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Genie in a bottle: Controlled release helps tame natural polypharmacology?

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Many drugs have a stereotyped target; Imatinib (Gleevec) (Figure 1) targets the oncogenic kinase Bcr-Abl. Yet, in reality, most drugs actually have multiple targets/interactions[1]. Of course, this promiscuity can cause undesired effects. But, sometimes, secondary targets are beneficial. The second-generation Bcr-Abl-kinase inhibitor, dasatinib, was developed as a Src-kinase inhibitor and also inhibits DDR2-kinase[2]. These additional targets partially explain why dasatinib fares better than imatinib[3], a “monogamous” Bcr-Abl inhibitor[4,5]. Unsurprisingly, many monogamous drugs are too constrained to effect prolonged relapse/cure of cancer. Several concepts have been proposed to combat the issues with monogamous drugs, including combination therapy (CT) and polypharmacology (drugs targeting numerous proteins). CT has been employed against cancer since the 1960’s[6] and a good deal of theory for development and experimental evidence supporting CT’s benefits is available[7,8]. Polypharmacology is arguably not as developed, but recent data are promising[9]. Here we propose a regimen to develop covalent polypharmacological drugs from existing electrophiles.

Combination therapy

There are many successful small-molecule CT regimens (Figure 2a)[10], including retinoic acid/arsenic trioxide that treats acute promyelocytic leukemia (Figure 2b)[11]. Small-molecule-and-antibody-based CTs are also approved: e.g., lung cancer patients now benefit from a pembrolizumab and pemetrexed/carboplatin combination, twice as effective as chemotherapy alone[12]. However, development of CT is not trivial. It is difficult to identify drugs functioning synergistically[13], and animal models are generally poor predictors of CT efficacy[14]. Combining drugs from different companies can be logistically difficult to coordinate[15]. CT can also change pharmacokinetics of individual drugs and there are multiple pathways to resistance[16]. Mutation is less common for CT than evolution of

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multiple drug-resistance, although several drug-resistant mutations in the oncogenic fusion-protein target of retinoic acid/arsenic trioxide, PML-RARA, are known (Figure 2b)[11].

Non-covalent polypharmacologic anti-cancer drugs

Several non-covalent anti-cancer drugs approved in 2017–2018 are poly- or oligo-pharmacological. These include ribociclib (CDK4/CDK6), binimetinib (MEK1/2) and midostaurin (a “multikinase” inhibitor)[17,18] (Figure 2a). Of these, only midostaurin is truly polypharmacologic; ribociclib and binimetinib target homologous sites within proteins with similar functions. In 2016, lenvatinib, a multireceptor tyrosine kinase inhibitor, and cabozantinib, a multitarget drug, were approved. Flavopiridol, a naturally-derived inhibitor of numerous cyclin-dependent kinases (orphan drug, 2014)[19] and sunitinib, a multiple receptor tyrosine kinase inhibitor (2006), were approved earlier[20]. Notably, many polypharmacologic drugs are inadvertent: imatinib is now approved to treat non-Bcr-Abl-positive tumors, since it targets the mast/stem cell growth factor receptor. Given the paucity of *truly* polypharmacologic anti-cancer drugs, and since these are predominantly ATP-competitive kinase inhibitors[9,21], new ways to design polypharmacologic drugs[22]—especially with novel target spectra—are needed. Several regimens have been proposed, but there are few guiding principles[23–25].

Polypharmacology and covalent binding

Until recently, pharma has shied away from covalent drugs because their reactive chemotypes were believed to amplify off-target effects, including drug-induced liver injury. We now appreciate that covalent drugs have numerous advantages over non-covalent binders, especially when targeting long-lived proteins. The go-to method to create covalent drugs is appending a low-reactivity electrophilic motif to a specific ligand, giving a more-or-less monogamous drug[1]; other examples of covalent inhibitors include mechanism-based inhibitors and reversible covalent drugs[26], although these are currently much less common.

However, the covalent Bruton-kinase inhibitor, ibrutinib (approved in 2013), which was developed using this common theme, inhibits numerous other kinases[27]. Furthermore, dimethyl fumarate (DMF) (Tecfidera)[28], a highly reactive molecule, is approved to treat relapsing multiple sclerosis, and has potential use in anticancer therapy. DMF—an analog of the oncometabolite fumarate[29,30]—taps into a mechanism called “electrophile signaling”. A thorough/exhaustive analysis of true *in vivo* pharmacological activities of DMF (or fumarate) is not technologically accessible currently, but it is likely that the targets of DMF and fumarate overlap. Fumarate targets several proteins including GAPDH[31] and Keap1. Additional targets have been proposed for DMF[28,32,33]. However, none of these fully explain the drug’s bioactivities. Sulforaphane—a polypharmacologic reactive compound present in vegetables[34] that is in Phase-II clinical trials—inhibits several deubiquitinating enzymes, and numerous other targets, although the principal target(s) are still debated[35]. Praeternatural sulforaphane analogs have been patented[36]. Similar arguments apply to other electrophilic pharmaceuticals in clinical trials, e.g., curcumin[37] and nitrooleic acid[38].

