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Mechanism of activation and the rewired network: New drug design concepts

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Abstract

Precision oncology benefits from effective early phase drug discovery decisions. Recently, drugging *inactive* protein conformations has shown impressive successes, raising the cardinal questions of *which targets can profit and what are the principles of the active/inactive protein pharmacology*. Cancer driver mutations have been established to mimic the protein activation mechanism. We suggest that the decision whether to target an inactive (or active) conformation should largely rest on the protein mechanism of activation. We next discuss the recent identification of double (multiple) same-allele driver mutations and their impact on cell proliferation and suggest that like single driver mutations, double drivers also mimic the mechanism of activation. We further suggest that the structural perturbations of double (multiple) *in cis* mutations may reveal new surfaces/pockets for drug design. Finally, we underscore the preeminent role of the cellular network which is deregulated in cancer. Our structure-based review and outlook updates the traditional Mechanism of Action, informs decisions, and calls attention to the intrinsic activation mechanism of the target protein and the rewired

tumor-specific network, ushering innovative considerations in precision medicine.

KEYWORDS
cancer network, driver mutations, drug discovery, inhibitor, kinases, KRAS, K-Ras4B

1 | INTRODUCTION

Drug discovery has encountered expensive and daunting failures. At the same time, it has been notable for its innovation, inspiring successes, and promising advancements. Among these is first, the successful drugging of the *inactive* state of an unyielding protein, the "then-undruggable" KRas4B, by thwarting its mechanism of activation.[1,2] An a priori identification of those proteins whose *inactive* state is more likely to be susceptible to drugs than their *active* state is expected to be immensely valuable to drug discovery decisions.[3] A second advancement with therapeutic potential is the discovery of the co-occurrence of multiple cancer driver mutations on the same allele, that is, *in cis*.[4–7] Even though expected,[8] only recently it has been validated on cancer genomes and shown to promote more vigorous oncogenic signaling and higher sensitivity to inhibitors. This is particularly notable since the observation was on proteins (e.g., phosphatidylinositol 3-kinase α [PI3Kα]) lacking effective isoform-specific pharmacology.[9,10] This observation heightened optimism of more effective and safer next-generation drugs by reining in hitherto overlooked conformations. In a third advancement, a combined orthosteric plus allosteric drugs strategy targeting the same protein has shown promise in drug resistance (e.g., Bcr-Abl kinase drugged with imatinib or nilotinib and GNF-5 compound).[9,11,12] As we discuss below, such promising observations are all structure-based and their theoretical basis can be straightforwardly understood. However, challenges lie in their practical implementations.

Here, we consider these drug discovery landscapes and review the concepts and literature along these lines. This leads us to suggest that the activation mechanism at the structural level can help guide drug discovery decisions.[3] Why should the activation mechanism of the enzyme (or receptor) be considered in making drug discovery decisions? Cancer driver mutations work by mimicking the activation mechanism of the wild-type protein—except that they override its regulation.[13–15] There are multiple pioneering examples indicating such mimicry.[15–17] Drug discovery may similarly benefit from deliberating the protein activation mechanism undertaken by nature. As to the co-occurrence of double (multiple) cancer driver mutations *in cis* on the protein target,[4–6] protein conformational behavior suggests that the additive effect of the mutations is unlikely to alter the activation mechanism which would still mimic that of the wild-type protein.[18] However, the more potent signaling that the multiple mutations abet argues that significant differences in structural details are likely to emerge.[7,19] These might be harnessed to yield more specific, safer drugs. Finally, for the third, orthosteric plus allosteric combination, extensive molecular dynamics (MD) simulations can couple with experiments to identify the allosteric drug that can mitigate drug resistance to enable the orthosteric drug to block the active site, thus ligand binding.

Much has already been said about precision oncology and its treatment decisions. It has also been postulated that they largely rest on genomic testing, next-generation sequencing which along with additional clinical data can lead to effective pharmacology. The challenging dilemma of the interpretation of the patient's cancer genome landscape has been deliberated as well (e.g., Nussinov et al.[20] and Schwartzberg et al.[21]). The literature is rife with reviews of cancer development and progression, and drug resistance linked to these.[22–25] Here, we consider the innovative drug discovery landscapes noted above, review the concepts, and propose new principles. This review distinguishes itself by providing an innovative structure- and mechanism-based drug discovery outlook for drug discovery decisions.[26,27] We discuss drug discovery scenarios that are based on activation mechanisms, their

advantages and caveats, and some possible guidelines as to when and how to implement them, updating the traditional phenomenological Mechanism of Action (MOA). Especially, we underscore the importance of heeding the activation mechanism of the protein designed by nature and the preeminent role of the rewired cellular network in cancer.

2 | THE TRADITIONAL MOA CLASSIFICATION

Initiating from target identification, drug discovery involves a broad range of considerations and decisions.[27–33] Traditionally, they may include diverse combinatorial screening strategies. Some are aided by reporters, medicinal chemistry, and optimization of candidates to increase affinity and selectivity, efficacy or potency, toxicity, metabolic stability (half-life), and oral bioavailability. In recent years screening and optimization of approved drugs for repurposing has been especially prominent.[34–37] Drugs have been grouped based on their therapeutic use and dominant MOA, which can be complicated since drugs can have multiple mechanisms of action which can also be defined at multiple scales. The drug's MOA also includes the molecular targets to which it binds, such as an enzyme or receptor, whether it produces a change in the cell function, such as cell growth, and how it produces the effect on the specific target in the cell.[29,38] A drug class has been defined as a set of medications (or compounds) that have similar chemical structures thus a likely related mode of action, and/or used to treat the same disease. A pharmacologic class has been defined as a group of active moieties that share properties defined on the basis of MOA, Physiologic Effect, and Chemical Structure.[39]

Yet, standard structural classification, such as competitive (orthosteric, binding at the active/functional site) or noncompetitive (allosteric, binding away from the active/functional site), covalent, or noncovalent has not been included. The structural classification is not based on the molecular mechanism of activation, that is, whether the drugs target the *inactive* state of the enzyme or the *active* state. Diverse types of structural approaches, here compiled for oncogenic Ras as examples, are not there either.[2,40–64] Feature-related structural classification such as drugs blocking membrane anchorage through, for example, inhibition of farnesyl transferase (FTase) and translocation to the plasma membrane (e.g., tipifarnib, deltasonamide)[65–68] are missing as are drugs stabilizing or disrupting protein–protein interactions (e.g., dimerization, Ras-effector interactions)[69–72] and monobodies.[46,61,73] Driver mutations commonly mimic these mechanisms, promoting membrane attachment, as in the case of PI3K[7,74–79] or debilitating it, as in phosphatase and tensin homolog (PTEN) tumor suppressor[15] where they reduce membrane association, like K13E, S10N, G20E, L42R, and F90S, near the phosphatidylinositol-3,4-bisphosphate (PIP_2)-binding pocket.[15,80–84] Notably, not all structural mechanisms commonly adopted by driver mutations can be directly targeted by drugs. A case in point is relieving the autoinhibition, another frequent mutation strategy.[13,85–100] The significance of a molecular view of MOA as compared with a traditional phenomenological outlook is evidenced from refocusing the therapeutics from tissue- or cancer type-based, to cancer genomics and accurate protein structural data. The latter perspective has been adopted by precision medicine, altogether arguing for an update and modernization of the traditional MOA.

