Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Elsevier hereby grants permission to make all its COVID-19-related research that is available on the COVID-19 resource centre - including this research content - immediately available in PubMed Central and other publicly funded repositories, such as the WHO COVID database with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the COVID-19 resource centre remains active.
6.14  Cell Signaling and Translational Developmental Therapeutics

Paul Dent, Department of Biochemistry and Molecular Biology, Massey Cancer Center, Virginia Commonwealth University, Richmond, VA, United States

© 2022 Elsevier Inc. All rights reserved.

6.14.1 Introduction: Early Days

The field of cell signaling and signal transduction dates back to the late 19th century. In 1895, epinephrine (adrenaline) was discovered (Yamashima, 2003). By the 1920s, insulin and glucagon had been discovered (Gilchrist et al., 1923; Kimball and Murlin, 1923). 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

Dedicated to Professor Sir Philip Cohen on the occasion of his 75th birthday.
unravelling how glycogen could be broken down by glycogen phosphorylase (Cori et al., 1939). 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 (Leloir et al., 1959). During the 1950s, Fischer, Krebs and Sutherland not only discovered and characterized the kinase which regulated glycogen phosphorylase, phosphorylase kinase, but defined for the first time that the phosphorylation of proteins could regulate enzyme activity (Fischer and Krebs, 1955; Rall et al., 1956; Sutherland and Wosilait, 1956; Wosilait and Sutherland, 1956).

### 6.14.2 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 or 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 second messenger (Haynes et al., 1960; Hughes et al., 1962; Sutherland and Robison, 1966). Subsequently, Fischer and Krebs isolated the kinase regulated by cAMP, protein kinase A (PKA) (Meyer et al., 1964). 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 (Cohen and Antoniw, 1973).

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) (George et al., 1970; Holian and Stickle, 1985; MacIntyre et al., 1985; Rapoport and Murad, 1983). 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 (Rapoport and Murad, 1983; Miki et al., 1975). During the 1970s and 1980s work by Leffkowitz, 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) adenyl 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 (Buxser et al., 1985; Daaka et al., 1997; Gilman and Nirenberg, 1971; Hepler et al., 1993; Huckle et al., 1990; Johnson et al., 1981; Kariya et al., 1989; Maguire et al., 1976; Qian et al., 1993; Ross et al., 1977; van Biesen et al., 1995). 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 (Lohse et al., 1990; Luttrell et al., 2018; Luttrell and Leffkowitz, 2002). Arrestin proteins prevent both the Gz 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 become phosphorylated on tyrosine. Studies in this field were focused on the insulin receptor (metabolism) and the epidermal growth factor receptor (EGFR, ERBB1) (growth, cancer) (Avruch et al., 1982; Hunter and Cooper, 1981; Kasuga et al., 1982; Ushiro and Cohen, 1980). Insulin caused the insulin receptor to become tyrosine phosphorylated, and a substrate for the receptor, insulin receptor substrate 1 (IRS1), was discovered (Stadtmauer and Rosen, 1983). 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 intratable to investigation (Larner, 1988). 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 (Larner et al., 2010; Sato et al., 1988; Villar-Palasi and Larner, 1960). Although at the time this concept was not widely supported, subsequent studies over the following 10 years demonstrated that insulin activated phosphatidylinositol 3-kinase whose product, phosphatidylinositol 3,4,5-trisphosphate (PI3,4,5P3), caused activation of the membrane-associated kinase, phosphoinositide-dependent kinase-1 (PDK1) (Alessi et al., 1996a,b, 1997; Cross et al., 1995; James et al., 1996). PDK1 was shown to phosphorylate AKT T308 causing enzyme activation, and AKT to phosphorylate and inactivate glycogen synthase kinase 3 (GSK3) (Alessi and Cohen, 1998). 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 (Dent et al., 1990). Thus, after 60 years of research, by the mid-1990s, the regulation of glycogen metabolism by epinephrine and insulin had largely been elucidated.
6.14.3 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 was initially based on studies using traditional biochemical methods (Facchinetti et al., 2020; Houslay, 1981; Keegan et al., 1991; Smart et al., 1981). 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 kDa protein whose tyrosine phosphorylation was increased after exposing cells to insulin (Sturgill and Ray, 1986). 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 (Ray and Sturgill, 1987). 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) (Sturgill et al., 1988). This enzyme should not be confused with p70 S6 kinase with is a component of the PI3K pathway (Alessi et al., 1996a,b). 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 (Haycock et al., 1992; Ray and Sturgill, 1988). 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 (Nakielny et al., 1992; Wu et al., 1992, 1993a,b). 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 (Errede and Levin, 1993; Marshall, 1994; Nishida and Gotoh, 1993). The mammalian MAPK renamed ERK1/2 (extracellular regulated kinase) pathway in yeasts regulates the yeast response to pheromones (Kurjan, 1993). 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) (Cobb et al., 1994). 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 (Dent et al., 1992; Kyrkiakos et al., 1992). Of note, prior to those studies it was believed that RAF-1 was downstream of ERK1/2 (Anderson et al., 1991). 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 (Bogoyevitch et al., 1996; Guan et al., 1998; Lee et al., 1997; Lu et al., 1997; Schlesinger et al., 1998; Uhlik et al., 2004). Concomitantly 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 (Engish et al., 1993; Gale et al., 1993; Li et al., 1993; Lowenstein et al., 1992; Rozakis-Adcock et al., 1992). It was demonstrated that the proteins GR2B (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 (Dent and Sturgill, 1994; Dent et al., 1995; Moodie et al., 1993, 1994). 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, 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 (Bettinger and Amberg, 2007). 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 (Fanger et al., 1997). 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 (English et al., 1995).