Thus, the pharmaceutical programs of reactive electrophilic molecules are an amalgam of multiple interactions, some beneficial, others possibly deleterious. How these behaviors fit together to produce a phenotypic output is complex. Improving our understanding of native electrophile signaling both in terms of defining key targets (those most likely/able to engage with the drug)[39] and linking specific ‘target engagement’ to pathway modulation(s) may promote better design of polypharmacologic drugs. Of course, it is not always easy to identify the key causative target(s) of compounds, especially polypharmacologic covalent binders. Indeed, the notion that key targets may exist for reactive electrophilic species (RES) was for a long time contentious. We and others have provided evidence that electrophiles hit specific sensor proteins that are key for their function. We called these sensor residues “privileged sensors” [26,39].

Mining privileged sensors

Insightful work from the Cravatt laboratory has shown that within ~1000 cysteines, 6 were hyperactive to the native lipid-derived RES, 4-hydroxynonenal (HNE)[40]. Only two of these proteins were kinases. 17 cysteines were reactive to an electrophilic prostaglandin (namely, 15-deoxy-^{12,14}-PGJ₂). Thus, only ~1% of the targets screened were reactive to these RES. Furthermore, there was little target overlap between the RES-chemotypes, even though they have similar reactive functional groups (enal/enone). Native RES are therefore discriminatory and likely have a built-in diverse repertoire of protein sensors. Despite the thoughtful developments from several laboratories[40–43], state-of-the-art chemoproteomics profiling strategies continue to face limitations as they only cover a fraction of the human cysteome (which contains ~200,000 unique cysteines in total[39]) and are restrained to target identification following bulk RES-exposure to live cells or isolated organelles. Whole-cell flooding with RES can affect redox homeostasis, label multiple proteins that are not typically modified, and/or upregulate apoptosis. Thus, bulk RES-exposure approaches pose formidable challenges in interlinking individual identified targets/sensor residues to compartmentalized/context-specific electrophile signaling[43–46]. Similar issues undermine efforts to understand promiscuous covalent drugs[39]. To understand electrophile signaling at the individual protein level with high spatiotemporal resolution, we developed a complementary target/ligand-pair-specific approach (T-REX)[47]. T-REX evaluates the RES sensitivity of a specific protein to a reactive enal/enone-based RES of choice in a largely unperturbed cell in live culture, worms, or fish, and for those targets that are RES-responsive, defines the precise RES-modification-dependent function directly in vivo [47–50]. Several privileged HNE-sensors that are known or potential drug targets were identified using T-REX. Relatively few were kinases and not all were enzymes. Interesting examples include: Akt3 (an oncogenic kinase; other isoforms were not as HNE-sensitive)[48]; PTEN (a tumor-suppressor phosphatase)[47]; and Ube2V1/Ube2V2 (allosteric stimulators of the ubiquitin-E2-conjugating enzyme, Ube2N)[50]. Importantly, mutation of the specific sensor cysteines [e.g., Akt3(C119), and Ube2V2(C69)] ablated HNE-sensing and signaling downstream. These privileged sensors showed hyper-reactivity to HNE *in vitro*[26]. Previously-reported HNE-sensors (e.g., glutathione-S-reductase) were folds less reactive to HNE *in vitro*[26,43]. These data underscore the complexities in pinpointing true electrophile “sensors” in uncontrolled conditions.

Low-occupancy RES-modifications determine cell response

Privileged sensors discovered by targeted RES-delivery in cells or fish altered their canonical activities as a function of RES-modification. By contrast, sensing-defective-but-otherwise-functional mutants did not. ~12% HNEylation of Akt3 elicited ~30% total Akt-inhibition in cells/fish (Akt3 expression was close to endogenous in fish)[48]; similar dominant inhibition occurred with PTEN[47]. Since PTEN and Akt function antagonistically (Figure 3), this result helps explain the difficulty to assign functional roles to HNE (and likely other) RES. The data also emphasize the importance of context to RES-modification-dependent cell responses.