3 | PROTEIN STRUCTURE-BASED MOA DRUG CLASSIFICATION

We list major protein structure-based drug classes along with brief descriptions of some of their attributes. Among these, (i) orthosteric drugs are the oldest category.[101] These are competitive drugs designed to dock into the active, or functional site. Their advantages include knowledge of the active site. Since, however, active sites are conserved in protein families, orthosteric drugs are prone to eliciting side effects. Their affinity will also need to be significantly higher than that of the native ligand or cofactor with which they will need to compete. At the same time, the dosage cannot be too high due to side-effects from binding to homologous active sites. They work by blocking the active

site. Drug resistance mutations commonly work by modifying the active site shape, sterically obstructing their binding. Mutations in Bcr-Abl kinase interfering with imatinib[12] are one example (Figure 1). (ii) Allosteric drugs bind away from the active or functional site.[102–111] Since these sites are not conserved across the family, they are typically more specific thus with lesser side effects. Furthermore, since they bind at a distance, they work by promoting a shift in the conformational ensemble toward a conformation with an altered active site, thus enabling modulation of protein activity.[112–115] They operate by impeding or fine-tuning high affinity ligand or cofactor binding. They do, however, require sufficiently large, or deep, pockets in the protein surface with chemically favorable residues lining it, with the pockets commonly a priori unknown.[116–118] At the same time, discovering appropriate surface pockets can be challenging, as the case of KRas4B has demonstrated.[1,119–122] An alternative approach involves discovering rescue mutations and mimicking them.[108,123] The allosteric mutations in the myristate-binding pocket of Bcr-Abl that were able to promote an inactive state that could bind the inhibitor can provide an example (Figure 1). They overcame the T315I gatekeeper drug resistance mutation to competitive drugs such as nilotinib that prevented it and were subsequently mimicked by allosteric inhibitor GNF-5.[12] Recently, (iii) combinations of orthosteric plus allosteric drugs have been shown to be successful in countering drug resistance that emerged to the orthosteric drug, and blocked its active site binding.[9,124] The modulation of the active site structure prompted by the allosteric drug restored effective binding to a competitive inhibitor.[12] Protease-activated receptor-2 (PAR2) provides another potential example[125] as does B-Raf.[126] (iv) The drug can bind non-covalently, which is the case most of the time, or especially in the absence of sufficiently deep pockets, it can be covalent.[121,127–137] Examples that target the KRas4B^{G12C} mutant include AMG510 (Sotorasib, the first-ever KRas drug to be approved by FDA),[138,139] MRTX849 (Phase I/II),[140–142] JNJ-74699157 (formerly ARS-3248; Phase I, earlier ARS-1620),[143] and LY3499446 (Phase I/II). MRTX849 is a promising clinical candidate[134,135] as are AMG and

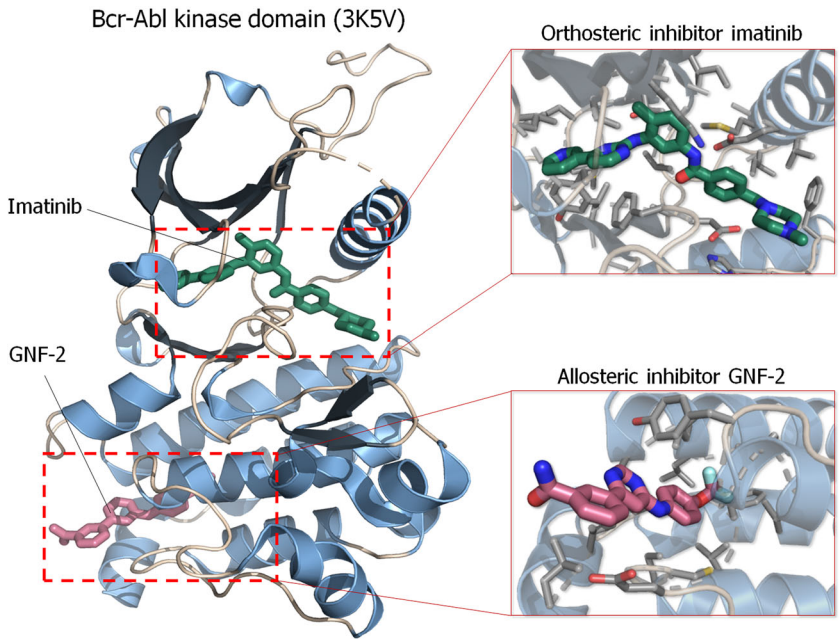

FIGURE 1 Bcr-Abl kinase domain structure. Bcr-Abl can be drugged with a combination of orthosteric and allosteric inhibitors to hinder the development of drug resistance. Crystal structure of Bcr-Abl kinase domain (PDB: 3K5V) with the orthosteric inhibitor imatinib (green) and the allosteric inhibitor GNF-2 (pink). Highlights of the ATP binding pocket with imatinib (upper right panel) and the myristate binding pocket with GNF-2 (lower right panel) [Color figure can be viewed at wileyonlinelibrary.com]

MRTX. Covalent attachment requires a cysteine and a sufficiently specific surrounding molecular surface, which allows a less active drug warhead. Since there are numerous cysteines in cavities in the proteome, this is likely to reduce toxicity. Covalent drugs have achieved remarkable successes in drugging the "undruggable" KRas4B. Recently, tyrosine has also been shown to anchor covalent drugs. A covalent inhibitor that forms a bond with a tyrosine was recently successfully placed on Ral which is almost identical to Ras GTPases.[144] Since covalent drugs are long-lived, protein degradation through covalently attached proteolysis targeting chimeras (PROTACs) are being pursued.[145–150]

4 | PROTEIN ENSEMBLES, DRIVER MUTATIONS, AND DRUGS

Biological functions are regulated by conformational states. Since proteins are highly dynamic molecules, evaluation of the protein structural ensembles is superior to that of a single state. Even if seemingly minor, a residue substitution would affect the conformational ensemble, and depending on the extent and type of the change, function.[151–153] Understanding the effects of single and double mutations on the conformational ensembles is crucial.[153–155] Intuitively, when a strong driver occurs on a protein, 90% of the conformations in the ensemble can be in an active state, and about 50%–75% of the conformations can be in an active state for a driver mutation. Weak drivers and strong latent drivers also can activate around 50% and 25% of the ensemble, respectively. A strong driver may be able to switch almost the entire set of the populations to a fully active state; but to facilitate such a fully activated state, other mutations need to cooperate. Ideally, personalized medicine would explore comprehensively such driver cooperation mechanisms across tissues.[156]

As we discuss below, recently, multiple driver mutations have been discovered in the same protein. They have shown larger sensitivity to orthosteric drugs.[4,5,9] No allosteric drug was tested since to date none exists for PI3K, the lipid kinase which was analyzed. It is however expected that the details and extents of the conformational changes that the mutations promote will differ, which may open new vistas for more specific drug discovery.[7]

5 | MOLECULAR ACTIVATION MECHANISM MATTERS IN DRUG DISCOVERY

Proteins act through structural changes and drugs aim to block their action. A competitive drug binding mechanism is powerful since it directly blocks ligand docking.[125,157–166] A noncompetitive drug binding can be powerful by altering the active site shape, leading to the same outcome.[167–177] These drug actions typically target the *active* conformation. Most drugs in the market work in this way. However, counter to intuition, drugs can also work by binding to the *inactive* state. These drugs can work by capturing the *inactive* or *nonfunctional* conformation and tampering with its mechanism of activation. The mechanism of activation can guide the decision on which type of drug to select, should it be one that targets the *active* or the *inactive* state.

A biological macromolecule exists not only in the shape captured in the crystal structure, but in a large ensemble of shapes.[178–184] Their distributions reflect their relative stabilities.[104,113,185–210] In the resting protein state, which is the state where most proteins (except for repressors) spend most of their lifetime, the protein is *inactive*. This is the more stable state under these conditions. Upon stimulation through some incoming cue, for example, a phosphorylated receptor motif as in the case of receptor tyrosine kinase (RTK), or a phosphorylated calmodulin,[211,212] or binding to another signal-activated molecule, as in the case of Raf binding to active Ras, or phosphorylation events as in the cases of AKT (a.k.a. protein kinase B) protein kinase,[213–216] mitogen-activated protein kinase kinase (MEK), and extracellular signal regulated kinase (ERK),[217,218] the relative stabilities of the *active* versus the *inactive* states change. This happens since binding events, noncovalent or covalent, and other changes in the macromolecular environment involve the formation and breaking of

interactions.[103,105,194,197,203,207,219–233] The alterations stabilize the *active* state (and/or destabilize the *inactive* state), leading to a shift in the ensemble from the *inactive* to the *active* state.[7,15,155,234] Driver mutations mimic the activation mechanism of the wild type. Like the native regulated scenarios, they also act by forming and/or breaking interactions incurred by the different chemical and geometrical properties of the substituted residue. The structural perturbations that they promote propagate in the structure just like the native scenarios do.[235,236] They hijack the same activation, and like them, can act to relieve the autoinhibition, expose surfaces to the membrane, and rearrange protein organization. However, the different mutations populate distinct conformations, thus preferred partners and signaling pathways as recently shown by the elegant work of Westover and his colleague[237] for $KRas^{Q61H}$ versus $KRas^{G12D}$ or $KRas^{G13D}$. That observation extends the group's earlier work[238] on $KRas^{Q61H}$ low GTPase activity, as well as higher affinity to Raf vs PI3Kα and the consequent enhanced mitogen-activated protein kinase (MAPK) signaling as compared to PI3Kα/AKT/mechanistic target of rapamycin (mTOR) signaling.

