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Cell Signaling and Translational Developmental Therapeutics[a]

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Abbreviations

AIF	Apoptosis inducing factor
AMPK	AMP-dependent protein kinase
ATM	Ataxia-telangiectasia mutated
ca	Constitutively active
dn	Dominant negative
EGFR	Epidermal growth factor receptor
ER	Endoplasmic reticulum
ERK	Extracellular regulated kinase
GPCR	G-protein coupled receptor
GRP	Glucose-regulated protein
HSP	Heat shock protein
IF	Immunofluorescence
IHC	Immunohistochemistry
IP	Immunoprecipitation
JAK	Janus Kinase
LKB1	Liver kinase B1
MAPK	Mitogen activated protein kinase
MEK	Mitogen/extracellular regulated kinase
mTOR	Mammalian target of rapamycin
PI3K	Phosphatidyl inositol 3 kinase
PTEN	Phosphatase and tensin homologue on chromosome ten
ROS	Reactive oxygen species
RTK	Receptor tyrosine kinase
SoS	Son of sevenless
STAT	Signal transducers and activators of transcription
ULK1	Kinase Unc-51 like autophagy activating kinase 1

1 Introduction: Early Days

The field of cell signaling and signal transduction dates back to the late 19th century. In 1895, epinephrine (adrenaline) was discovered.[1] By the 1920s, insulin and glucagon had been discovered.[2,3] Collectively, these discoveries paved the way for researchers to explore how these hormones acted to regulate glucose metabolism in the liver and skeletal muscle. The laboratory of Dr. Carl Cori played a seminal role in partially unravelling how glycogen could be broken down by glycogen phosphorylase.[4] He, his wife Gerty and Bernardo Houssay received the 1947 Nobel Prize in Physiology or Medicine for their work. Although the Cori laboratory had discovered and described glycogen phosphorylase, it was not until 1959 that Leloir discovered the enzyme that made glycogen, glycogen synthase.[5] During the 1950s, Fischer, Krebs and Sutherland not only discovered and characterized the kinase which

[a]Dedicated to Professor Sir Philip Cohen on the occasion of his 75th birthday.

regulated glycogen phosphorylase, phosphorylase kinase, but defined for the first time that the phosphorylation of proteins could regulate enzyme activity.[6–9]

2 Further development of signal transduction

Up until the late 1950s, however, no-one had been able to elucidate how insulin signaled to make a cell store glucose as glycogen nor how epinephrine and glucagon activated phosphorylase kinase/glycogen phosphorylase to break down glycogen. Sutherland and colleagues during their investigations into glycogen phosphorylase discovered a heat-stable factor in liver sections whose levels were regulated by epinephrine and glucagon: cyclic AMP, the first second messenger.[10–12] Subsequently, Fischer and Krebs isolated the kinase regulated by cAMP, protein kinase A (PKA).[13] For these discoveries, Sutherland, as well as Fischer and Krebs, received the Nobel Prize. Sutherland, Fischer, and Krebs during their studies also discovered an enzyme activity which could remove phosphate from glycogen synthase, i.e. a protein phosphatase. A postdoctoral researcher from the laboratory of Fischer in the late 1960s, Philip Cohen, focused their independent career upon characterizing the many protein phosphatases in cells and above all understanding how phosphatases regulated glycogen metabolism, naming the ser/thr protein phosphatases.[14]

Over the 20 or so years after the discovery of PKA, multiple additional small molecule second messengers were discovered including: calcium ions, diacyl glycerol and IP3; and nitric oxide and cyclic GMP (cGMP).[15–18] Signaling by cGMP in the eye was shown to be essential for the perception of light and cGMP as well as with nitric oxide in the regulation of smooth muscle contractility resulted in the Nobel Prize being awarded to Murad in 1998.[18,19] During the 1970s and 1980s work by Lefkowitz, Gilman and Johnson led to the discovery of serpentine plasma membrane receptors for hormones, e.g. the beta-adrenergic receptor for epinephrine, as well as receptor-associated large GTP binding protein complexes on the inner leaflet of the plasma membrane which transduced receptor signals to intracellular effectors such as: (1) adenylyl cyclase leading to the generation of cAMP; (2) activation of phospholipases leading to the generation of diacyl glycerol and inositol 1,4,5-trisphosphate (IP3), with IP3 triggering the release of calcium ions into the cytosol.[20–30] Diacyl glycerol and calcium ion then activated multiple protein kinase C (PKC) isoforms. This resulted in the award of additional Nobel Prizes. Serpentine G-protein coupled receptor (GPCR) signaling can be down-regulated by proteins called Arrestins.[31–33] Arrestin proteins prevent both the Gα Gβγ proteins interacting with the GPCR and cause the GPCR to be internalized. Internalization can result either in receptor degradation or recycling back to the plasma membrane.

