells defective in BRCA1 or BRCA2 demonstrate increased mutational rates due to the breakdown of DNA repair and thus are likely to become tumorigenic.

Poly(ADP-ribose) polymerases (PARPs), are a family of proteins whose primary function is to locate and repair single strand DNA breaks (SSB). PARPs, and in particular PARP1, bind to both single-strand and double-strand breaks and initiate DNA repair (87–89). PARP1 can also reactivate stalled replication forks (90). In cases where BRCA1 or BRCA2 function is compromised, PARPs play an important role in the cellular response to this loss of function. In addition to repairing SSBs and restarting stalled forks, in the absence of BRCA1/BRCA2 it is believed that PARPs can serve a further role in DSB repair (91,92). Therefore, in patients with defective BRCA homologous recombination mediated DNA repair, PARPs can be key proteins in regulating the amount of DNA damage accumulated within cancer cells.

The interplay of BRCA1/2 and PARPs in DNA repair, offers a unique target for clinical treatment. PARP inhibitors were first developed after the observation that PARP1 is activated by radiation and DNA-methylating agents (89). It was thought that combining inhibition of PARPs with radiation, or DNA damaging agents may increase the efficacy of these more traditional cancer treatments. PARP inhibitors are effective anti-cancer treatments, not only in combination with DNA damaging agents, but also as single agents in cells defective in HR. Cells lacking HR, due to defective BRCA1, BRCA2 or other repair proteins such as PALB2 have proven to be excellent targets for PARP inhibition. In these cells, loss of PARP activity will increase the number of SSB, eventually leading to DSB when replication forks become stalled (93). The vast increase in damaged DNA in cells lacking both HR and PARP activity initiates a synthetic lethal effect (91). Additionally, it is thought that PARP inhibitors “trap” PARP proteins at the site of DNA damage and that this PARP “trapping” is also toxic to the cell (94). Both modes of action for PARP inhibitors are selective only for cancerous cells that lack HR and not for the surrounding normal cells with functional DNA repair. Many PARP inhibitors have been developed including olaparib, veliparib, rucaparib, and MK4827.

Mechanisms of PARP inhibitor resistance are just beginning to be understood. It has been found that isoforms of BRCA2 can arise in culture that restore some HR function to cancer cells harboring BRCA2 mutations, allowing these cells to bypass the synthetic lethality of PARP inhibition (95,96). It is likely that such a mechanism of resistance could also be discovered in BRCA1 mutant cells. It is also possible that other methods of acquired or innate resistance to PARP inhibitors may arise, such as mutations in the active site of PARP1 that decreases drug affinity, changes in PARP expression or a decreased dependence on substrate for activity. In fact, many BRCA1 defective cancers have low or no PARP1 expression and resistant or refractory cancers often show decreased PARP activity relative to cancers that show high PARP inhibitor sensitivity (97). PARP inhibitor resistance can also arise from differential expression of other redundant DNA repair pathways. For example, it has been shown that some BRCA1 deficient breast cancers lose 53BP1 expression, a change that can lead to restoration of HR repair and PARP inhibitor resistance (98,99). Variability in clinical response to PARP inhibition has also been linked to the potency and specificity of the drugs being used, as greater than 90% inhibition of PARP activity may be needed to significantly affect DNA repair (89). Nonetheless, PARP inhibitors either as single agents or in combination with DNA damaging agents continue to demonstrate results in many selected patients enrolled in clinical trials.

HER2

HER2 (ErbB2) expression is amplified and the protein overexpressed in approximately 15-20% of breast cancers (100). These “HER2 positive” cancers have traditionally been associated with aggressive disease and poor patient outcome. HER2 is a transmembrane receptor tyrosine kinase belonging to the ErbB/HER family. This family includes EGFR (HER1/ErbB1), and HER 2, 3 and 4. While each member of the family shares common tyrosine kinase domain motifs, they each contain unique ligand binding domains (101). HER2 is

activated by autophosphorylation upon homo- and heterodimerization, and can dimerize with other members of the ErbB family. In fact, HER2 has been shown to be the preferred binding partner of the other ErbB receptors (102). Overexpression of HER2 alone is often adequate to trigger receptor phosphorylation and activation even in the absence of ligand. HER2 activates several proliferative pathways upon phosphorylation, including the MAP Kinase and PI3 Kinase pathways. Overexpression of HER2 leads to upregulation of these pathways and a proliferative and anti-apoptotic effect on cells.