As an example, protein kinase activation involves switching the αC-helix-out to αC-helix-in (Figure 2A). This involves rotation and shift, with a salt bridge between the β3-Lys and the αC-Glu, and R-spine assembly.[104] The hydrophobic R-spine, with two aromatic residues in the C-lobe and two aliphatic residues in the N-lobe, is assembled in the active state, parallel to the C-spine, and disassembled in the inactive state (Figure 2B). Stabilized R-spine promotes activation. Driver mutations can stabilize the αC-helix-in and/or break interactions that stabilize the αC-helix-out. The L858R driver in epidermal growth factor receptor (EGFR) within a hydrophobic region is such a case. Replacing a hydrophobic by a positively charged residue breaks the hydrophobic interactions destabilizing the inactive αC-helix-out conformation and stabilizes the active αC-helix-in organization through heterodimerization. The T790M mutation in EGFR, T315I in Bcr-Abl, T334I in Abl1 (c-Abl), T338(341)I in Src, T670I in c-Kit, and T674I in platelet-derived growth factor receptor α (PDGFRα), all introduce a hydrophobic residue that stabilizes the hydrophobic R-spine, similarly shifting the ensemble toward the active conformation.[232,239] In PI3Kα lipid kinase, activation involves the binding of the nSH2 domain in the p85α subunit to RTK's phosphorylated tyrosine motif pYXXM in the C-terminal, leading to exposure of the active site in the kinase domain in the p110α subunit at the membrane. With charge reversal,[240] major driver mutations E542K and E545K in the helical domain relieve the nSH2 autoinhibition,[86] and through a series of conformational changes, lead to the same outcome.[7] Oncogenic replacements of Glu81, Gly106, Arg108, Lys111, and Gly118 in the adapter binding domain (ABD) also promote exposure.[14] These mutations act by lowering the transition state barrier (*ka*). The H1047R hotspot acts by increasing the population time (*km*) of the PIP_2 in the active site.

Thus, activation of kinases involves a shift in the ensemble from the *inactive* to the *active* state. Can drugs work by reversing this shift? Designing allosteric drugs that would shift the ensemble back to the *inactive* state is challenging. Allosteric drugs for kinases commonly aim at altering the shape of the active site to enable occluded orthosteric drugs to dock.[12] That is, kinase inhibitors work by *blocking the mechanism of activation*—not by targeting the active or inactive states. Nonetheless, there are exceptions that are based on the location to which the drug binds. Raf is one example[126,241,242] where a drug can bind to the inactive monomer at the dimeric interface to block activation by interfering with dimerization.

6 | INHIBITION OF AN ACTIVATION MECHANISM INVOLVING AN INACTIVE STATE

Kinases provide an example of inhibition of the *active* state. This however is not the case for the superfamily of Ras GTPases where a pioneering strategy has recently shown that inhibitors targeting the *inactive*, *nonfunctional* state can work and to date successfully advance through clinical trials. This disparity between kinases and small GTPases reflects the distinction of the activation scenarios between the two classes of proteins.[3]

Different from kinases, Ras activation cannot be described by the free energy as a shift of the ensemble from the *inactive* to the *active* state and neither can its activation by driver mutations. Instead, Ras is activated by

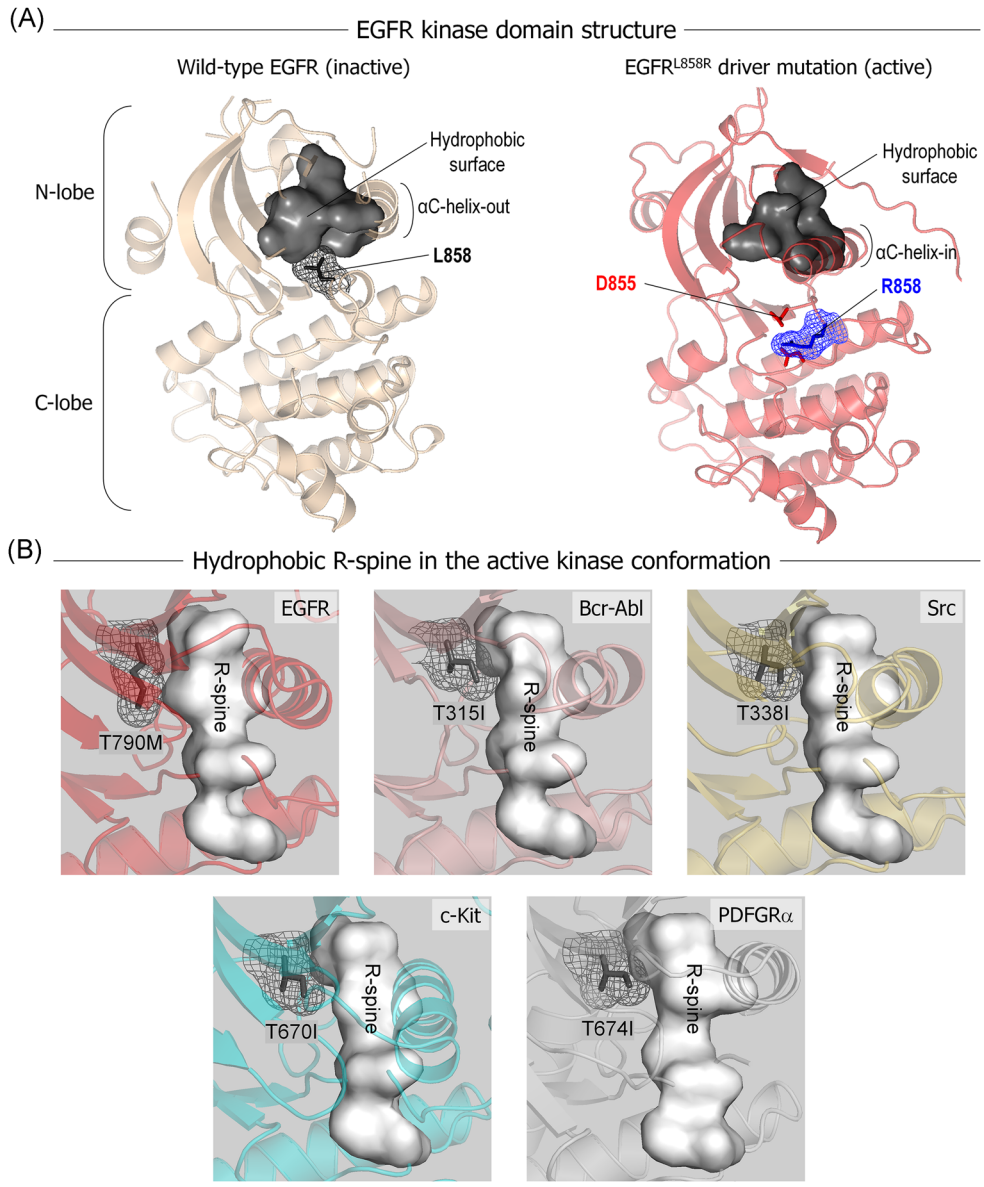

FIGURE 2 Structural insights into the driver mutations in kinases. (A) The L858R driver mutation in EGFR destabilizes the inactive structure (PDB: 1XKK) and stabilizes the active conformation (PDB: 6JX4). (B) The "gatekeeper" mutations in EGFR (PDB: 6JX4), Bcr-Abl (PDB: 2GQG), Src (PDB: 1YI6), c-Kit (PDB: 1PKG), and PDFGRα (PDB: 6JOI) stabilize the R-spine for the active conformation. The mutated residues were modeled based on the crystal structures [Color figure can be viewed at wileyonlinelibrary.com]