In another example, upregulation of antioxidant response (AR) triggered as a result of Keap1-HNEylation alone under T-REX was as high as that achieved following whole-cell HNE-exposure[47]. Several other α,β -unsaturated electrophiles showed similar behaviors[51]. The parity of AR outputs between T-REX and bolus dosing is surprising, since multiple regulators of AR are RES-sensitive. Subtle differences were observed between T-REX and bolus dosing for HNE, such as signal latency and sub-population-specific response[47]. To us, this suggests that the reasons for this apparent parity are complex and worthy of further study, although by analogy to Akt3 versus PTEN above, antagonistic effects are likely at play. Conversely, Ube2V2-specific HNEylation in cells and fish stimulated activity of its cognate binding-partner, Ube2N, promoting homoallosteric upregulation of the DNA-damage response[50]: sensing-defective-mutant did not show such response. Thus, tractable responses are realizable following low stoichiometry RES-modification of privileged sensors. Such effects are often lost or suppressed in the cacophony of bolus RES-dosing, possibly due to triggering antagonistic pathways.

We thus propose the same muting effect occurs during administration of reactive/bioactive drugs/natural products. Such a dampening of phenotypic response would limit efficacy and render dosing strategies and individual responses difficult to predict, likely decreasing efficacy. Could we therefore “train” native RES to target (a) specific protein(s)? Could these targets be “hand-picked” to give a drug (or drug-like compound) which interacts with target(s) whose modification promotes intended phenotypes/signaling and eschews target(s) triggering phenotypes opposed to the intended outcomes?

Training HNE: monogamy vs. polypharmacology

One strategy is to design hybrid small-molecule modulators housing an attenuated HNE and a high-selectivity ligand. Such a strategy, by analogy to modern covalent-drug design based on “ligandable interactions”, may deliver “monogamous HNEs”. Such a drug should confer benefits of both HNE (covalent binding linked to dominant outputs) and the ligand (specificity). For instance, an Akt3-targeted monogamous HNE would avoid PTEN, and so would not trigger counteracting responses, unlike bolus HNE-exposure. Akt3 selectivity would be instilled by HNE-chemotype-implicit Akt3-isoform-specific reactivity. This proposed pipeline is similar to the development of imatinib, a “Bcr-Abl-monogamous” analog of the promiscuous natural product staurosporin. However, this monogamous-HNE design may not take full advantage of HNE’s intrinsic polypharmacology.

Several semi-selective, non-covalent ligands are known. Some bind similar sites (e.g., ATP-binding site) in a family of enzymes, while others inhibit seemingly-unrelated targets. Such promiscuous ligands could be coupled with an attenuated HNE (or similar covalent handle with tailored target repertoire). The ideal setup would lock in the ligandable interactions to targets, yielding phenotypically-matched outcomes, whereas antagonistic interactions would be transient or not possible. One could consider this pipeline analogous to the polypharmacological staurosporin analog, midostaurin, but because of HNE's broad binding promiscuity, not necessarily kinase focused. In addition, the inherent mechanism of privileged sensing would elicit phenotypes at modest occupancy on the target and response would be sustained, due to covalent binding. By biasing binding to chosen sensors whose pharmacological outputs are reinforcing/synergistic (critical attributes for polypharmacologic inhibitors)[23,24], and/or by choosing a covalent appendage only capable of interacting with a subset of ligand binders, synergistic polypharmacologic inhibitors could be generated. One element that sets this design apart from traditional therapeutics is that signaling-output is engendered by the electrophilic motif, as well as/or more than the ligand. Thus, chemotype- and function-guided target engagement could be iteratively modeled and an optimized drug motif developed using T-REX.

Interestingly, some cancers are more susceptible to RES-stimulated pathways than normal cells. Furthermore, target-specific responses differ from responses upon bolus RES-dosing[47,48,50,52]. The former can also be masked in studies using bolus approaches[52]. Thus, limiting reactivity to a subset of the targets of the parent compound could engender new and/or potentially more beneficial outputs than have been observed with traditional "untamed" polypharmacologic molecules.

A chemical genetic means to uncover pathway intersections: Achilles heels in cancers

T-REX-specific HNEylation of Keap1 and Akt3 are some of the most selective, effective, and least-invasive ways to modulate canonical signaling pathways. T-REX thus serves as a chemical genetic method to modulate pathway output, or to model target engagement of an oligo-/polypharmacologic RES/electrophilic drug. We illustrate this concept using our newly-uncovered link between AR and Wnt axes, pathways hyperstimulated in cancer. T-REX examined how Keap1-specific AR-signaling was upregulated in lines where proteins that regulate AR orthogonally to Keap1-specific signaling were knocked down. Lines in which β -TrCP1 or GSK3 β (proteins in the same pathway) were knocked-down did not mount AR when Keap1 alone was selectively HNEylated. However, these lines upregulated AR upon bolus HNE. We postulated that an HNE-sensitive β -TrCP1/GSK3 β -regulated protein may affect AR. One protein that fit this bill was β -catenin, a positive regulator of the Wnt pathway and a driver of cancer.