The overexpression of HER2 in breast cancers (and now other cancers such as stomach cancers), has made it a strong candidate for targeted therapies. HER2 overexpression is present largely in cancer cells, but not in surrounding normal cells. Targeting HER2 would then limit toxicity exclusively to cancer cells in theory. In 1998, the FDA approved trastuzumab (Herceptin) as the first targeted treatment against HER2 positive breast cancers. Trastuzumab is a monoclonal antibody specific to the juxtamembrane domain of HER2 and results in a downregulation of the protein (103–105). Trastuzumab also has other proposed anti-proliferative effects such as inhibiting transcription and decreasing the levels of a constitutively active truncated form of HER2 (p95-HER2) (104,106). Also, similar to the method of action for rituximab, trastuzumab can trigger antibody dependent cellular toxicity (ADCC) through an interaction between the antibody and Fc receptors on immune cells (77).

Trastuzumab is administered to patients with cancers overexpressing HER2 as a single agent or in combination with chemotherapy, such as paclitaxel. Combination therapies using trastuzumab have shown to greatly improve response rates, median survival and disease free survival (107,108). Like all targeted therapies, the successes of trastuzumab are balanced by occurrences of innate or acquired resistance. There are three proposed pathways of trastuzumab resistance, each inherently linked to the nature of HER2. These mechanisms of resistance include HER2-independent activation of the PI3 Kinase pathway, upregulation of the other HER family member receptors, and increased production of p95-HER2 (109,110).

One of the primary signaling pathways activated by ErbB receptors is the PI3 Kinase pathway. PI3K is composed of two subunits, the p85 regulatory subunit and the p110 catalytic component. These subunits interact with activated HER2, thus beginning a signaling cascade, which ultimately results in cell proliferation, growth, invasion and other cancerous phenotypes. In many cancers, PI3K or downstream targets, such as AKT and MAPK can become constitutively activated independent of the signaling dependence from HER2 activation (111–113). For example, mutations that lead to loss of PTEN function, or activation of the *PIK3CA* gene (*PIK3CA* encodes for p110α) are frequent in breast cancer (111). In these cases, targeting HER2 overexpression with trastuzumab potentially lessens its anti-proliferative effects, as these cancers can grow without HER2 activation and have in preclinical models been associated with resistance (114)(115).

The nature of HER2 as a family member of receptor tyrosine kinases, inherently allows for cells with inhibited HER2 to find alternative pathways for proliferation. Redundancy in the downstream signaling pathways of HER2, HER3, EGFR and IGF-1R indicates that loss of signal from one of these receptors alone may not be enough to impede MAPK and PI3K activation (116). As example, the HER2-HER3 heterodimer has been shown to be an essential interaction for HER2 dependent tumorigenesis (117). HER2 independent activation of HER3 or EGFR may lead to HER2 positive cancers that lack sensitivity to trastuzumab (117–119). In addition to redundancy within the members of the ErbB family of membrane receptors, IGF-1R has been demonstrated to activate PI3K through crosstalk with HER2 (120). It has been purposed that IGF-1R stimulation in certain cancer cells may be a source of trastuzumab resistance, and inhibition of IGF-1R can restore trastuzumab sensitivity (121).

As previously mentioned, a truncated form of HER2, p95-HER2, can also be expressed in cells. This protein lacks the amino terminus of HER2 and therefore lacks the binding site for trastuzumab. Moreover, p95-HER2 is constitutively active, and downstream targets become phosphorylated independent of ligand interaction. While trastuzumab treatment has been shown to decrease p95-HER2 expression, 30% of HER2 positive tumors overexpress p95-HER2 (104,122). Thus it has been proposed that cancers with increased levels of p95-HER2 are relatively resistant to trastuzumab's anti-neoplastic effects.