exchange of GDP by GTP through the Son of Sevenless (SOS), a Ras-specific guanine nucleotide exchange factor (GEF), which is recruited by Grb2 adapter protein[243–247] and is deactivated by hydrolysis of GTP to GDP by GTPase-activating proteins (GAPs).[246] Ras drivers work by interfering with the deactivating mechanisms. Stronger drivers block hydrolysis. Weaker, or tissue-specific drivers work by meddling with the nucleotide exchange. The strong drivers involve substitution of Gly12 and Gly13 by residues with long side chains which prevent GAP from inserting its arginine finger. The "finger" mediates proton transfer from an attacking water molecule to another, and

a subsequent different proton from that water molecule to GTP.[239,248,249] Gln61, also a strong driver, stabilizes the transient OH^- and $H3O^+$ ions to reduce the transition state barrier.[250] Weaker driver mutations, for example, $KRas4B^{A146T}$, aid nucleotide exchange back to GTP. Multiple approaches have been attempted for inhibition of Ras *active* states which to date have proven challenging. Apart from the GTP binding site, Ras lacks a deep pocket. However, the high millimolar range concentration of GTP in the cell and its picomolar affinity, results in approximately 75% of $KRas^{G12C}$ molecules interacting with it, making it formidable to compete even though hydrolysis is the highest for the $KRas4B^{G12C}$ mutant.[238] Approaches that target the active state include blocking cysteine farnesylation at the C-terminal, translocation from the endoplasmic reticulum and thus Ras anchoring to the membrane,[65–68] Ras–GEF interaction, dimerization,[69,70] Ras–Raf interaction,[71,72] synthetic single domain monobodies,[46,73] and more.[44,221,251–257] These were all extensively reviewed.[3] Several possible factors may have contributed to failures, including membrane liquidity, toxicity (e.g., FTase farnesylates additional proteins), lack of specificity and/or replacement by another prenyl moiety (e.g., geranylgeranylation), as in the case of blocking farnesyl transferase.

In an innovative strategy, Shokat and his colleagues covalently linked an inhibitor to the cysteine in *inactive* $KRas^{G12C}$ tampering with nucleotide exchange.[1] Their millimolar affinity inhibitor prompted subsequent higher-affinity drug development,[59] including AMG510 (Sotorasib, the first-ever KRas drug to be approved by FDA),[138,139] MRTX849 (Phase I/II),[140–142] JNJ-74699157 (formerly ARS-3248; Phase I, earlier ARS-1620),[143] and LY3499446 (Phase I/II) (reviewed in Nussinov et al.[3]). Responses of patients harboring the mutation that were given MRTX849 have been promising[134,135] as were those of Phase 1 AMG510 with advanced colorectal cancer and several other tumors.[139] To make the inhibited $KRas^{G12C}$ degradable, a C12 covalently linked PROTACs molecule (a proteolysis targeting chimera consisting of two linked molecules where one end binds ubiquitin ligase, and the other binds Ras) with a potent MRTX849 warhead (LC-2) was developed. LC-2 is an E3 ligase VHL-mediated degrading agent.[145] Exploiting cysteine disulfide tethering,[1] Shokat and his colleagues synthesized drugs binding to a pocket in the Switch II region near the nucleotide-binding pocket. This SII-P pocket is present only in the GDP-bound KRas4B, but not in the active, GTP-bound state. Binding to the inactive KRas4B promoted conformational changes in the Switch I and II regions which disfavored binding of Ras regulators and effectors, indicating that inhibitor binding to the *inactive* conformation is a feasible pharmacological route.[46] ARS-1620[143] has shown a more potent drug action in quenching Raf activation thus MAPK signaling.

A druggable pocket between Switch I and II[69,72,258,259] that exists in both active and inactive conformations of KRas4B proteins have also been targeted.[119] This SI/II-pocket is shallow and polar thus previously considered "undruggable." Initiating from weak binding candidates and structure-based drug design Kessler and his colleagues discovered BI-2852 (compound 1), a nanomolar inhibitor that curtails MAPK and PI3K/AKT signaling decreasing cellular proliferation. Inspection of the neighboring unit cell in the crystal structure of KRas4B–BI-2852 complex suggested that the inhibitor promotes KRas4B dimerization with two inhibitors with rotational symmetry.[260] Further structural scrutiny[3,119] pointed to formation of a nonfunctional KRas4B dimer, stabilized by two molecules of the BI-2852 inhibitor. Subsequent dimeric Switch I/II compound 2 pocket binders stabilized the active $KRas4B^{G12D}$ dimers with a K_D of 3.8 μM.[120] Soaking the crystal with compound 2 yielded a 1.9 Å resolution structure.[261] Co-crystallization obtained 1.57 Å resolution dimer, with an interface resembling that observed with BI-2852 and earlier proposed by modeling and MD simulations of active KRas4B molecules.[262]

Mutant Ral GTPases were also targeted by drugs binding to their GDP-bound state exploiting a new pocket[263] which displays a $KRas^{G12C}$-like mutation. A subsequent covalent Ral inhibitor with a novel tyrosine linkage was developed.[144] Ral proteins belong to the Ras superfamily and are almost identical to Ras. Blocking Rheb GTPase has also been explored albeit not targeting its inactive state.[264]

Thus, kinases and GTPases have distinct mechanisms of activation of their wild-type species. Their driver mutations mimic their respective mechanisms, and their drugs target their mechanisms of activation—rather than their active or inactive states.

7 | HOW TO IDENTIFY CANDIDATES FOR ACTIVE (OR INACTIVE) STATE PHARMACOLOGY?

As an example, we consider the PI3Kα lipid kinase. PI3Kα is activated at the membrane by activated RTKs and Ras proteins.[14,211,265–267] It's catalytic p110α subunit contains the kinase domain. The nSH2α and cSH2α domains in the p85α regulatory subunit have high affinity to the phosphorylated tyrosine motif (pYXXM) in the C-terminal of RTKs.[268,269] nSH2 autoinhibits the catalytic p110α by precluding its contact with the membrane where the PIP_2 signaling lipid substrate resides. The RTK–nSH2 interaction outcompetes that between the p85α and the p110α subunits, initiating a series of conformational changes that culminate in PI3Kα activation.[270] Hydrogen/deuterium exchange mass spectrometry (HDX-MS) data point to four prerequisites in activation: releasing the interaction of nSH2 with the helical domain of the p110α, breaking the interaction of the iSH2α domain in the p85α with the p110α C2 domain, movement of the ABD p110α domain which exposes the catalytic kinase domain surface to the membrane, and finally, lipid interaction.[17] nSH2 release also promotes structural rearrangement in the C-lobe of the kinase domain, resulting in a reduced ATP-substrate distance that permits phosphoryl transfer from ATP to the PIP_2 to generate phosphatidylinositol-3,4,5-bisphosphate (PIP_3). Notably, the regions where these actions take place do not interact directly with the catalytic sites. Whereas the crystal structures provide the key data and HDX-MS capture key activation events, modeling and molecular dynamics simulations can outline exactly how these events which are far away from the catalytic site regulate activation, and how the decisive conformational changes switch the inactive to the active state at the membrane. It can also elucidate how mutations promote activation and offer an allosteric inhibitor strategy.[7,9,10,15,15,240,271–275] This also holds for Ras and its other effectors (e.g., Refs.[236,276–278]).