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 (English et al., 1995; Chen et al., 1996; Xia et al., 1995). 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 (Auer et al., 1998a,b; Qiao et al., 2003). 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 TGFβ, 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 (Li et al., 1989; Sorkin et al., 1988, 1989; Wiley et al., 1991). Signaling by p38 MAPK regulated chaperone functions but also could cause cell cycle arrest and DNA damage repair (Aviv et al., 2014; Roy et al., 2018). 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 (Auer et al., 1998a,b; Qiao et al., 2003). 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) (Beyfuss and Hood, 2018; Carón et al., 2005; Jiang et al., 2020; Rutkowska et al., 2019).
6.14.4 Autophagy

The cellular process of autophagy was discovered in the 1960s (De Duve and Wattiaux, 1966; Levine and Klionsky, 2017). 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 (Klionsky et al., 1992; Takeshige et al., 1992; Tsukada and Ohsumi, 1993). 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 (Corona Velázquez and Jackson, 2018; Liu and Sabatini, 2020; New and Thomas, 2019; Shi et al., 2019; Tamargo-Gómez and Mariño, 2018). 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 (Jhanwar-Uniyal et al., 2019; Kim and Guan, 2019; Sridharan and Basu, 2020). 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 (de Souza Almeida Matos et al., 2019; González et al., 2020; Li and Chen, 2019). The AMPK is itself regulated by phosphorylation, with the most notable regulators being Liver kinase B1 (LKB1) and ataxia-telangiectasia mutated (ATM) (Ciccarese et al., 2019; Kullmann and Krahn, 2018; Liang et al., 2019; Li et al., 2015; Puustinen et al., 2020; Tripathi et al., 2013). 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) (Liu et al., 2020; Turco et al., 2020; Wang and Kundu, 2017; Zachari and Ganley, 2017). 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 (Wang and Kundu, 2017; Zachari and Ganley, 2017; Gong et al., 2018). 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 (Klionsky et al., 2016; Yang et al., 2018). 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 (Morgan et al., 2018; Tompkins and Thorburn, 2019). 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 (Yacoub et al., 2006).

6.14.5 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 (Park et al., 2009; Yacoub et al., 2008a,b).