Thus, by the mid- to late-1980s a large body of literature existed which argued that signal transduction pathways consisted of a receptor linked to a large GTP-binding protein which in turn regulated an enzyme that generated "second messengers;" the second messengers would then diffuse throughout the cytosol activating cellular processes, predominantly for metabolism.

In parallel to the study of serpentine receptors, other investigators were focused on the relatively few proteins who became phosphorylated on tyrosine. Studies in this field were focused on the insulin receptor (metabolism) and the epidermal growth factor receptor (EGFR, ERBB1) (growth, cancer).[34–37] Insulin caused the insulin receptor to become tyrosine phosphorylated, and a substrate for the receptor, insulin receptor substrate 1 (IRS1), was discovered.[38] For many years prior to the 1990s, diagrams of insulin receptor signaling would include the receptor and IRS1, together with downstream insulin targets such as glycogen synthase. In-between the receptor and synthase was drawn a "black box" as the pathway by which insulin regulated glycogen synthase appeared to be intractable to investigation.[39] Studies by the laboratory of Larner and Villar-Palasi argued that insulin caused the generation of a "mediator" second messenger which was an inositol phospholipid, that regulated glycogen synthase.[40–42] Although at the time this concept was not widely supported, subsequent studies over the following 10 years demonstrated that insulin activated phosphatidyl inositol 3-kinase whose product, phosphatidylinositol (3,4,5)-trisphosphate (PI(3,4,5)P3), caused activation of the membrane-associated kinase, phosphoinositide-dependent kinase-1 (PDK1).[43–47] PDK1 was shown to phosphorylate AKT T308 causing enzyme activation, and AKT to phosphorylate and inactivate glycogen synthase kinase 3 (GSK3).[48] Reduced GSK3 activity results in reduced glycogen synthase phosphorylation, leading to activation of synthase activity. One additional component within this process was activation of protein phosphatases to facilitate the dephosphorylation and activation of glycogen synthase.[49] Thus, after 60 years of research, by the mid-1990s, the regulation of glycogen metabolism by epinephrine and insulin had largely been elucidated.

3 MAP kinase pathways

For the EGFR and other subsequently discovered membrane associated tyrosine kinases, e.g. the non-receptor SCR family and the fibroblast growth factor receptor (FGFR) family, understanding how these enzymes signaled into the cell again initially rested on studies using traditional biochemical methods.[50–53] In the mid-1980s, a postdoctoral researcher in the laboratory of Dr. Ora Rosen, Thomas Sturgill, was given a project in which he was to identify a 42 kilo Dalton protein whose tyrosine phosphorylation was increased after exposing cells to insulin.[54] As an independent investigator Sturgill continued his studies into the enzyme he called MAP2-kinase, microtubule associate protein 2 (MAP2) being the substrate used to measure its kinase activity.[55] It was subsequently renamed to be "mitogen activated protein kinase" (MAPK) after it was discovered to not only be regulated by many growth factors, but also that it was an intermediary kinase in the regulation of another insulin-activated kinase p90 ribosomal S6 kinase (p90rsk).[56] This enzyme should not be confused with p70 S6 kinase with is a component of the PI3K pathway.[43,44] By the end of the 1980s, it

had been determined that there was another MAPK isoform (p44) and that these kinases were regulated by tyrosine/threonine joint phosphorylation.[57,58] At that time, kinases were considered to be specific for either serine/threonine or for tyrosine. The discovery of MEK1 and MEK2 (mitogen/extracellular regulated kinase), kinases that phosphorylated the MAPKs on both tyrosine and threonine was considered biochemically novel.[59–62] From work in yeast (cerevisiae, pombe), however, was in parallel demonstrating that they also expressed MAPK-like and MEK-like enzymes, and that their MEK-like enzymes phosphorylated the MAPK-like enzymes on tyrosine and threonine.[63–65] The mammalian MAPK/renamed ERK1/2 (extracellular regulated kinase) pathway in yeasts regulates the yeast response to pheromones.[66] This understanding facilitated the further characterization of MEK1 and MEK2.