Lipid kinase domains in PI3Ks coincide with the kinase domains in protein kinases. In protein kinases, the signature features of the DFG motif, αC-helix, and the activation loop (a-loop) specify the activity status of the enzyme.[85,279] In PI3K lipid kinases, signature features include the activation loop and the kinase domain helix 11 (kα11). In the inactive state the activation loop is collapsed and the kinase domain helix 11 is in the IN state. In the active state, the loop is extended and kα11 is in the OUT state. nSH2 regulates activation, catalysis, and autoinhibition through the a-loop. In the wild type, the inactive state is more stable. The altered interactions in the mutants render the active state of higher stability, driving the conformational change and activation. That however is not the case for Ras proteins whose activation status involves binding to GTP and retaining it.[3]

8 | DRUGS CAN ACT ON INACTIVE KINASES AND ON ACTIVE GTPASES

There are exceptions. Drugs can act on inactive kinases and on active GTPases. As an example, B-Raf mutations have been grouped into three classes[241] (Figure 3). B-Raf^{V600E} mutations belong to Class I. Mutations falling into Class I activate Raf by mimicking activation loop phosphorylation, causing B-Raf to adopt an active configuration.[280–282] Class II includes constitutive dimers. The activating mutations increase the dimer binding affinity, thus also relinquishing Ras help. A combination of strong latent drivers[154,155] at or close to the dimer interface can lead to this outcome. Class III features mutations that enhance dimerization, but still need active Ras. A drug that binds the *inactive* monomer at the dimer interface and maims dimerization is a successful inhibitor.[126,242] In another remarkable kinase example, allosteric compounds that bind to the myristate-binding pocket of Bcr-Abl can promote an inactive state (Figure 1), overcoming drug resistance mutations in the ATP-binding pocket as well as the T315I gatekeeper mutation (Figure 2B) restoring the inhibitory activity of ATP-competitive drugs in cellular and murine models of chronic myelogenous leukemia (CML).[12] At the same time, an inhibitor binding at the effector binding site of *active* Ras can still cripple Raf binding and MAPK signaling.

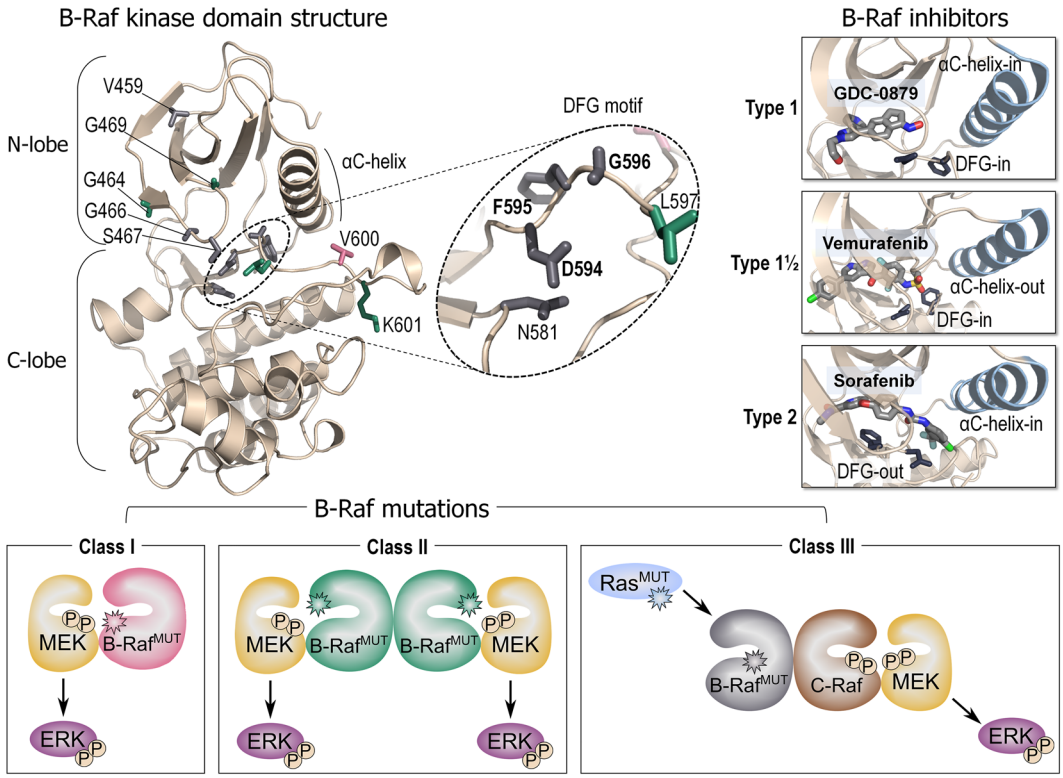

FIGURE 3 B-Raf mutations and inhibitors. B-Raf kinase domain structure with highlighted ^{594}DFG^{596} motif (left panel). Examples of B-Raf inhibitors (right panels). Inhibitors can bind to active or inactive B-Raf. GDC-0879 is a Type 1 inhibitor that binds to the active form of B-Raf with αC-helix-in and DFG-in. Vemurafenib is a Type 1½ inhibitor that binds to an inactive form of B-Raf with αC-helix-out and DFG-in. Sorafenib is a Type 2 inhibitor that binds to an inactive form of B-Raf with αC-helix-in and DFG-out. The αC-helix and the side chains of DFG motif are colored blue and black, respectively. In the cartoons, the crystal structures (PDB: 4MNE, 4MNF) were used to model the protein structures. The mechanism of activation for B-Raf mutation classes (bottom panels). B-Raf mutations are grouped into three classes based on activation mechanisms. B-Raf kinase domain with Class I (pink), Class II (green), and Class III (gray) mutation sites highlighted. Class I mutations are Ras and dimer independent. Class II mutations are Ras independent but require homodimerization. Class III mutations require activation via mutated Ras and dimerization with wild-type C-Raf [Color figure can be viewed at wileyonlinelibrary.com]

There can also be the same allele (*in cis*) double (multiple) mutations. Double driver mutations have been discovered in *PIK3CA* in human breast cancers.[4] They hyperactivated PI3K signaling and enhanced tumor growth which was more responsive to PI3K inhibitors as compared with single mutation tumors. A pan-cancer analysis of 60,954 samples identified 14 pan-cancer and six cancer-type-specific oncogenes where multiple *in cis* mutations occur more frequently than expected.[5] In PI3Kα, combinations included E453K/Q, E726K, and M1043V/I with E542K, E545K, and H1047R.[4] The first are weaker while the second stronger. As examples of the mechanisms, E453K/Q is in the C2 domain; E726K in the N-lobe of the kinase domain, and M1043V/I in the C-lobe (Figure 4). Like H1047R, with a positive charge E726K assists in membrane interaction. H1047 and M1043 are at the regulatory arch of kinase domain C-lobe. M1043 is buried, strengthening the hydrophobic core. E542K and E545K are in the helical domain, replacing RTK binding to the p85α to relieve the autoinhibition.[7] Altogether, they promote oncogenic cell growth and proliferation by contributing additively.[4,6] Since they mimic the activation mechanism,

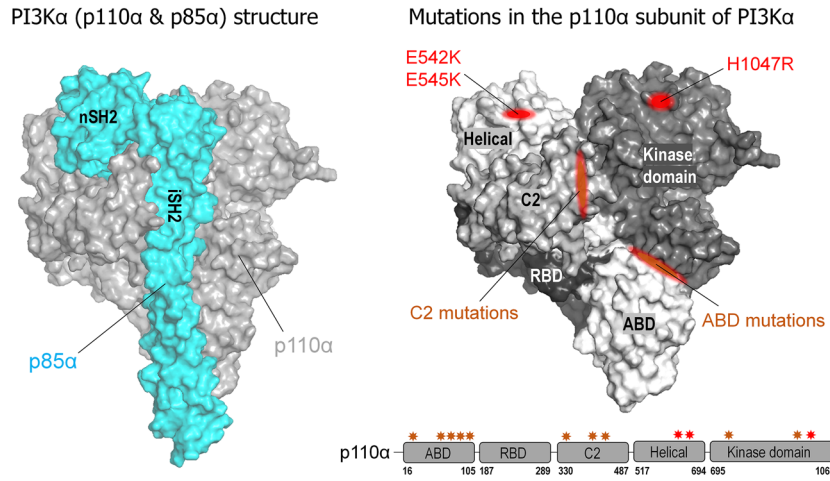

FIGURE 4 PI3Kα structure and mutations. A modeled PI3Kα structure (left panel) based on the crystal structure (PDB: 4OVV). PI3Kα is an obligate heterodimer composed of the p110α catalytic and p85α regulatory subunits. Mutations in the p110α subunit of PI3Kα (right panel). The p110α subunit in PI3Kα contains the hotspot (E542K, E545K in the helical domain; H1047R in the kinase domain) and weak (R38H/C, R88Q, R93Q, R108H, and G118D in the ABD; N345R/K, C420R/K, and E453K/Q in the C2 domain; and E726K, M1043V/I in the kinase domain) driver mutations [Color figure can be viewed at wileyonlinelibrary.com]

they are unlikely to change pharmacological decisions. However, structural perturbations incurred by two mutations likely differ from those incurred by one, which may affect drug designs.