Targeting HER2 with trastuzumab is now standard treatment for HER2 amplified/overexpressing breast cancers and as mentioned, some GI cancers as well. On the heels of this success, alternative treatments against HER2 have been developed. Pertuzumab is another monoclonal antibody directed against HER2 that was approved for use in 2012. However, pertuzumab interacts with the dimerization domain of HER2, blocking the homo- and heterodimerization of HER2 (110). This antibody shows promise over trastuzumab, in that it can inhibit not only HER2 stimulated growth, but also the dimerization dependent activation of the other members of the ErbB family, specifically HER3 (123,124)(125). Also, unlike trastuzumab, pertuzumab may be able to inhibit p95-HER2 mediated growth (126). Clinical data indicates that trastuzumab and pertuzumab are complementary and can be highly effective when used in combination (127). These benefits, along with retaining the ability to stimulate ADCC, make pertuzumab an attractive option for HER2-positive cancers.

There have also been studies to increase the effectiveness of trastuzumab, leading to the development of TDM-1, which was approved by the FDA in 2013. TDM-1 is a conjugate of trastuzumab with DM1 (derivative of maytansine 1), a microtubule inhibitor. This antibody-drug conjugate (ADC) is designed to combine the anti-proliferative effects of trastuzumab with targeted delivery of the cytotoxic compound DM-1 directly to HER2 overexpressing cells. TDM-1 shows promise in attacking cells that may be resistant to trastuzumab, and demonstrates retention of trastuzumab's other anti-neoplastic functions such as PI3K signal inhibition, decreases in p95-HER2 mediated signaling and ADCC (128,129).

In addition to these antibody-based approaches for targeting HER2, small molecules that inhibit the kinase activity of HER2 have been developed, most notably lapatinib. Lapatanib, approved in 2007, is a small molecule inhibitor of EGFR and HER2, which competitively binds to the ATP-binding domain thus blocking autophosphorylation. Lapatinib is indicated for HER2 positive breast cancers that have progressed with standard trastuzumab containing regimens. Due to the specificity for the ATP-binding pocket, lapatinib may have distinct mechanisms of resistance compared to trastuzumab. Nonetheless, lapatinib resistance can still occur via several mechanisms. Activating mutations of the PI3 Kinase pathway may also contribute to lapatinib resistance, as can loss of PTEN function (130). A mutation in the ATP binding pocket of HER2 (L755S) has also been reported to confer lapatinib resistance *in vitro* (131). It has also been purposed that activation of IGF-1R, ER or other receptor tyrosine kinases (such as AXL) may lead to tumors resistant to lapatinib treatment (132,133).

EGFR inhibitors

EGFR (HER1, ErbB1) is overexpressed and/or mutated in many cancers including lung and colon cancers. EGFR is a receptor tyrosine kinase activated by binding with epidermal growth factor (EGF), transforming growth factor α (TGFα), and other ligands (116). Activation of EGFR initiates several cell proliferating signal transduction cascades including the MAP Kinase, PI3 Kinase, JNK and STAT pathways (116). The tendency for EGFR to be upregulated or mutated in nearly 30% of epithelial cancers, demonstrates its high therapeutic potential for targeted therapy. To date, several compounds have been developed for treatment against EGFR-activated cancers including small molecule inhibitors such as gefitinib, erlotinib and antibody based therapies such as cetuximab.

Gefitinib (Iressa) and erlotinib (Tarceva) are small molecule inhibitors of EGFR which were approved for the treatment of EGFR positive lung cancer in the past decade. Both compounds reversibly inhibit EGFR kinase activity by competitively binding the ATP binding site (134,135). It was soon discovered that these drugs not only block EGFR activation, but that the best predictors of response were specific EGFR activating mutations (136). These sensitizing mutations were found in the tyrosine kinase domain, either in deletions within exon 19 between codons 746 and 759, or in exon 21 at L858R (136,137). However, while these mutations can increase the sensitivity of tumor cells to gefitinib or erlotinib, subsequent analyses have identified mutations that can confer drug resistance. The most clinically relevant resistance mutation in EGFR is in exon 20 at T790M (138,139). This mutation is thought to block the binding of inhibitors to the ATP binding site, thus leading to the constitutive activation of EGFR (140). This mechanism of resistance may be overcome with the introduction of irreversible EGFR inhibitors (141). Interestingly, another EGFR mutation within exon 19 at D761Y has been linked to gefitinib and erlotinib resistance in tumors that contain the L858R mutation (142,143). As in the case with many kinase inhibitors, resistance can also occur through activation of downstream pathways, such as PI3K (144). Likewise, activation of other receptor tyrosine kinases such as MET, IGFR-1, PDGF and additional ErbB family members has been linked to acquired gefitinib and erlotinib resistance (125,144).