The next question in the development of the "MAPK pathway" was to define the kinase(s) upstream of MEK1/2. Based on data from yeasts, this kinase should have been similar to the mammalian MAP3K, known as MEKK1 (mitogen/extracellular regulated kinase kinase).[67] However, in 1992, two groups linked c-RAF-1 and its truncated oncogenic variant v-RAF as the kinase activity which enhanced MEK1/2 phosphorylation and activity; there are no yeast homologues of the RAF family proteins.[68,69] Of note, prior to those studies it was believed that RAF-1 was downstream of ERK1/2.[70] The function of MEKK1 subsequently, and with its family members, was linked in mammalian cells to the regulation of the c-Jun NH2-terminal kinase (JNK1/2) and p38 MAPK pathways.[71–76] Contemporaneously with these studies, researchers were determining how receptor tyrosine kinases regulated RAS family small GTP binding proteins, and other groups determining how RAS proteins signaled downstream off the plasma membrane and into the cytosol.[77–81] It was demonstrated that the proteins GRB2 (Growth factor receptor-bound protein 2) and SoS (Son of sevenless homolog 1) linked receptor tyrosine phosphorylation to the exchange of GTP for GDP in RAS proteins. Within months of these discoveries being published, it was shown that GTP-bound RAS would associate with the NH2-terminal domain of RAF-1.[82–85] GDP-bound RAS proteins did not associate with RAF-1. Thus, within the period between 1986 and 1994, the first of the "MAP kinase pathways" had been delineated. Because of extant data from yeasts, other parallel mammalian MAP kinase pathways were rapidly discovered and delineated. For example, as mentioned previously, the p38 MAPK pathway in mammalian cells is a stress-induced signaling pathway and was the equivalent of the HOG osmo-sensing pathway in yeasts.[86] The JNK pathway has similarities to several yeast and mammalian MAPKs, but only a ~60% best-fit to ERK1 and ERK2. It was discovered as a UV-activated kinase that bound to the NH2-terminus of the transcription factor c-Jun.[87] A parallel MAPK pathway, the ERK5 "big MAP kinase pathway" was discovered and inhibitors of MEK1/2 also inhibit MEK5, demonstrating the close functional alignment of both pathways.[88]

Hence, by the mid-1990s the basic structures of multiple MAP kinase as well as the PI3K pathway were in place. Broadly, over the past 25 years, signaling by ERK1/2 and ERK5 were most often linked to tumor cell growth whereas signaling by p38 MAPK and JNK were linked to cell death.[88–90] However, coordinated ERK/JNK signaling strongly promoted growth and under prolonged high activity ERK1/2 signaling would cause growth arrest via the induction of cyclin dependent kinase inhibitor proteins or tumor cell death.[91–93] These were also reflected at the level of receptor tyrosine kinases, comparing different ligands for the same receptor with different on-/off-rates, e.g. EGF and TGFα, as well as associated with ligand concentration. High ligand levels permanently down-regulate the receptor, and ligands such as EGF that remain with the receptor in endosomes cycle the receptor for degradation.[94–97] Signaling by p38 MAPK regulated chaperone functions but also could cause cell cycle arrest and DNA damage repair.[98,99] What also became readily apparent was that activation of the same pathway to the same extent in different tumor cells could result in different changes in tumor cell biology, with some cells exhibiting growth/growth arrest and other cells becoming moribund either through apoptosis, necrosis or autophagy.[91–93] Some of these behaviors could in part be explained due to the differential expression of driving oncogenes such as mutation of p53, RAS proteins, receptor tyrosine kinases or the lipid phosphatase: phosphatase and tensin homologue on chromosome ten (PTEN).[100–103]