9 | IN DRUG RESISTANCE, CO-OCCURRING MUTATIONS CAN BE ON THE SAME ALLELE AND PATHWAY, OR ON DIFFERENT PATHWAYS

More challenging scenarios include combinations of mutations harbored in different proteins in the same or distinct signaling pathways. Such combinations are likely to have emerged during cancer progression and metastasis calling for combinatorial drug regimes. No recurring cancer-causing mutations that were specific to metastatic tumors were observed, with most (96%) of the driver mutations being clonal.[283] Large-scale pan-cancer analyses on metastatic cancer tissue identified cancer drivers and mutation hotspots, observing that the mutational landscapes of metastatic genomes do not differ from those of primary tumors.[283,284] This suggested that metastasis-specific mutations are not responsible for the spreading of cancer. Forecasting the emergence of pathways harboring drug resistance mutations may involve detecting regulatory genomic regions with sparser chromatin density[285] and scanning and analyzing pre-existing and emerging mutations.[286]

Large-scale cancer genome sequencing projects including The Cancer Genome Atlas (TCGA)[287] and the International Cancer Genome Consortium (ICGC) obtained genomic profiling of more than 10,000 tumors. The AACR Project Genomics Evidence Neoplasia Information Exchange (GENIE)[288] led to the accumulation of a large volume of mutational profiles in human cancers. Transformation of these high-volume data to clinically interpretable knowledge and optimizing the treatment strategies based on the findings derived from these data are proceeding at a considerable pace. Recent statistical analysis[156] on somatic mutation profiles of approximately 80,000 tumors from pan-cancer data sets of TCGA and GENIE detected significant double mutations occurrences on the same alleles.[288–290] The tumor samples are from 671 cancer subtypes and 34 tissues. 228 significant double mutations

are identified on 35 genes including of 20 tumor suppressor genes (TSG), 12 oncogenes (OG), and the rest labeled as both.

Figure 5A presents tumors in brain, bowel, stomach, bladder, uterus, breast, and lung tissues harboring double mutations on four TSGs (*TSC1*, *APC*, *NF1*, and *PTEN*) and four OGs (*BRAF*, *KRAS*, *PIK3CA*, and *EGFR*). Although double mutations are extremely rare, the accumulation of the same allele double mutations is tissue specific. *PIK3CA* double mutations, for instance, are prevalent in breast, bowel, and uterine tumors; *EGFR* and *KRAS* double mutations accumulate mostly in lung tumors. *APC* double mutations are populated in bowel tissue; *BRAF* and *TSC1* double mutations are prominent in brain tissue.[156] Pan-cancer data revealed that double mutation components on the same protein rarely belong to the same domain. This can be attributed to the fact that oncogenic signaling can be boosted by co-occurring mutations on different domains; but on the same domain, it may not be the optimal way

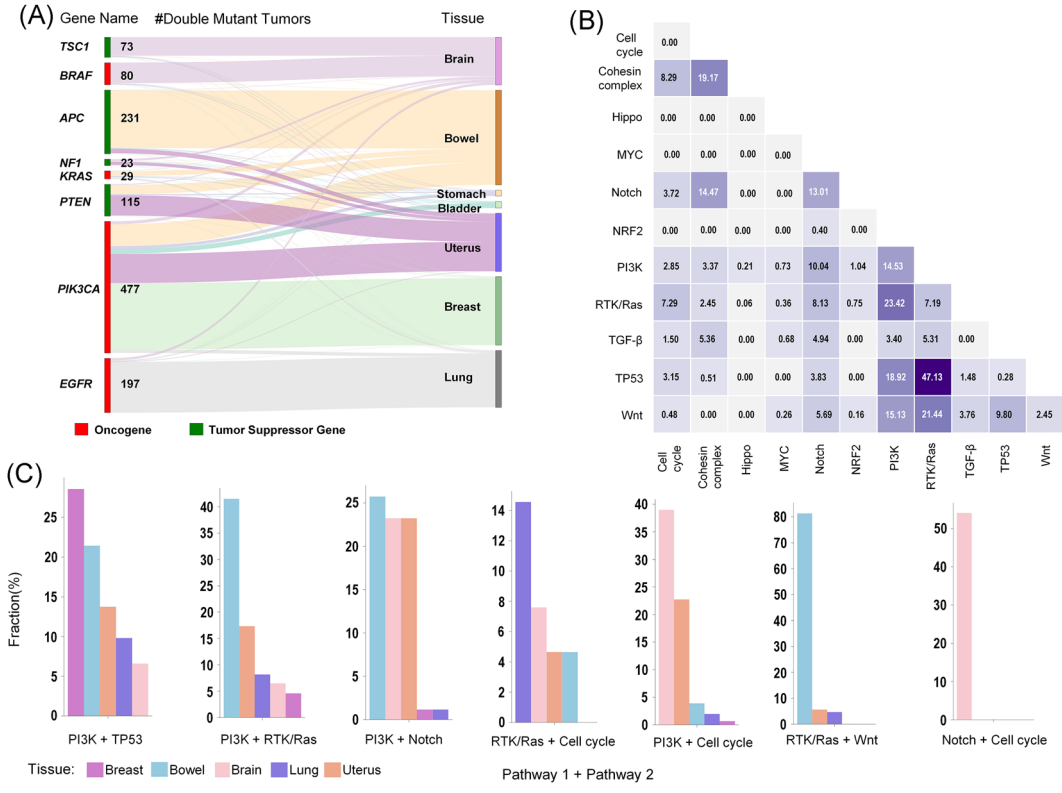

FIGURE 5 Co-occurrence patterns of mutations on same genes and pathways. (A) Prevalence of the double mutant tumors on four tumor suppressor genes, TSGs (*TSC1*, *APC*, *NF1*, and *PTEN*) and four oncogenes, OGs (*BRAF*, *KRAS*, *PIK3CA*, and *EGFR*) among the tumors in brain, bowel, stomach, bladder, uterus, breast, and lung tissues. Source nodes are genes harboring significant double mutations, and target nodes are the tissues enriched with double mutant tumors. Green source nodes are tumor suppressors, red source nodes are oncogenes. Size of the arc proportional to the number of double mutant tumors, arc color is compatible with the target node color. (B) Heatmap shows fraction of different gene double mutant tumors where constituents of the double mutations belong to the pathways on the *x*-axis and y-axis. Fractions are calculated based on the ratio of the double mutant tumors from pathway 1 and pathway 2 to the number of double mutant tumors where one component from pathway 1 or pathway 2. (C) Fraction of different gene double mutant tumors in breast, brain, bowel, lung, and uterus tissues. More than 25% of double mutant tumors where one component from PI3K and the other from TP53 pathways are accumulated in breast tissue. The fraction of double mutant tumors with components from PI3K and RTK/Ras pathways is ~5% [Color figure can be viewed at wileyonlinelibrary.com]

having another functionally equivalent mutation to provide growth advantage. In terms of precision oncology and drug resistance mechanisms, Nussinov et al.[155,285] proposed that it is vital to understand whether signaling pathways are redundant or parallel. Redundant pathways involve members of the same protein family and perform the same functions; pathways that involve evolutionary-independent proteins, are parallel. This distinction helps clarify the mechanisms of network rewiring and hence drug resistance. Remarkably, examination of the tendency of co-occurring mutations among genes belonging to the 10 canonical signaling pathways[287] and subunits of the cohesin complex (RAD21, STAG2, and SMC3) revealed that although not all genes in the listed pathways are covered by the data, genes belonging to the same pathway rarely harbor co-occurring mutations. Figure 5B indicates that only cohesin complex subunits, Notch and PI3K pathways co-mutate between 10% and 20% with a gene belonging to the same pathway; this fraction is below 10% for the remaining pathways. Single mutations in approximately 47% of the double mutant tumors are in genes in the RTK/Ras and TP53 pathways.