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 (Park et al., 2008c). 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 (Park et al., 2010). 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 (Blano and Prehn, 2018). 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 (Park et al., 2008d). 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 (Haherty, 2007; Limvorasak and Posadas, 2009). For both drugs, we demonstrated that they synergized with HDAC inhibitors to kill liver, kidney, pancreatic and sarcoma tumor cells (Booth et al., 2012, 2019a; Dent et al., 2019a). Contemporaneously with these studies, we were also studying the celecoxib derivative developmental drug, OSI-03012. Originally OSI-03012 was proposed to inhibit PDK1 within the PI3K/AKT pathway (Yacoub et al., 2006; Park et al., 2008d). OSI-03012 has an order of magnitude anti-cancer efficacy than the parent compound. The key, arguably single, mechanism by which we found OSI-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 OSI-03012 was an inhibitor of chaperone proteins, in particular GRP78 (Booth et al., 2012). 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 (Zhu and Lee, 2015). As we compared the chemical structures of OSI-03012, pazopanib and sorafenib we realized that had many similarities. Compared to OSI-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 (Booth et al., 2015a,b, 2016a,b; Roberts et al., 2015). 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 OSI-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 (Booth et al., 2015a, 2016a). All human pathogenic viruses require cells express functional GRP78 (He, 2006; Levy et al., 2017). In a virus-dependent manner, different viruses also recruit other additional chaperone proteins to facilitate their replication and life cycle (Booth et al., 2016a,b). OSI-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 (Huang et al., 2020; Jung et al., 2020). However, because the drug inhibits multiple HSP90 and HSP70 family GRP78 dependant chaperones within its clinically relevant safe concentration range, OSI-03012 could potentially become a broad spectrum anti-viral drug. OSI-03012 prevented the reproduction of viruses including Mumps, Influenza, Measles, Coxsackie virus B4, Junin, 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 (Booth et al., 2016a). Very recently we discovered, to some extent not surprisingly, that OSI-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 (Chan et al., 2018; Chen et al., 2017; Hassandarvish et al., 2017). 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 (Roberts et al., 2015). 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) (Kobayashi et al., 2005; Kwak et al., 2005; Lynch et al., 2004; Sordella et al., 2004). 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 EGF (Kwak et al., 2005; Arteaga, 2006; Inukai et al., 2006; Johnson and Jänne, 2005). Second and third generation EGFR inhibitory drugs such as afatinib and osimertinib potently inhibit double mutant EGFR and are in first-line clinical use (Andrews Wright and Goss, 2019; Thongprasert et al., 2019; Yamamoto et al., 2020). At the time of these discoveries we had several research projects determining whether we could combine afatinib with other agents to kill NSCLC cells (Booth et al., 2016c,d; Tavallai et al., 2016). 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 expressed a double mutant EGFR, so we were expecting to discover novel experimental 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 (Booth et al., 2017a). Furthermore, the ability of neratinib as a single agent or when combined with other drugs, including afatinib, was enhanced in the afatinib-resistant cells (Dent et al., 2019b). 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 (Davis et al., 2011; Klaeger et al., 2017).

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 (Booth et al., 2017a,b, 2018). 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 (Booth et al., 2017b, 2018).

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 (Dent et al., 2020). 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 (Jiang et al., 2017; Secomb et al., 2019). 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 (Dent et al., 2019b).

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 (Dent et al., 2019b, 2020). 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 (Pocaterra et al., 2020; Thompson, 2020). 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 (Kapoor et al., 2014; Zhang et al., 2014). 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.

### 6.14.6 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 (Mark and Rapp, 1984). 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 an “anti-angiogenic” agent rather than per se an inhibitor of RAF-1 (Clark et al., 2005; Strumberg et al., 2002). Finally, sorafenib was shown to be an inhibitor within its physiological range of HSP90 and HSP70 chaperone proteins (Booth et al., 2016a; Roberts et al., 2015). Similarly, neratinib was developed solely with the intention of inhibiting the receptor tyrosine kinase HER2 (ERBB2) as a putative therapeutic for HER2+ breast cancer (Bose and Ozer, 2009). 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 (Davis et al., 2011; Klae格尔 et al., 2017).

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-intensive 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, 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 (Hotte and Hirte, 2002). 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.

6.14.7 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 been shown considerable clinical utility. However, many of the newly developed “specific” targeted drugs have had little to no testing to fully define off-target effects 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.

See Also: 2.15: Pharmacogenomics of Anti-Cancer Drugs; 6.25: Mitochondria and Tumor Metabolic Flexibility: Mechanisms and Therapeutic Perspectives
References

Alessi, D.R., Cohen, P., 1998. Mechanism of activation and function of protein kinase B. Current Opinion in Genetics & Development 8, 55–62.

Alessi, D.R., Caudwell, F.B., Andjelkovic, M., Hemmings, B.A., Cohen, P., 1998a. Molecular basis for the substrate specificity of protein kinase B; comparison with MAPKAP kinase-1 and p70 S6 kinase. FEBS Letters 399, 333–338.

Alessi, D.R., Andjelkovic, M., Caudwell, B., Cron, P., Morrice, N., Cohen, P., Hemmings, B.A., 1996b. Mechanism of activation of protein kinase B by insulin and IGF-1. The EMBO Journal 15, 6541–6551.

Alessi, D.R., James, S.R., Downes, C.P., Holmes, A.B., Gaffney, P.R., Reese, C.B., Cohen, P., 1997. Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase B alpha. Current Biology 7, 261–269.

Anderson, N.G., Li, P., Marsden, L.A., Williams, N., Roberts, T.M., Sturgill, T.W., 1991. Raf-1 is a potential substrate for mitogen-activated protein kinase in vivo. Biochemical Journal 277, 573–576.

Andrews Wright, N.M., Goss, G.D., 2019. Third-generation epidermal growth factor receptor tyrosine kinase inhibitors for the treatment of non-small cell lung cancer. Translational Lung Cancer Research 8 (3), S247–S264.

Arteaga, C.L., 2006. EGFR receptor mutations in lung cancer: From humans to mice and maybe back to humans. Cancer Cell 9, 421–423.