4 Autophagy

The cellular process of autophagy was discovered in the 1960s.[104,105] The primary purpose of the process is to recycle cellular components into their elemental building blocks during times of metabolic stress, permitting the cell to survive. Materials are first encapsulated in a double membrane, called an autophagosome.[106–108] Autophagosomes fuse with lysosomes, the interior acidifies, and they become autolysosomes where materials are digested, ready for recycling. The regulation of autophagy and with it the sensing of nutrient and ATP energy levels within a cell are regulated by mammalian target of rapamycin (mTOR) and the AMP-dependent protein kinase (AMPK), respectively.[109–113] The regulation of mTOR is complex as it integrates upstream signaling from AKT in the PI3K pathway, together with other signals that sense amino acid, lipid and carbohydrate levels. There are two complexes of proteins which associate with mTOR, with the kinase being termed mTORC1 or mTORC2 based on the members of the protein complex.[114–116] The AMPK senses AMP levels, which are high when the cell is depleted of ATP; high AMP levels cause allosteric activation of the AMPK, and activated AMPK then acts to phosphorylate and inactivate mTOR.[117–119] The AMPK is itself regulated by phosphorylation, with the most notable regulators being Liver kinase B1 (LKB1) and ataxia-telangiectasia mutated (ATM).[120–125] LKB1 is often mutated in tumor cells, leading to dysregulation of energy sensing and autophagy regulation. In the nucleus ATM senses DNA damage and cytosolic ATM senses the levels of reactive oxygen species; ATM at both cellular locations phosphorylates and activates the AMPK.

The key regulatory target for both mTOR and the AMPK is the kinase Unc-51 like autophagy activating kinase (ULK1/2).[126–129] ULK1 is a classic example of a protein whose function is regulated by multi-site phosphorylation. Phosphorylation of ULK1 at specific sites by mTOR inactivates the kinase. Phosphorylation of ULK1 at different specific sites by the AMPK activates the

kinase.[128–130] The primary substrate of ULK1 is the gate-keeper protein for autophagosome formation, ATG13. Phosphorylation of ATG13 leads to the formation of multi-protein complexes which act to form a double membrane around the cellular materials that will be digested. Autophagic flux occurs where a fully-formed autophagosome fuses with an endosome/lysosome to form an autolysosome.[131,132] Autolysosomes acidify their interior, activating a variety of proteases and other enzymes required to break down the vesicle's contents. Many tumor cells exquisitely rely on autophagy to survive, which explains why drugs such as chloroquine, which prevent autophagosome lysosome fusion, have been trialed as cancer therapeutics.[133,134] Alternatively, as tumor cells utilize autophagy for survival, drugs which profoundly stimulate autophagosome formation and autophagic flux cause the over-digestion of cellular proteins and cause the cytosolic release from the autolysosome of active proteases, which collectively leads to a multi-factorial form of tumor cell death.[135]

5 Using our understanding of autophagy and cell signaling to therapeutically kill tumor cells

In all scientific studies, experiments should be performed from an agnostic standpoint. That is, follow the data wherever it may lead, regardless of prior opinions or perceptions. Twenty years ago, in collaboration with Dr. Paul Fisher, we began to investigate the molecular mechanisms by which the cytokine IL-24 acted to kill tumor cells.[136–138]

At that time, the mechanisms by which tumor cells died were not particularly sophisticated, with death receptor signaling via caspases 8/10 (the extrinsic apoptosis pathway) and mitochondrial dysfunction via caspase 9 (the intrinsic apoptosis pathway) being the two pathways then considered most important in the causation of tumor cell death. Because we had observed the cytokine was inactivating mTOR, studies were performed to define if "autophagy" played any role in the cytokine's biology. Molecular knock down of key autophagy regulatory proteins, ATG5 or Beclin1, profoundly suppressed IL-24 lethality. Our studies with autophagy and IL-24 resulted in other laboratory projects exploring the role of autophagy in their biology and killing mechanisms. For example, in hepatoma cells, the combination of the multi-kinase inhibitor sorafenib with the histone deacetylase (HDAC) inhibitor vorinostat killed cells by activating the death receptor CD95, and in hepatoma cells, knock down of ATG5 or Beclin1 enhanced drug combination lethality, i.e. autophagy was acting as a protective cellular response.[139] However, in pancreatic cancer cells, knock down of ATG5 or Beclin1 significantly reduced the ability of this drug combination to kill, i.e. autophagy played a role in the killing process.[140] Subsequent studies in the laboratory over the past decade have almost invariably discovered that autophagosome formation was playing an essential role in the tumor cell killing process.