Figure 5C shows that PI3K pathway genes are co-mutated with the TP53 pathway (~25%) in breast tissue, RTK/Ras (~40%), and Notch (~25%) pathways in the bowel, and cell cycle pathway (~40%) in brain tissue tumors. The RTK/Ras and the cell cycle pathways are also co-mutated in the lung (~15%) and Wnt pathway in bowel (~80%) tumors. 50% of the tumors with mutations on genes from Notch and cell cycle pathways belong to brain tissue. Thus, certain pathways are co-mutated depending on the tissue in which the tumor is located; this offers a preliminary evaluation scale worth further investigation.

10 | DOUBLE MUTATIONS ON THE SAME ALLELE CAN RESULT IN DRAMATIC PHENOTYPIC ALTERATIONS

In "oncogenic addiction" the growth of the cancer cell can be targeted through a single oncogene. In certain genes, such as *PIK3CA*, double mutations increase the growth rate of the tumor, which can be dramatically slowed upon treatment with drugs targeting these genes.[4] Availability of the cell line and patient-derived xenograft data sets with treatment response information enables comparison of responses to drug therapy for different mutation status. Xenografts are especially well suited for the observation of wild-type, single and double mutation effects, as xenografts often provide untreated and treated versions with tumor volume information. Drug treatment responses for same-allele double mutations in cell lines and xenografts, which contain a single mutation and a wild type, differ. Figure 6A presents the *PIK3CA* wild-type, single (H1047), and double mutant (H1047/P539) breast cancer gene (BRCA) cell lines obtained from Cell Model Passports[291] and Cancerrxgene[292] databases. In AlloDB, the mutation P539R is cataloged as an allosteric mutation.[293] *PIK3CA* wild-type cell line does not contain same/different allele double mutation, and the single mutant cell line does not have any same allele double mutation. The three cell lines were treated with 39 common drugs targeting the six signaling pathways including the PI3K/mTOR, RTK/Ras, EGFR, Wnt, ERK, and cell cycle pathways. Drug responses of wild-type, single mutant, and double mutant cell lines are significantly different from each other (Mann–Whitney U Test, $p = 0.05$). The analysis included drugs with z-score < −0.5 in the double mutant cell line (BT-20). Moreover, it helps to observe the changes in tumor volumes after individual drug therapies. For double mutations in patient-derived BRCA xenograft models,[294] the volume of the tumor in the untreated double mutant xenograft increases more aggressively compared with the wild-type and single mutant xenografts (Figure 6B). The growth rate of the double mutant xenograft (*PIK3CA*$^{H1047/E726}$) is larger than the wild-type and the single mutant (*PIK3CA*^{H1047}). Also, treatments with drugs such as Paclitaxel (microtubule stabilizer), LEE011 (Ribociclib, CDK inhibitor), Trastuzumab (anti-HER2), BYL719 (Alpelisib, PI3K inhibitor), LJM716 (anti-HER3), and Tamoxifen (antiestrogen) slow down the tumor growth rate of the double mutant xenograft. As the available clinical drug treatment data increase, it might be possible to test the effects of double mutations in tumor suppressors that may cause loss of function on tumor growth and drug response.

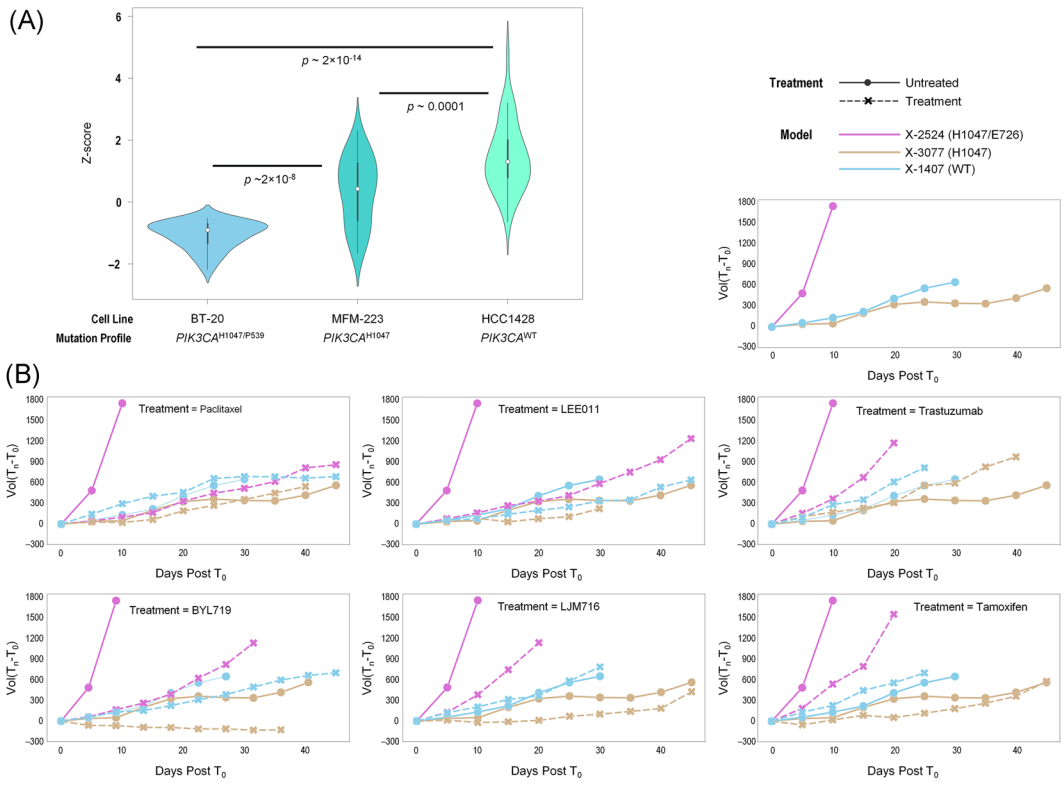

FIGURE 6 BRCA cell lines and xenografts drug responses. (A) Violin plot showing drug response distributions of *PIK3CA* wild-type, single (H1047), and double mutant (H1047/P539) cell lines. Drugs with z-score < −0.5 in the double mutant cell line (BT-20) are covered. 18 drugs (out of 39 common drugs) target the PI3K/mTOR signaling pathway. (B) Comparisons of tumor growth rates of wild type, single, and double mutant xenografts before and after treatments with several drugs. Double mutant xenograft shows better treatments with drugs, slowing down tumor growth rate [Color figure can be viewed at wileyonlinelibrary.com]

11 | PRECISION ONCOLOGY DECISIONS: THE CELLULAR NETWORK IS A CHIEF ACTOR IN CANCER

Even though commonly not emphasized, the rewired cell-specific cellular network emerging in drug resistance is a chief player in cancer.[20] Advances in genomics and interactomics are now making it possible to observe how disease mutations perturb protein–protein interaction networks within human cells.[295] The rewiring is driven by the altered cellular environment and the dynamic chromatin density at the regulatory regions of genes encoding proteins in the same or alternative pathways. Regions with lower chromatin density are more likely to be accessible to the transcription machinery[285,296,297] or to undergo some conformational remodeling leading to a similar outcome. Cell transformation involves alterations in genes regulating cell growth, division, and apoptosis and in pathway cross-talks. Cancer typically emerges from genetic changes involving uncontrolled growth and broken antiproliferative cellular responses. Both point to a rewired cellular network. That the network is a chief player can also be seen from genome-wide analysis of oncogene signaling. One example involves melanoma *BRAF* data which indicated that distinct pathways promote distinct melanomas.[298] In another, PI3K (*PIK3CA*), also a highly mutated gene in cancers such as breast, colon and endometrial cancer[299,300] merges incoming growth and survival cues from RTKs and Ras and acts on them by converting signaling lipid PIP_2 to PIP_3, and transmits the signals to the mTOR, MAPK, FOXO1 (forkhead box protein O1), and GSK3β (glycogen synthase kinase 3β) pathways. However, the gene

encoding the p85α subunit of PI3Kα is mutated in only approximately 10% of colorectal cancer patients raising the question whether this points to altered signaling that could be harnessed in drug resistance by the cancer cell.[249]