Cetuximab (Erbitux) and panitumumab (Vectibix) are monoclonal antibodies against EGFR that are clinically used for the treatment of colorectal and other cancers. These antibodies bind to the extracellular domain of EGFR and are thought to change the conformation of the dimerization domain thereby simultaneously blocking ligand binding and preventing receptor dimerization (145). The resultant inhibition of EGFR phosphorylation combined with several other mechanisms of action contribute towards cetuximab's and panitumumab's efficacy against EGFR positive tumors (146).

One well classified determinant of EGFR antibody therapeutic sensitivity is the mutational status of *KRAS*. Constitutive activation of the MAP Kinase signaling pathway downstream of EGFR bypasses the effectiveness of cetuximab and panitumumab. In fact, patients with *KRAS* activating mutations have a poorer predicted outcome than those with wild type *KRAS* (147–149). As a result, *KRAS* mutational status is screened for prior to the administration of these therapeutic antibodies. More recently, two independent groups have demonstrated the appearance of *KRAS* mutations within sites of progressive disease in metastatic colon cancer patients treated with anti-EGFR antibody therapies (150,151). Mutations in *BRAF*, a serine-threonine kinase in the MAP Kinase pathway immediately downstream of KRAS, have also been linked to EGFR antibody resistance. Specifically, the V600E activating mutation is strongly associated to poor response to EGFR antibody therapy (152). Activating mutations of other downstream effectors of EGFR have also been linked to antibody resistance. For example, constitutive activation of AKT or PI3K, or loss of PTEN can diminish responses to EGFR antibody therapies (146,153). Finally, MET amplification has also been recently shown to be associated with acquired resistance to EGFR antibody therapies in colon cancers that are wild type for *KRAS* (154).

There are other documented mechanisms of EGFR antibody resistance. One method has been the production of VEGF leading to EGFR antibody resistance. The increased vascularization and activation of growth pathways by VEGFR is thought to bypass much of the anti-migratory and anti-proliferative effects of EGFR inhibition (155,156). Localization of EGFR to the nucleus may also be a contributor to sensitivity versus resistance (146). As in EGFR small molecule inhibitor resistance, changes in expression levels of the receptor tyrosine kinase family members HER2, HER3 and IGFR1 can also impart resistance to EGFR antibody therapies [143,145].

BRAF

The cytoplasmic serine/threonine protein kinase BRAF is a critical member of the MAP Kinase signal transduction pathway. Oncogenic mutations in both RAS and BRAF have been thoroughly described, with activating RAS mutations reported in ~15% of cancers, and BRAF mutations described in several cancer types including melanoma, colorectal, ovarian and papillary thyroid carcinomas (37). Nearly 66% percent of melanomas harbor BRAF mutations and of those, 80% of the mutations are a single base pair substitution in the kinase domain yielding V600E (157). The extremely high frequency of V600E mutations in malignant melanoma along with its role in resistance to other "upstream" therapies demonstrates high clinical potential as a target for treatment and predictor of response to targeted therapies. Currently, there are FDA approved therapies that target the V600E and other V600 mutations in clinical use: vemurafenib and dabrafenib. Vemurafenib, approved for clinical use in 2011, is a kinase inhibitor that selectively targets cells harboring V600E mutations, but has little effect on wild-type BRAF cells (158). When interacting with mutated BRAF, vemurafenib locks onto the ATP-binding site and will downregulate ERK signaling, inducing cell cycle arrest and activating apoptosis (158,159). Treatment with vemurafenib can be highly effective and many patients demonstrate significant (up to 80%) clinical response (160). More recently, dabrafenib in combination with the MEK inhibitor trametinib has also been shown as effective therapy for metastatic melanomas that harbor BRAF V600 mutations (161).