One consideration when discussing the role of autophagy in causing cell death is whether the autophagic process caused killing directly, or indirectly by causing, e.g. mitochondrial dysfunction, followed by release of cytochrome c and apoptosis inducing factor (AIF) into the cytosol. AIF moves to the nucleus to cause DNA fragmentation in a fashion similar to necrosis.[141] Cytochrome c binds to Apoptotic protease activating factor 1 (Apaf-1) which together with ATP causes the cleavage of pro-caspase 9. Activated caspase 9 cleaves and activates caspase 3, which moves to the nucleus to cause apoptotic DNA fragmentation, with DNA fragments encapsulated in membranes. Alongside the apoptotic processes, cathepsin proteases released from autolysosomes can cleave and activate the pro-apoptotic protein BID that is upstream of mitochondria, and which will lead to mitochondrial dysfunction and death.[142] However, it is possible that release of activated proteases by themselves into the cytoplasm can also cause death, without involvement of the mitochondria.

We will now illustrate in more detail the role of autophagy in the development of anti-cancer therapeutics and in the development of anti-viral therapeutics. The multi-kinase inhibitor drugs sorafenib and pazopanib are approved for the treatment of liver/kidney cancers and soft tissue sarcoma, respectively.[143,144] For both drugs, we demonstrated that they synergized with HDAC inhibitors to kill liver, kidney, pancreatic and sarcoma tumor cells.[145–148] Contemporaneously with these studies, we were also studying the celecoxib derivative developmental drug, OSU-03012. Originally OSU-03012 was proposed to inhibit PDK1 within the PI3K/AKT pathway.[135,142] OSU-03012 has an order of magnitude anti-cancer efficacy than the parent compound. The key, arguably single, mechanism by which we found OSU-03012 acted to kill tumor cells was by causing the generation of autophagosomes followed by autophagic flux and the cytotoxic actions of autolysosome localized proteases such as cathepsin B. Ultimately, we determined that OSU-03012 was an inhibitor of chaperone proteins, in particular GRP78.[145] GRP78 is an endoplasmic reticulum (ER) localized chaperone that plays an essential role in regulating ER stress signaling during times of protein overload and protein denaturation.[149] As we compared the chemical structures of OSU-03012, pazopanib and sorafenib we realized that had many similarities. Compared to OSU-03012 which had IC50 values of inhibiting the ATPase activities of HSP90 and HSP70 in the ~200 and ~300 nM range, respectively, the chaperone inhibitory activities of sorafenib were found to be similar, and the inhibitory activity of pazopanib significantly stronger with IC50 values of ~50 and ~100 nM, respectively.[150–154] Thus, drugs that had been developed and marketed as "multi-kinase inhibitors" for many years also had multiple unknown *chaperone targets*. Hence, just because a drug company states on their packaging that a drug inhibits enzymes A, B and C to cause a therapeutic effect, does not mean that the drug also inhibits unknown enzymes Y and Z. Furthermore, it is probable that without inhibition of Y and Z, the inhibition of A, B and C together will only have a modest therapeutic effect.

In the case of OSU-03012, despite a phase I trial in cancer patients (NCT00978523), further studies with drug took an unexpected turn away from cancer therapeutics, and towards infectious disease and the development of the drug as an anti-viral agent.[150,152] All human pathogenic viruses require cells express functional GRP78.[155,156] In a virus-dependent manner, different viruses also recruit other additional chaperone proteins to facilitate their replication and life cycle.[152,153] OSU-03012 is not a high-affinity inhibitor of a single chaperone or chaperone family, unlike many chaperone inhibitors developed for use in the cancer

therapeutics field.[157,158] However, because the drug inhibits multiple HSP90 family and HSP70 family chaperones within its clinically relevant safe concentration range, OSU-03012 could potentially become a broad spectrum anti-viral drug. OSU-03012 prevented the reproduction of viruses including Mumps, Influenza, Measles, Coxsackie virus B4, Junín, Rubella, West Nile, Yellow Fever, HIV (wild type and protease resistant), and Ebola, effects that were replicated by molecular knock down of multiple chaperone proteins, alone or in combination.[152] Very recently we discovered, to some extent not surprisingly, that OSU-03012 could also prevent synthesis of the SARS-CoV-2 spike protein. In three separate animal model systems, rabbit hemorrhagic fever virus, Zika and Dengue OSU-03012 prolonged animal survival and significantly reduced the negative sequelae of virus infection.[159–161] Subsequent studies using the FDA approved cancer therapeutic drugs sorafenib and pazopanib also demonstrated that these FDA approved drugs also have potent anti-viral properties.[154] Thus, a project which began as development of an anti-cancer drug became a project developing broad spectrum anti-viral drugs.