Wnt upregulation often occurs in cancers through mutation or deletion of the β -catenin N-terminus, preventing GSK3 β / β -TrCP1(2) from causing β -catenin degradation (Figure 4). We established that β -catenin/Wnt upregulates AR; however, AR strongly downregulates Wnt. Loss of β -catenin's N-terminus or knockdown of β -TrCP1 sensitized Wnt signaling to

inhibition by upregulated AR. We thus established that prevention of β -TrCP1 binding to β -catenin sensitizes the Wnt-pathway to AR-mediated inhibition. These data uncovered a dichotomy that must be resolved during oncogenesis: Wnt signaling is often upregulated due to loss of β -catenin N-terminus, but AR is also upregulated. We concluded that cancers with mutated β -catenin are likely more susceptible to electrophiles/AR-inducers than cells with wild-type β -catenin. This is because flux through the cancer-promoting Wnt signaling pathway will be more significantly downregulated upon AR in cells in which β -catenin cannot bind β -TrCP1.

Conclusion

There remains a pressing need for actionable molecular targets and new modes of action. With the growing suite of broad-specificity electrophilic drugs such as DMF, inherently reactive molecules usher a gateway to bona-fide pharmacophores with new and improved properties. The pharmaceutical programs of promiscuous drugs may thus be modified by focusing their specific target spectra. T-REX can parse synergistic vs. antagonistic responses by either executing T-REX simultaneously on two proteins, or measuring outputs that accompany individual protein/electrophile-specific perturbations. By iterative screening, undesired off-target effects may be prevented or winnowed.

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Abbreviations

Abl	Abelson murine leukemia viral oncogene homolog
Akt	RAC-alpha serine/threonine-protein kinase
APC	Adenomatous polyposis coli protein
ARE	Antioxidant response element
AXIN1	Axis inhibition protein 1
Bcr	Breakpoint cluster region protein
β-TrCP	Beta-transducin repeats-containing proteins
CDK	Cyclin-dependent kinase
CK1	Casein kinase 1
c-KIT	KIT Proto-Oncogene Receptor Tyrosine Kinase
DAG	Diacylglycerol
DMF	Dimethyl fumarate

ERK	Extracellular signal-regulated kinase
FGFR	Fibroblast growth factor receptor
FLT3	Fms Related Tyrosine Kinase 3
FOXO	Forkhead box protein
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GSK3	Glycogen synthase kinase 3
Halo	Modified bacterial dehalogenase
HNE	4-Hydroxynonenal
IKK	IκB kinase
IP₃	Inositol 1,4,5-trisphosphate
Keap1	Kelch-like ECH-associated protein 1
MDM2	E3 ubiquitin-protein ligase MDM2
MEK	Mitogen-activated protein kinase kinase
mTOR	Mammalian target of rapamycin
mTORC	Mammalian target of rapamycin complex
Nrf-2	Nuclear factor (erythroid-derived 2)-like 2
PDGFR	Platelet-derived growth factor receptor
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PIP₂	Phosphatidylinositol 4,5-bisphosphate
PIP₃	Phosphatidylinositol (3,4,5)-trisphosphate
PLC	Phospholipase C
PML-RARA	Promyelocytic leukemia/retinoic acid receptor alpha
PTEN	Phosphatase and tensin homolog
RA	Retinoic acid
RAF	RAF proto-oncogene serine/threonine-protein kinase
RAS	GTPase RAS protein
RES	Reactive electrophilic species
TCF/LEF	Transcription factor TCF/LEF
T-REX	Targetable reactive electrophiles and oxidants

Ube2N	Ubiquitin Conjugating Enzyme E2 N
Ube2V1	Ubiquitin Conjugating Enzyme E2 V1
Ube2V2	Ubiquitin Conjugating Enzyme E2 V2
VEGFR	Vascular endothelial growth factor receptor

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Synopsis

Ability to faithfully report drug—target interactions constitutes a major critical parameter in preclinical/clinical settings. Yet assessment of target engagement remains challenging, particularly for promiscuous and/or polypharmacologic ligands. Drawing from our improved insights into native electrophile signaling and emerging technologies that profile and interrogate these non-enzyme-assisted signaling subsystems, we posit that “trained” polypharmacologic covalent inhibitors can be designed. The accumulating evidence indicates that electrophile-modified states at fractional occupancy can alter cell fate. Thus, by understanding sensing preferences and ligandable regions elected by the seemingly-promiscuous Nature’s electrophilic signals at individual protein–ligand resolution, we can begin to achieve better evaluations of target engagement and function-guided understanding of polypharmacology.