There are several described mechanisms of vemurafenib resistance, which eventually occurs in virtually all metastatic patients. Upregulation of PDGFRβ has been recognized as a mode of acquired resistance in a subset of melanomas (162). Alternatively, the up-regulation or mutation of oncogenic *NRAS* can also contribute to loss of vemurafenib sensitivity (162), further substantiated by a recent clinical report demonstrating mutations in *NRAS* and *MEK* being associated with vemurafenib resistant melanomas (163). The tumor micro-environment can also play a role in BRAF inhibitor resistance. Secretion of hepatocyte growth factor can stimulate MET leading to activation of the PI3 Kinase and MAP Kinase pathways independent of RAF inhibition (164). In addition, early attempts using vemurafenib in BRAF V600E mutant colon cancers did not demonstrate any appreciable response. Although mutant BRAF has been shown to predict for resistance against EGFR mediated therapies as mentioned above, recent work suggests an even more complex interplay. It appears that mutant BRAF suppression leads to feedback activation of EGFR in colon cancer cells resulting in vemurafenib resistance, suggesting the need to simultaneously target both proteins (165). Interestingly, it has been shown that vemurafenib treatment can paradoxically stimulate MAP Kinase signaling and may promote tumorigenesis in cells with wild type RAF (159).

EML4-ALK

Nearly 5% of non-small cell lung cancer cases are estimated to harbor a somatic translocation between echinoderm microtubule-associated protein-like 4 (EML4) and anaplastic lymphoma kinase (ALK) (166). The resultant EML4-ALK protein and others produced from ALK translocations, are hyperactive versions of the ALK receptor tyrosine kinase (167,168). Inspired by the success of BCR-ABL targeting with imatinib, a selective inhibitor of ALK was quickly developed. Crizotinib is a competitive inhibitor of the kinase activity that interacts with the ATP binding domain of ALK, ROS1 and MET (169). Crizotinib inhibits cell proliferation and angiogenesis, initiates cell arrest and can induce apoptosis (170). With a response rate of ~60%, acquired and innate resistance to crizotinib has also been documented (168).

Several mutations in ALK have been linked to crizotinib resistance. The most well documented of these is L1196M which impedes inhibitor binding to the active site (171). Other resistance mutations have been

identified in the kinase domain, as well as mutations that increase ATP affinity thus decreasing the effectiveness of competitive inhibition (168). Copy number increases in ALK can also decrease the efficacy of competitive inhibition by crizotinib (172). Alternatively, activation of other proliferative mechanisms can decrease tumor sensitivity to crizotinib. Specifically, upregulation of EGFR, HER2 or HER3, constitutive activating mutations in EGFR (L858R and S7681) as well as KRAS G12C and G12V mutations have been reported to be associated with a decrease in ALK inhibitor sensitivity (168,173).

Other Targeted Therapies and Conclusions

The past success and future potential of targeted therapies for cancer are evident, and many new drugs are currently in development. An obvious candidate for targeted therapy is PI3K, or specifically the p110α component encoded by the *PIK3CA* gene. *PIK3CA* is the second most commonly mutated gene in the cancer genome with a relatively high frequency found in lung, breast, brain, gastric, colon and other cancers (111,174). A high frequency of three "hotspot" *PIK3CA* mutations are found in two coding regions of the gene: the kinase domain in exon 9 (E542K, E545K) and the helical domain in exon 20 (H1047R) (175). These are not only common oncogenic mutations, but as described above, they also have been closely linked to resistance with receptor tyrosine kinase antibody targeted therapies. As such, there is currently much interest in developing PI3K inhibitors for cancer therapy (176–178). However, due to numerous classes and isoforms of PI3Ks, the isolation of compounds specific for activated p110α has proved challenging, and early generation PI3K inhibitors had predicted side effects due to off target effects of inhibiting other PI3K isoforms. Thus, the ability of using mutant *PIK3CA* as a predictor of response to these early compounds has not been uniformly observed and as a consequence knowledge of genetic effectors of resistance (and sensitivity) is lacking.