Auer, K.L., Contessa, J., Brenz-Verca, S., Pirola, L., Rusconi, S., Cooper, G., Abo, A., Wymann, M.P., Davis, R.J., Birrer, M., Dent, P., 1998a. The Ras/Rac1/Cdc42/SEK/JNK/c-Jun cascade is a key pathway by which agonists stimulate DNA synthesis in primary cultures of rat hepatocytes. Molecular Biology of the Cell 9, 561–573.

Auer, K.L., Park, J.S., Seel, F., Coffey, R.J., Darlingston, G., Abo, A., McMahon, M., Depinho, R.A., Fisher, P.B., Dent, P., 1998b. Prolonged activation of the mitogen-activated protein kinase pathway promotes DNA synthesis in primary hepatocytes from p210Gv-1/WAF1-null mice, but not in hepatocytes from p180Nk4a-null mice. Biochemical Journal 336, 551–560.

Avruch, J., 2000. AMPK in nutrition and metabolism: How does an ancient pathway regulate metabolism in modern cells? Cell Signaling and Translational Developmental Therapeutics 15, 6541.

Avruch, J., Nemenoff, R.A., Blackshear, P.J., Pierce, M.W., O’Rahilly, C.N., Cornwell, C.N., Dent, P., 2015a. GSK3B regulates the activity of AMPK in liver cells. FEBS Letters 589, 2850–2857.

Beyfuss, K., Hood, D.A., 2018. A systematic review of p53 regulation of oxidative stress in skeletal muscle. Redox Report 23, 100–117.

Bogoyevitch, M.A., Gillespie-Brown, J., Ketterman, A.J., Fuller, S.J., Ben-Lyvy, R., Ashworth, A., Marshall, C.J., Sugden, P.H., 1996. Stimulation of the stress-activated mitogen-activated protein kinase subfamilies in perfused heart. P38/RK mitogen-activated protein kinases and c-Jun N-terminal kinases are activated by ischemia/reperfusion. Circulation Research 79, 162–173.

Booth, L., Cazanave, S.C., Hanned, H.A., Yacoub, A., Ogetrenner, B., Chen, C.S., Grant, S., Dent, P., 2012. OSI-0312 suppresses GPR7/B/FP expression that causes PERK-dependent increases in tumor cell killing. Cancer Biology & Therapy 13, 224–238.

Booth, L., Roberts, J.L., Cash, D.R., Tavallai, S., Jean, S., Fitranza, A., Cruz-Luna, T., Srivastava, P., Cycon, K.A., Cornelissen, C.N., Dent, P., 2015a. GPR7B/B/FP/HSFA5/Cna K is a universal therapeutic target for human disease. Journal of Cellular Physiology 230, 1661–1676.

Bettinger, B.T., Amberg, D.C., 2007. The MEK kinases MEKK4/Ssk2p facilitate complexity in the stress signaling responses of diverse systems. Journal of Cellular Biochemistry 101, 315–324.

Bano, D., Prehn, J.H.M., 2018. Apoptosis-inducing factor (AIF) in physiology and disease: The tale of a repentant natural born killer. eBioMedicine 30, 29.

Booth, L., Roberts, J.L., Tavallai, S., Cazanave, S.C., Hamed, H.A., Yacoub, A., Albers, T., Roberts, J.L., Tavallai, M., Proniuk, S., Zukiwsky, A., Chen, C.S., Bottaro, D., Ecroyd, H., Lebedyeva, I.O., Dent, P., 2016a. Multi-kinase inhibitors interact with selenofluorinated and ERBB1/2/4 inhibitors to kill tumor cells in vitro and in vivo. Oncotarget 7, 40398–40417.

Buxser, S., Puma, P., Johnson, G.L., 1985. Properties of the nerve growth factor receptor. Relationship between receptor structure and activation. Proceedings of the National Academy of Sciences 82, 4168–4172.

Ciccarese, F., Zulato, E., Indraccolo, S., 2019. LKB1/AMPK pathway and drug response in cancer: A therapeutic perspective. Oxidative Medicine and Cellular Longevity 2019, 3425487.

Clark, J.W., Eder, J.P., Ryan, D., et al., 2005. Safety and pharmacokinetics of the dual action Raf kinase and vascular endothelial growth factor inhibitor, BAY 43-9006, in patients with advanced, refractory solid tumors. Clinical Cancer Research 11, 5472–5480.

Cobb, M.H., Xu, S., Hepler, J.E., Hutchison, M., Frost, J., Robbins, D.J., 1994. Regulation of the MAP kinase cascade. Cellular & Molecular Biology Research 40, 253–256.