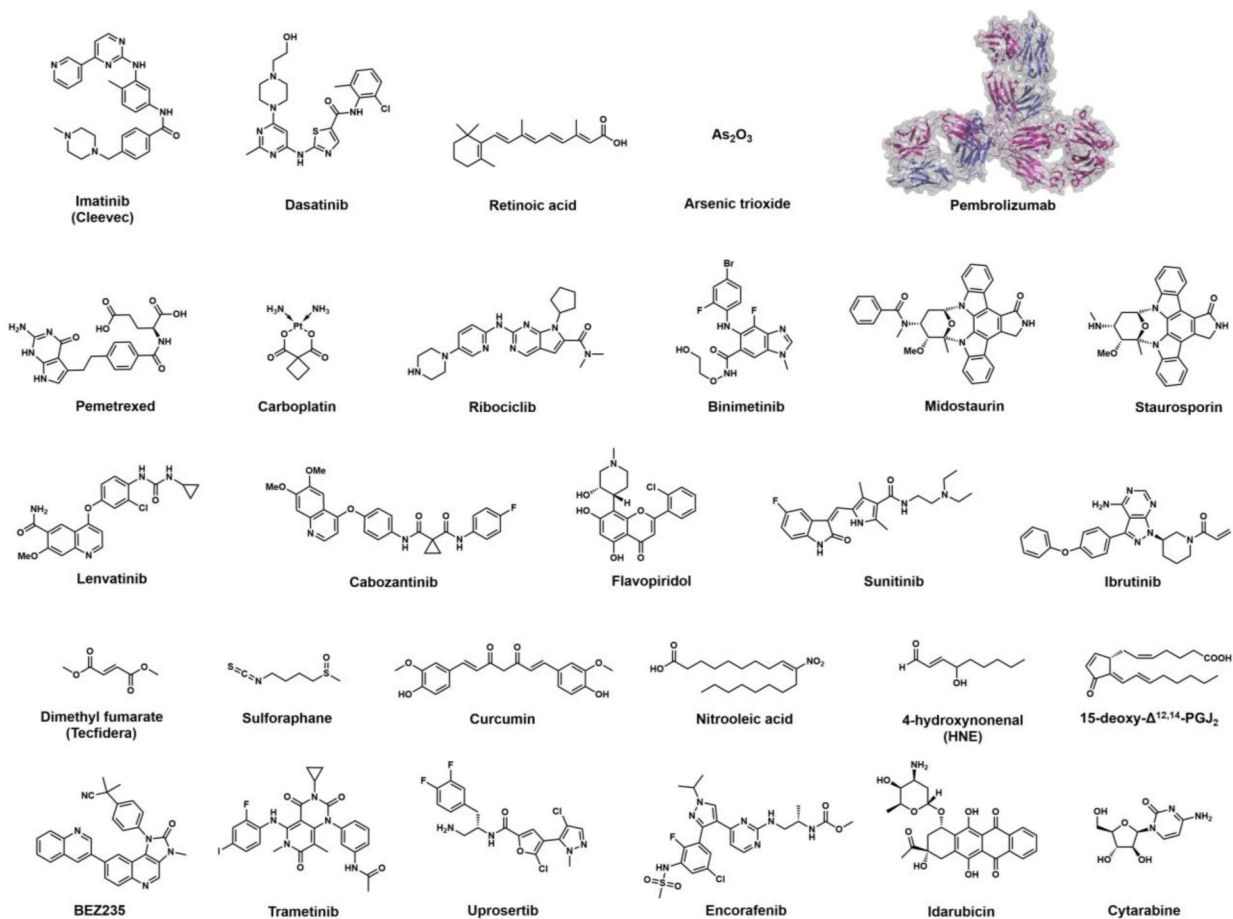


Figure 1. Small-molecule drugs and antibody discussed in this perspective.

The protein structure of pembrolizumab is displayed in ribbon and surface representation (PDB: 5DK3). The heavy and light chains are shown in magenta and blue, respectively. (The order of the small-molecule drugs and antibody in the figure is based on their first appearance in the corresponding text followed by their appearance in subsequent figures).