Other targets within the PI3 Kinase and MAP Kinase pathways are currently being pursued. AKT (protein kinase B) is a serine/threonine kinase in the PI3K/AKT/mTOR pathway that is often mutated and/or activated in human cancers (179). The three isoforms of AKT play key roles in activation of the cell cycle, angiogenesis, cell metabolism and cell survival (180). Similar to *PIK3CA*, AKT is a heavily pursued target for targeted therapies with numerous inhibitors at various stages of clinical development. MEK is another kinase downstream of RAS-RAF signaling within the MAP Kinase pathway. Targeting of this kinase has resulted in the development of several small molecule inhibitors including trametinib as mentioned above, and selumetinib (AZD6244). Based upon laboratory data and early clinical trials, there is a high level of optimism that targeted therapies against the above described kinases either as single agents or in combination will lead to new approved therapies over the next few years for a variety of human cancers.

An emerging area for targeted cancer therapies is the Hedgehog pathway. Hedgehog (Hh) signaling is key to embryogenesis, regulating the patterning and polarity of developing cells. Hh expression is silenced in mature cells, but can be activated by overexpression of ligand in certain cancer cells (181). Aberrant Hh signaling has been demonstrated in lung, pancreas, colorectal, and prostate cancers, as well as melanomas, lymphomas, multiple myelomas and glioblastomas (181). This activation is thought to support cell proliferation, angiogenesis, cell survival, cell migration and metastasis (181–183). Hh signaling is also a major component in the control, proliferation and differentiation of cancer stems cells (183). Thus, members of this pathway are strong candidates for anti-cancer targeted therapies. As example, vismodegib (GDC-0449), a small molecule inhibitor of the Hh pathway, has demonstrated significant activity in basal cell carcinoma, leading to its recent approval (184). Similar to many small molecule inhibitors, vismodegib is initially remarkably effective, but prolonged treatment often leads to acquired resistance. Resistance to this drug has been linked to mutation of

the ligand binding pocket of the target receptor (SMO), gene duplications and overexpression of cyclin D or Gli2, and activation of the PI3 Kinase pathway (185–187).

Over the past two decades, targeted therapies for cancer have made a significant impact on the morbidity and mortality inflicted by this group of diseases. These historic and recent examples demonstrate that the future of cancer medicine lies in the pursuit of better patient and tumor-specific therapies, and that genetic knowledge of cancer genomes can help guide these efforts. Much attention has been given to the clinical development of targeted therapies in the form of antibodies or small molecule inhibitors. The relative specificity of these drugs, combined with lower toxicity when compared to radiation and chemotherapy make them attractive for both clinicians and patients. Currently most efforts have been focused on the inhibition of kinase activity, and most drugs/compounds to date have similar mechanisms of action. Newer research emerging from academic labs and industry partners are now focused on novel methods and companion diagnostics to best match patients with the optimal targeted therapies. Importantly, the identification and validation of new types of targets remains as an important and high priority. Despite the past successes, drug resistance continues to be a common problem among targeted therapies limiting their use. As one might have predicted, the mechanisms of drug resistance are as complex and diverse as each individual's cancer genome. Activation of redundant pathways as well as selection for genetic alterations such as mutations and amplifications all contribute to the phenomenon of resistance to targeted therapies. Future research will therefore emphasize the use of multiple therapies that block cancer cell signaling across many pathways, as well as nodal points within each of these pathways. How this can be accomplished while maintaining a tolerable toxicity profile presents a challenge for future drug development. Further complicating this problem, the genetic instability of cancer cells leads to tumor heterogeneity which predicts a Darwinian selection process in metastatic disease favoring the emergence of drug resistant populations. This calls for not only the continued development of targeted therapies, but a better understanding of the moving landscape of a patient's cancer genome. Current and future technological breakthroughs in DNA sequencing and expression profiling may allow scientists and clinicians to better understand their patient's individual disease at a given time point allowing for tailored treatment regimen to prevent or combat drug resistance. Clearly, great strides have been made in the assessment and treatment of cancer, yet much work remains in finding true cures for this disease.

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We apologize to our colleagues whose important work could not be cited due to space constraints.

Author contributions
B.G.B., A.B. and B.H.P. wrote and revised the manuscript.

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