The role of an activating point mutant in the EGF receptor was first demonstrated in non-small cell lung cancer (NSCLC).[162–165] Subsequently, as patient tumors carrying the activated EGFR were treated for prolonged periods with EGFR inhibitors such as gefitinib, it became evident that drug resistance, when it eventually evolved, was mediated by the evolution of a second point mutation in the EGFR.[163,166–168] Second and third generation EGFR inhibitory drugs such as afatinib and osimertinib potently inhibit double mutant EGFR and are in first-line clinical use.[169–171] At the time of these discoveries we had several research projects determining whether we could combine afatinib with other agents to kill NSCLC cells.[172–174] As part of this work, we generated afatinib-resistant H1975 NSCLC cells by treating tumors in mice until the tumor completely regressed and then had begun to regrow. H1975 cells already express a double mutant EGFR, so we were expecting to discover novel evolutionary survival signals. Initial characterization of the resistant cells demonstrated they had permanently up-regulated signaling by the receptors c-KIT, c-MET and ERBB3 to survive during exposure to afatinib. Additional characterization studies then delivered unexpected data; whilst afatinib-resistant H1975 cells were resistant to the irreversible ERBB receptor inhibitor afatinib, they were not resistant to the irreversible ERBB inhibitor neratinib.[175] Furthermore, the ability of neratinib as a single agent or when combined with other drugs, including afatinib, was enhanced in the afatinib-resistant cells.[176] Ostensibly, both drugs should mechanistically "do" exactly the same thing to a tumor cell. Thus, by implication, in addition to ERBB family receptors, neratinib had to have additional "targets" to cause killing in the resistant cells. Two molecular modeling manuscripts had stated neratinib, in addition to inhibiting ERBB family tyrosine kinases could also inhibit MAP4K and MAP3K serine/threonine kinases.[177,178]

In parallel to the studies described above, from our loading control data, we observed that neratinib but not afatinib, could rapidly reduce the protein expression of ERBB family receptors in a wide variety of tumor cell types.[175,179,180] We also included negative controls in our studies; c-MET and c-KIT. To our surprise, neratinib also reduced c-MET and c-KIT levels, albeit in a delayed fashion. To down-regulate the EGFR required a ubiquitination step whereas to down-regulate c-MET did not. Growth factor receptors localize in large quaternary structures in the plasma membrane and we hypothesized that if neratinib was reducing the levels of the EGFR, c-MET and c-KIT, could it also reduce the levels of an important signal transducer on the inner leaflet of the plasma membrane: RAS. In pancreatic cancer cells neratinib not only caused internalization and degradation of the EGFR, it also caused the degradation of the key oncogenic driver in this disease, mutant K-RAS. Subsequently, in melanoma cells expressing a mutant N-RAS, similar findings with neratinib were obtained.[179,180]

The convergence of the afatinib-resistance studies and the RAS down-regulation studies was a project to define the roles of MAP4K and MAP3K enzymes in the biological actions of neratinib.[181] From the modeling studies, two potential neratinib targets were MST3 and MST4. This caught our interest because the dose-limiting sequela for neratinib is diarrhea, and MST3 and MST4 play important roles in regulating the integrity of the epithelial brush boarder in the gut.[182,183] Because we did not know what effects would be observed, we agnostically examined the activities of multiple MAP4K enzymes, as well as associated chaperone/docking proteins following neratinib exposure. As MAP4K/MAP3K enzymes are expressed in carcinoma cells which express high levels of ERBB family receptors as well as in blood cancer cells that express none or very low levels of that receptor family, we performed studies in both tumor cell types. Regardless of ERBB family receptor expression, neratinib reduced the expression of RAS proteins and reduced tumor cell viability.[176]

Neratinib reduced the phosphorylation of MST1/2, MST3 and MST4 in carcinoma and blood cancer cells; this would a priori predict that phosphorylation of their downstream substrates such as LATS1/2 or the cytoskeletal protein Ezrin, would be reduced.[176,181] As was a priori expected, the phosphorylation of Ezrin *was* reduced. However, the phosphorylation of LATS1/2 was *enhanced*, as were the downstream substrates of these enzymes, the co-transcription factors YAP and TAZ. YAP and TAZ are Hippo pathway effectors and when phosphorylated leave the nucleus which is followed by degradation in the cytoplasm.[184,185] As YAP and TAZ cooperate with mutant K-RAS to drive pancreatic cancer growth and metastasis, our data suggest that neratinib could be a useful drug to employ in the treatment of this disease.[186,187] This data also suggests that inhibition of the MST "MAP4K" kinases probably caused a compensatory activation of another "MAP4K" kinase(s) which phosphorylated LATS1/2.