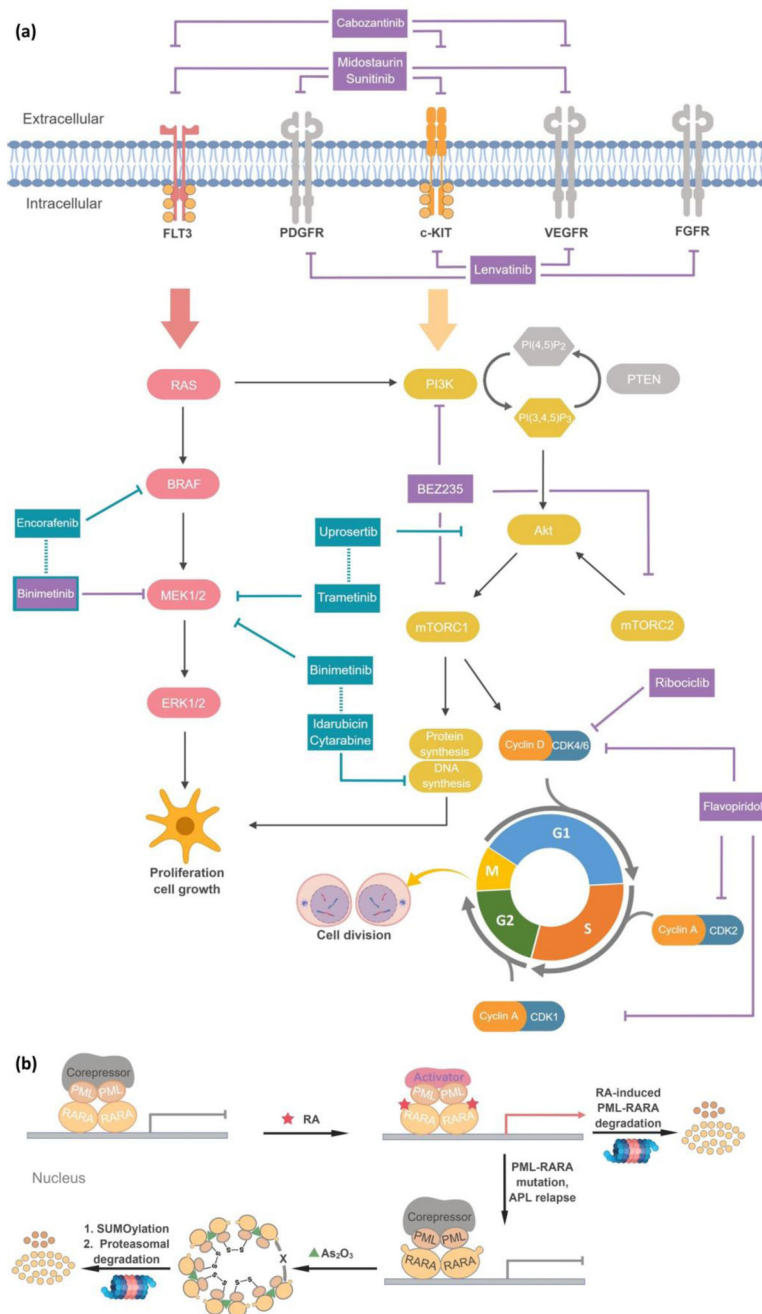


Figure 2. (a) Examples of combination therapies (CT) and polypharmacologic drugs[10]. This illustration focuses on combination therapies and polypharmacologic anti-cancer therapeutics (either approved or in clinical trials) that intercept Ras/RAF/MEK/ERK and PI3K/Akt/mTOR signaling. Drugs in aquamarine boxes with dotted connecting lines indicate CT and those in magenta boxes indicate drugs with more than one bioactive target. In particular, the MEK1/2 inhibitor binimetinib in combination with encorafenib has been approved by FDA to treat patients of BRAF-mutated melanoma, whereas the combination therapy (also shown in figure) of binimetinib, idarubicin and cytarabine has recently been terminated owing to logistical problems. Blunt-end and classical arrows (either originating

from the drugs or within the pathways) indicate inhibition and activation, respectively. **(b) Molecular actions of retinoic acid (RA) and arsenic trioxide (As₂O₃) CT in the treatment of acute promyelocytic leukemia (APL)[11].** Promyelocytic leukemia/RA receptor alpha (PML-RARA) together with transcription co-repressor mediate transcriptional silencing which impedes APL differentiation. RA induces cell differentiation by promoting the interaction between PML-RARA and its coactivator, eliciting target-gene expression. PML-RARA degradation can also be triggered upon prolonged exposure to RA in high concentrations. However, mutations in the RARA-domain often resist RA-assisted gene expression. As₂O₃ is used as a second-line of defense, which induces PML-RARA multimerization (denoted by “X” here) of both wild-type and RA-resistant mutants through interprotein disulfide bond formation, with consequent SUMOylation and proteasomal degradation. This CT-approach affords a high cure rate for APL patients.