Networks emerge to coordinate key processes during development, including proliferation, apoptosis, and differentiation. Cancer cells rewire them. Cancer commonly involves co-occurring mutations and deregulation of signaling pathways can take place upstream or downstream.[20] If upstream it can be the outcome of, for example, overexpression of growth factors[301,302] or deregulating mutations in cell surface receptors. If downstream, activation mutations can bypass the requirement of incoming signals, as in the case of activating PI3K mutations that release the autoinhibition or obliviate the need for active, GTP-bound Ras signals. Another example involves PKC (protein kinase C) which can activate the MAPK pathway bypassing Ras activation[303] as can Raf by multiple classes of activating mutations.[304,305] The molecular checkpoint switches are often crippled in cancer, with the cell losing control of regulated progress through the cell cycle phases.[232]

The cell-specific network is a map of protein–protein interactions.[285,306] The proteins are nodes and the network controlled by network motifs and signal integration mechanisms. Cell- and state-specific networks vary, with some signaling pathways becoming more populated whereas others less so. Each node may receive several allostery-promoting signals through, for example, binding proteins or small ligands such as cofactors, and post-translational modifications such as phosphorylation, methylation, and ubiquitylation. The protein (node) integrates the signals and transmits a response.[225] Protein expression is controlled by the accessibility of the corresponding chromatin segments.[285,297] It is organ (tissue) specific and influenced by the cell state which is the outcome of stimuli and development. The accessibility is retained as the cell goes through mitosis,[307] emphasizing the cell-specific profiles of signaling pathways. Chromatin accessibility, thus protein expression, is a key factor determining the populated signaling pathways.

Cancer drivers often display tissue-specific mutational frequencies.[308,309] The distinct distributions of Ras isoforms, KRas, NRas, and HRas, and the statistics of their mutations are one example.[285,309–313] $KRas^{G12D}$ expression and its consequences in colorectal adenocarcinoma (CRC) development as compared with $NRas^{G12D}$ provide a mutant-specific case.[314,315] Distinct occurrences and outcome have also been observed in aggressive myeloproliferative disorder,[316] in intestine carcinoma,[317] and in cancers of the pancreas, colon, and lung as well. However, these may reflect the high signaling levels of MAPK pathway in these cancers. Metastasis-specific mutations were also discovered in *DCC*, *ABCA13*, *TIAM2*, *CREBBP*, *BCL6B*, and *ZNF185* genes, with signaling through distinct pathways during malignant progression.[318] Specific combinations were observed as well in metastatic CRC versus primary cancers.[319]

The cell-specific accessible chromatin regions and the protein–protein interaction networks of a skin cell differ from those of a pancreatic cell. This may explain the distinct functions of specific isoforms among tissues. They are expressed and preferentially interact with proteins which are available in those cells. Mutations emerging in these isoforms are then likely to be preferentially distributed in the specific tissues as well, demonstrating distinct distributions. That is, the mutants of isoforms operate within this landscape clarifying the observed tissue-specific tendencies.[285,308,320,321] Cellular perturbation following inhibition of a mutated target may advance drug resistance. Resistance may give rise to a mutant protein upstream which can bypass the drugged target by recruiting a family member that can substitute for the targeted protein. Higher expression of family proteins may evolve through shifts of the chromatin ensemble which alter genome accessibility. Alternatively, pioneer transcription factors can expose regulatory regions of genes that are tightly packed in the differentiated cell. The expressed proteins can signal through parallel proliferation pathways.[322] These proteins may not be expressed in the tissue-specific differentiated cell states or act in other cell types.[285,308,320,323] BRCA in pancreatic adenocarcinoma can serve as examples. Mutations to BRCA can increase the risk of developing pancreatic cancer and impact treatment decisions.[324]

Cancer cells draw on the chromatin organization to make expression of proteins in alternative signaling pathways possible by modulating their accessibility status.[325,326] They adopt cell lineage principles. Analysis of single-cell chromatin[327] and chromatin dynamics observed stage-specific transcriptional networks,[328] which can be activated in parallel proliferation pathways.[329] Modulation of the chromatin conformational landscape was shown

to relate to developmental and tumor-specific signaling pathways.[330–333] Additional examples emerged from Hi-C experiments[334] and more.[285,335]

Tumors showcase highly heterogeneous populations derived from a common progenitor.[336,337] They challenge pharmaceutical strategies and incite more resistant aggressive cells. Forecasting tumor-specific networks in drug resistance can be powerful in helping physicians select drug combination. A "pathway drug cocktail"[249] can be supported by a redundant pathway resource.[20,323] The National Cancer Institute assembled and made available drug combinations, many of which have been tested.[338] The massive genome sequencing data facilitated oncological drug discovery and a comprehensive database, My Personal Mutanome, was constructed for accelerating the development of precision cancer medicine protocols.[339] A strategy that forecasts the emerging proliferation pathway and the specific proteins based on high-resolution chromatin maps can be immensely useful. New high-resolution electron microscopy techniques which can image promoter regions of oncogenes are making this feasible[323] obtaining more accurate and deliberate targeting of specific cancers at a fraction of the cost.

12 | CONCLUSIONS

Precision medicine is challenging. Efficiency and potency of early decisions are vital for successful late phase clinical trials.[20,323] Recently, inhibition of inactive protein states has shown impressive successes, raising the question which targets can profit and what are the principles and guidelines for pharmacology of the protein inactive state. This has led us to provide a structure-based MOA classification, which updates the traditional phenomenological MOA, including orthosteric and allosteric drugs, and their combination, covalent and noncovalent drugs, and the innovative inactive/active category. *That is - should the active or the inactive state of the protein be targeted*? We suggest that the decision as to which conformation to take up in the design should largely rest on the protein's mechanism of activation. If activation involves switching the conformational ensemble from the inactive to the active state as in kinases, targeting an inactive state conformation has lower chances of success. However, if activation involves another mechanism, e.g. blocking nucleotide hydrolysis or promoting GDP/GTP exchange as in small GTPases, it is more promising. We consider the discovery of double same-allele mutations and their impact on cell proliferation and suggest that like single drivers, *in cis* double drivers also mimic the mechanism of activation although the conformational changes that they promote may differ. Collectively, these emphasize that drug discovery may benefit from deliberating and heeding the natural activation mechanism of the protein designed by nature. Finally, we underscore preeminent role of the cellular network which is deregulated in cancer. Altogether, our classification extends and updates the classical MOA, informs pharmacological decisions, and heralds innovative ingredient consideration offering new concepts in drug design.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

DATA AVAILABILITY STATEMENT

Data are available from the corresponding author upon reasonable request.

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AUTHOR BIOGRAPHIES

Ruth Nussinov pioneered the "conformational selection and population shift" (1999) as an alternative to the "induced fit" textbook model to explain the molecular mechanism of recognition and posited that population shift underlies allosteric regulation. She extended this pre-existing ensemble model to catalysis (2000), and oncogenic activation, contributing to extraordinary advancements in understanding structure and function. Nussinov received her PhD in 1977 from Rutgers University and did post-doctoral work in the Structural Chemistry Department of the Weizmann Institute. Subsequently, she was at the Chemistry Department at Berkeley, the Biochemistry Department at Harvard, and a visiting scientist at the NIH. In 1984 she joined the Department of Human Genetics, at the Medical School at Tel Aviv University. In 1985, she accepted a concurrent position at the National Cancer Institute of the NIH, Leidos Biomedical Research, where she is a Senior Principal Scientist and Principle Investigator heading the Computational Structural Biology Section at the NCI. She has authored over 650 scientific papers. She is the Editor-in-Chief of *Current Opinion in Structural*

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