Thus, the key take-home messages from this section are that without a full appreciation and understanding of *ALL* potential targets of a particular drug, its mechanisms of action cannot be properly understood. Because neratinib inhibits MAP4K/MAP3K enzymes besides ERBB family receptors and particularly HER2/ERBB2, very few pre-clinical studies were performed in cells that did not over-express HER2/ERBB2 and none in cells that express mutant RAS proteins or in blood cancer cells. These findings emphasize that in developmental drug and therapeutics studies, a broad agnostic approach is essential so as not to miss potential unknown off targets. This is diametrically different to almost all cell biology research projects where intense focus on a particular pathway, or even a component of a pathway is a standard approach. Similarly, studying the mechanisms of cell killing by a drug by their nature have to be conceptually broad because very frequently drug-induced killing is not "pure" with only one pathway to tumor cell death

being engaged. The drug-induced killing mechanism, for example, could include death receptor signaling, mitochondrial dysfunction and autophagosome formation, all interacting in a contemporaneous fashion. Again, this approach is diametrically different to almost all basic science cell biology research projects.

6 Conceptual developmental therapeutics strategies

Developing a compound into a putative drug and eventually into an agent that can be tested in humans is a long process that generally costs in the region of $200–300 million dollars. To some extent, the high cost of all prescription drugs to the consumer is influenced by this math. The screening of millions of compounds may result in the discovery of a new agent with anti-cancer, anti-viral or anti-bacterial properties. Alternatively, compounds are screened against a specific target until molecules are defined that potently act to inhibit the target's biological activity. Optimization of these compounds, either by computer aided design, or by traditional organic chemistry methods, results, hopefully, in a series of compounds all with a low nanomolar IC50 inhibitory activity. Drug development companies will then determine which of the drugs has the greatest apparent bioactivity in a range of tumor cell lines, alongside determination of in-animal stability and bioactivity against tumors. These studies collectively will deliver one or two compounds that are considered worthy of further investigation and development. It is at this point where drug companies will often seek outside academic collaborators to assist in their drug development studies. The first thing the independent academic collaborator needs to know is what was the highest safe dose of the compounds used in prior mouse studies? And, ideally, if pharmacodynamic and pharmacokinetic studies were performed, what was the safest peak plasma concentration of the compound, termed the C max and often listed as ng/mL (which requires conversion into a Molar value). Thus, if the highest safe dose of a compound is 10 mg per kg of animal, with a plasma C max of 1 μM, then all preliminary in vitro cell-based investigative studies MUST use the compound at concentrations well below 1 μM.

To further understand the biology of the compound, preliminary in vitro dose-response studies against tumor cells are most often performed on a log-scale, e.g. 1, 3, 10, 30, 100 and 300 nM. The first question the academic investigator should ask is, in their hands, does the dose-response effect on tumor cell growth/viability correspond to the claimed inhibitory IC50 of the compound against its purified specific target? i.e. if the protein target has an IC50 inhibition of 1 nM and an IC50 for growth inhibition and cell killing of 300 nM, it suggests the compound may be binding tightly to the serum in the culture media, resulting in a very low concentration of free "active" drug. On the other hand, if the target inhibition IC50 is 100 nM but the IC50 for growth arrest/killing is 3 nM, the data implies the compound may have additional unknown higher affinity targets in addition to its primary target which all collectively contribute to the biological efficacy of the agent.

In this article we have discussed the FDA approved drugs sorafenib and neratinib. Sorafenib was originally developed to inhibit RAF-1 and B-RAF. Prior to the discovery that RAF-1 phosphorylated MEK1/2, it was noted that the catalytic site of the RAF-1 serine/threonine kinase most closely resembled the active sites of SRC family non-receptor tyrosine kinases.[188] Hence, it was no surprise that within a few years sorafenib was also shown to also inhibit Class III receptor tyrosine kinases, and investigators now considered the biology of drug to be as an "anti-angiogenic" agent rather than per se an inhibitor of RAF-1.[189,190] Finally, sorafenib was shown to be an inhibitor within its physiological range of HSP90 and HSP70 chaperone proteins.[152,154] Similarly, neratinib was developed solely with the intention of inhibiting the receptor tyrosine kinase HER2 (ERBB2) as a putative therapeutic for HER2+ breast cancer.[191] Yet, within several years of neratinib entering the clinic, two groups demonstrated it could inhibit MAP4K and MAP3K serine/threonine kinases with low nanomolar IC50 values.[177,178]