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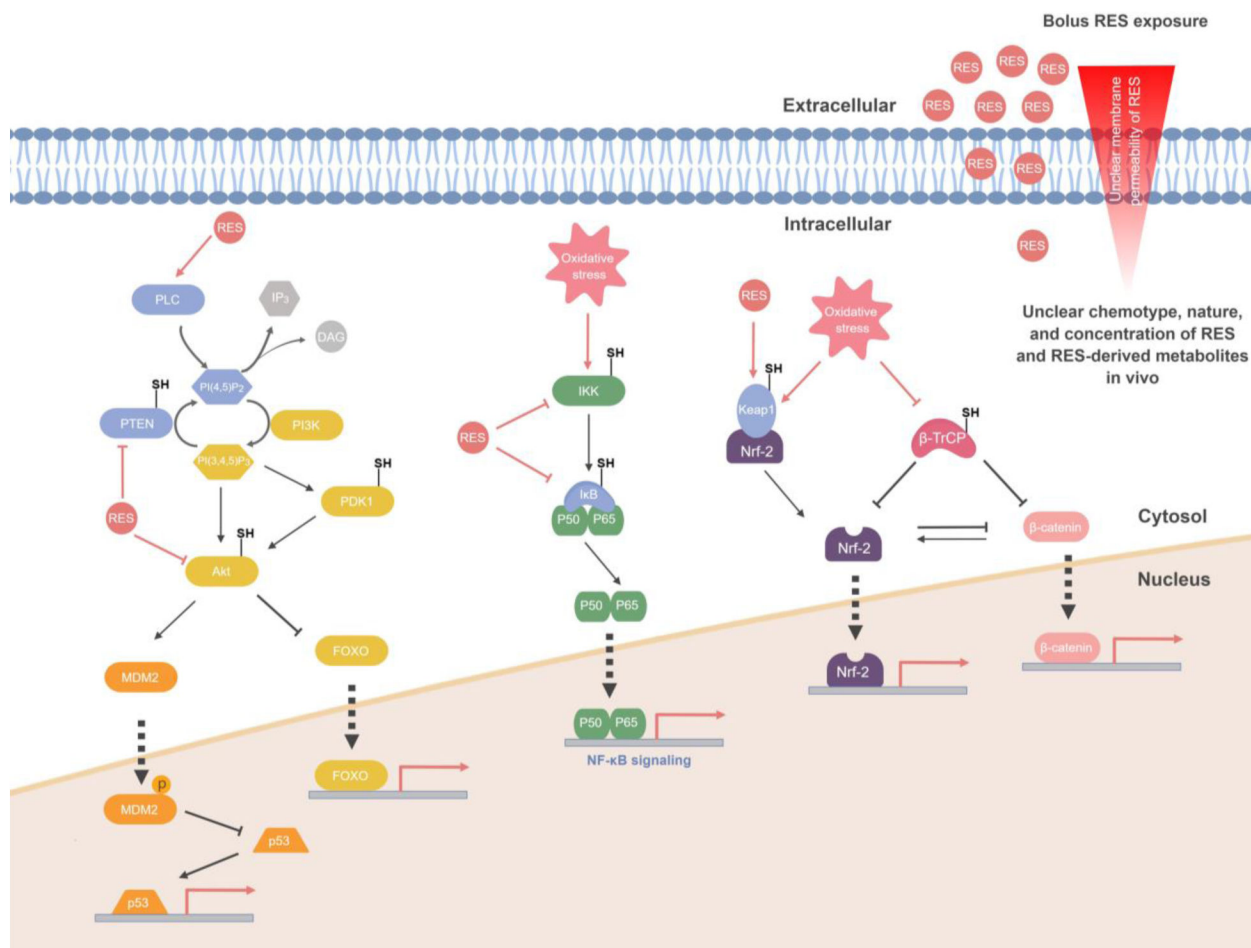


Figure 3. Challenges in deconstructing multicompartment cell responses triggered as a result of uncontrolled RES-exposure.

RES-flooding approaches modify various known and postulated redox-sensitive players (labeled with “-SH”) regulating manifold pathways. Examples shown include PI3K/Akt/mTOR[47,48], NF-κB[44], Keap1/Nrf-2[45] and β-catenin/Wnt signaling[52]. Both cooperative and antagonistic events are elicited: e.g., PTEN and Akt3 are each inhibited by RES adduction, but the effects of each inhibition are antagonistic, suggesting that the change in signaling is dominated by the relative importance of flux through the pathway on PTEN/Akt3 and PTEN/Akt3’s relative RES-sensitivities. RES also modulates PLC activity and accelerates PIP₂ hydrolysis[46], further complicating the RES-dependent regulation. Similar issues are encountered in NF-κB stress-responsive pathway. Oxidative stress induced by RES or ROS triggers inflammatory NF-κB signaling through upstream receptors/kinases activation, leading to IKK phosphorylation. The activated IKK phosphorylates IκB to release NF-κB transcription factor (p50/p65-complex), triggering transcription[44]. On the other hand, RES also reportedly covalently modifies (and suppresses the activity of) IKK to impede NF-κB signaling[44]. The key negative regulator of NF-κB signaling IκB also is covalently inactivated by RES upon bulk RES-exposure conditions. These conflicting RES regulatory events in NF-κB signaling occur predominantly at low to modest HNE concentration (1–5 μM). Bolus approaches can also mask RES-regulated pathway

intersections, such as the crosstalk between Keap1/Nrf-2 and β -catenin/Wnt pathways[52]. See text and Figure 4 for discussion.

