The Importance of Ras in Drug Resistance in Cancer

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Abstract

In this review, we analyse the impact of oncogenic Ras mutations in mediating cancer drug resistance, and progress made in the abrogation of this resistance, through pharmacological targeting. At a physiological level, Ras is implicated in many cellular proliferation and survival pathways. However, mutations within this small GTPase can be responsible for the initiation of cancer, therapeutic resistance and failure and ultimately disease relapse. Often termed 'undruggable', Ras is notoriously difficult to target directly, due to its structure and intrinsic activity. Thus, Ras-mediated drug resistance remains a considerable pharmacological problem. However, with advances in both analytical techniques and novel drug classes, the therapeutic landscape against Ras is changing. Allele-specific, direct Ras-targeting agents have reached clinical trials for the first time, indicating there may, at last, be hope of targeting such an elusive but significant protein for better more effective cancer therapy.

Abbreviations: AML, acute myeloid leukaemia; CRCs, cancer stem cells; FLT3, fms-like tyrosine kinase 3; FTI, farnesyltransferase inhibitor; HNSCC, head and neck squamous cell carcinoma; EGFR, epidermal growth factor receptor; GAP, GTPase activating protein; GEF, guanine nucleotide exchange factor; lncRNAs, long non-coding RNAs; MAPK, mitogen-activated protein kinase; mCRC, metastatic colorectal cancer; MEK, MAPK kinase; NF-κB, nuclear factor κB; NRF2, nuclear factor erythroid 2-related factor 2; NSCLC, non-small cell lung cancer; PI3K, phosphatidylinositol-3-kinase; PPI, protein-protein interaction; RBD, Ras binding domain; ROS, reactive oxygen species. RTK, receptor tyrosine kinase; SOS, son-of-sevenless; TKI, tyrosine kinase inhibitor.

Introduction

Resistance to conventional therapeutic agents is an increasingly concerning issue across all areas of disease, including cancer. Whilst the heterogenous nature of cancer means there are many mechanisms resulting in drug resistance, Ras mutations underpin resistance to a variety of therapies (Hobbs, Der & Rossman, 2016; Prior, Hood & Hartley, 2020; Prior, Lewis & Mattos, 2012). Oncogenic mutations in this small GTPase, which occur in approximately 19% of all cancers, cause constitutive activation of proliferative and survival pathways (Prior, Hood & Hartley, 2020). This can abrogate the effects of standard chemotherapy and newer, receptor-targeted therapies. Examples of such resistance is seen across a wide range of cancers, including metastatic colorectal cancer (mCRC), non-small cell lung cancer (NSCLC), pancreatic cancer, acute myeloid leukaemia (AML) and basal cell carcinoma (Li, Xie, Wolff & Abbruzzese, 2004; McMahon et al., 2019; Misale et al., 2012; Tao et al., 2014). Thus, there is a distinct clinical need to target Ras pharmacologically. Whilst this has been particularly challenging due to structural difficulties and very high levels of intrinsic activity, significant developments have been made within the last decade. Allele-specific, direct Ras-targeting reaching clinical trials present a new era of potential Ras therapeutics and increases the likelihood of overcoming this resistance mechanism.

What is Drug Resistance?

There are two general classes of resistance: intrinsic and acquired. Intrinsic resistance occurs due to overt pre-existing factors, including variations in protein expression levels (such as increased expression of the P-gp (MDR1) transporter), epigenetic modifications (including by the long non-coding RNA HAND2-AS1 and chromatin modifier Jarid1A) and somatic mutations (such as in Ras) (Burrell, McGranahan, Bartek & Swanton, 2013; Gruber et al., 2012; Marusyk, Almendro & Polyak, 2012; Sharma et al., 2010). Changes conferring drug resistance often co-exist, resulting in a resistance heterogeneity similar to that seen in the original disease itself (Gerlinger et al., 2012; Ramirez et al., 2016).