So, if the compound under investigation is considered by a drug company to be a "specific" inhibitor of a particular protein kinase, regardless as to whether the agent is also FDA approved, the in vitro studies the academic investigator should perform are an agnostic wide-ranging series of assessments, over a clinically-relevant drug dose-response range and over a time course. These studies will define, in your own hands, the changes in phosphorylation of the proposed target but also of multiple other cellular signaling pathways. This involves studying components of each specific pathway, e.g. the regulatory phosphorylation and total expression of ERBB1, ERBB2, ERBB3, ERBB4, RAF-1, B-RAF, MEK1/2 and ERK1/2, as well as of downstream nuclear transcription factors whose functions are controlled by each pathway, such as cAMP response element-binding protein (CREB). Such wide-ranging data-intense screening studies are difficult to perform using traditional SDS PAGE and western blotting approaches, and more advanced methods such as dot-blots or staining fixed cells in situ and measuring the intensity fluorescent staining, using validated antibodies, which permit a high-throughput approach, are required.

An old phrase in science is: "the data is what it is." Thus, if signaling from the primary target in one signaling pathway is only partially inhibited by the drug under examination, but signaling through an unrelated pathway is almost abolished, one would therefore tentatively conclude that the compound has an unknown target in a different signaling pathway. Or, if at a low concentration of the drug no inhibition of the primary target is observed, but that this occurs alongside changes in the activities of other pathways, an effect which is also associated with significant levels of growth arrest and tumor cell death, one would conclude that the biological primary target is not the key functional target which regulates tumor cell biology. These examples for drug actions are binary, and in reality, the differential effects upon signaling and tumor cell biology of any drug are more subtle and nuanced.

A different set of concepts come into play when rationally combining FDA approved drugs to develop a novel anti-cancer therapeutic approach. First, the safe C max values for both agents in patients should be determined alongside their plasma half-lives, C min at 24 h values and serum binding properties. The C max/C min values alongside the drug's half-life should inform the

researcher that, for example during a 24 h in vitro time course assay, a drug concentration considerably less than the C max but above the C min should be used to approximate for a physiologic treatment concentration. Many clinically relevant drugs are stated to be 99% protein bound in 100% serum; most in vitro studies are performed using 10% (v/v) fetal calf serum. What is self-evident, however from extant data, is that for a drug such as sorafenib, with a safe C max of ~13 μM and a stated 99% plasma protein binding, is that a free sorafenib concentration of ~130 nM in vitro has a very modest impact on altering tumor cell biology, i.e. the partitioning on-off rate for drug association with plasma proteins and with tumor tissue must also be taken into consideration when deciding the most in vitro physiologic drug concentration.[192] Thus, taking all of these parameters into account, studies in the author's laboratory, in vitro with cells in 10% (v/v) serum, and in an attempt to remain within the physiologic range, do not use sorafenib above 2 μM. An additional consideration for drug combination studies is to determine from the literature the dose-limiting toxicities of each drug. Regardless of excellent laboratory-based data, if the two drugs being combined both have dose-limiting toxicities (DLTs) in the same tissue, e.g. the gastrointestinal tract (GI), the likelihood that both agents can be safely and successfully combined in a patient is considerably reduced.

7 Conclusions

Studies to define hormonal signaling and intracellular signal transduction are almost 100 years old. Although much essential biological information was gleaned from work performed in the 1920s to the late 1980s, it was only with the widespread use of more modern molecular biology approaches combined alongside classic biochemical approaches that the signal transduction landscape of the last 25 years evolved. Today, essentially all of the building blocks of all signal transduction pathways are known. What is still under investigation are the complex protein-protein interactions which define nuanced signaling during growth, development, and various pathologies. Small molecule therapeutic interventions have been and are being developed using ever more sophisticated technologies, of which some have shown considerable clinical utility. However, many of the newly developed "specific" targeted drugs have had little to no testing to fully define off-target effectors of their biology. It is very probable that this on-target/off-target issue for all drugs will never be fully resolved. Hence, the step-wise approaches described in this article will still be required to fully understand the use and application of all new drugs.

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