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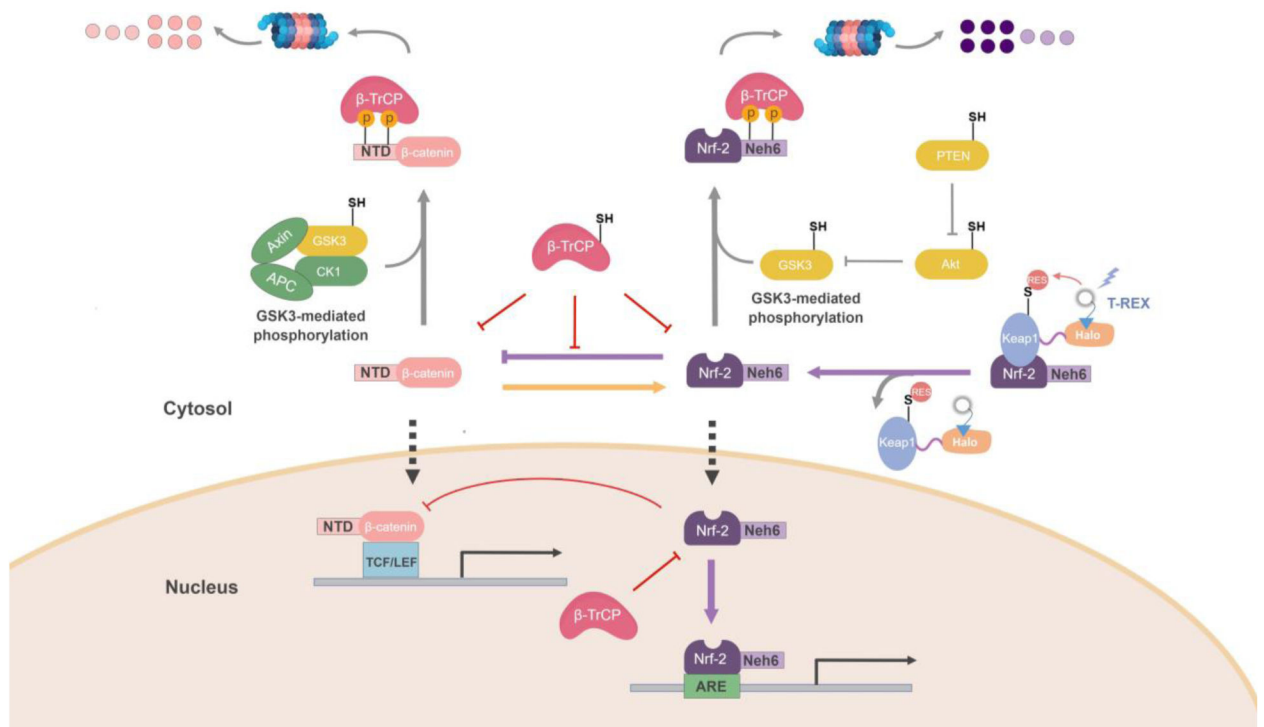


Figure 4. Study of AR signaling under Keap1-specific RES-modification reveals an unexpected intersection between β -catenin/Wnt and Keap1/Nrf-2 axes, in which β -TrCP sensitizes Wnt signaling to AR-mediated Wntpathway-inhibition[52].

Nrf-2 strongly inhibits β -catenin/Wnt signaling, whereas β -catenin overexpression upregulates AR. Both Nrf-2 and β -catenin are subject to proteasomal degradation mediated by β -TrCP. β -TrCP's binding occupancy at the N-terminal domain (NTD) of β -catenin protects β -catenin against Nrf2-mediated inhibition. To prevent β -TrCP-promoted degradation of β -catenin, the β -catenin-NTD is frequently mutated in cancers: these NTD-mutations upregulate Wnt-signaling. However, impeding β -TrCP-binding to β -catenin renders β -catenin signaling more susceptible to Nrf2/AR-mediated inhibition. This novel regulatory mechanism was uncovered only as a result of studying RES-induced AR-upregulation in a Keap1-specific manner. This delicate regulation and crosstalk between β -catenin/Wnt and Keap1/Nrf-2-signaling pathways are masked during whole-cell RES-stimulation. Known/postulated redox-sensitive players are labeled with “-SH”.