Acquired resistance is thought to occur for a number of reasons, including through pre-existing (but initially undetectable) and *de novo* mutations (Bhaduri et al., 2020; Russo et al., 2019).This is often identified weeks to months after treatment has commenced (Santoni-Rugiu et al., 2019). In recent times, the concept of disease clonal heterogeneity has provided greater insight into the causes of acquired resistance, suggesting that chemoresistance and disease relapse occur as a result of minor sub-clonal populations. Given that these likely contribute to the heterogenous nature of cancer, it seems likely that these also contribute to disease re-emergence and relapse (Bonnet & Dick, 1997; Gerlinger et al., 2012; Pattabiraman & Weinberg, 2014; Roy & Cowden Dahl, 2018; Seth et al., 2019). Within these minor clonal populations, mutations conferring drug resistance may exist at the early stages of disease, but remain dormant and undetectable upon first presentation (Pietrantonio et al., 2017; Russo et al., 2018). When the bulk of the cancer is eliminated as a result of initial chemotherapy targeted at overt mutations, cells from this minor subclone proliferate and become dominant in the tumour bulk (Jones et al., 2019; McMahon et al., 2019).

A second, related, resistance mechanism involves a very rare subpopulation of cells, known as cancer stem cells (CSCs). CSCs were first described in AML, but have since been applied to many other cancers including (but not limited to) breast cancer, colorectal cancer, myeloma and pancreatic adenocarcinoma (Bonnet & Dick, 1997; Koury, Zhong & Hao, 2017; Lapidot et al., 1994). CSCs have unique properties compared to bulk tumour cells: they are undifferentiated and have strong self-renewal and proliferative capabilities. They typically remain in a quiescent state, thus avoiding chemotherapy targeted at rapidly dividing cells (as in bulk tumour cells). However, they do have the proliferative capacity to maintain and expand the tumour burden, as bulk tumour cells are eliminated

(Jordan, Guzman & Noble, 2006). These 'stemness characteristics' render this subset of cells intrinsically resistant to chemotherapy, with distinct immunophenotypic and molecular signatures (Bonnet & Dick, 1997). This includes increased expression of efflux transporter P-gp, and the ability to repair damaged DNA, a common method of inducing cancer cell death (Dean, Fojo & Bates, 2005; Pattabiraman & Weinberg, 2014).

Certain pathways upregulated in CSCs have been linked to the increased self-renewal capacity seen in this subset of cells. Key examples include the Wnt/β-catenin and Hedgehog (Hh) pathways, as well as the Ras-dependent MAPK and PI3K/AKT pathways. Of these, the Wnt/β-catenin pathway is perhaps the most associated with CSCs. Briefly, at a physiological level, Wnt signalling is not active, and β-catenin is ubiquitinated and sent for proteasomal degradation following interaction with GSK3β, APC and Axin. In CSCs however, activation of Wnt signalling inhibits formation of the β-catenin-GSK3β-APC-Axin complex, stabilising β-catenin. This translocates to the nucleus whereby it stimulates transcription of proliferative genes, including c-Myc. This promotes proliferative signalling, increasing the self-renewal capacity of CSCs (Krausova & Korinek, 2014; Moon, Jeong, Park, Kim, Min do & Choi, 2014). Although still ambiguous, it has been reported that β-catenin stabilisation can promote Ras stabilisation and protects Ras against proteasomal degradation (Jeong, Ro & Choi, 2018; Lee et al., 2018). This in turn promotes MAPK and PI3K pathway signalling, further contributing to the self-renewal capacity of CSCs.

Alternatively, upregulation of the Hh signalling pathway has been shown in CSCs. Whilst there are many facets to this pathway, its activation can stimulate upregulation of stemness-associated transcription factors including NANOG, OCT4 and SOX2, thus promoting self-renewal (Boyer et al., 2005; Po et al., 2010; Takahashi & Yamanaka, 2006; Zhu et al., 2019). Increased activation and stabilisation of these genes (through either Hh signalling or biochemical stresses) have been implicated in the dedifferentiation of bulk tumour cells to CSCs (Herreros-Villanueva et al., 2013; Kumar et al., 2012).

Interest in epigenetic remodelling has grown considerably in the last decade, and it has considerable effects in promoting drug resistance. Long non-coding RNAs (lncRNAs) are also implicated in survival of CSCs. In hepatocellular carcinoma, the lncRNA HAND2-AS1 is upregulated and stimulates the self-renewal capacity of CSCs, through activation of the BMP signalling pathway. BMP activation has in turn been shown to induce chemoresistance in other cancer models, such as lung cancer (Gruber et al., 2012; Wang et al., 2015). Indeed, this pathway is also regulated by the fusion gene *CBFA2T3-GLIS2* and the Hh and JAK-STAT signalling pathways (Gruber et al., 2012; Okada et al., 2014).

Taken together, it is evident that many signalling pathways and associated factors play a critical role in mediating drug resistance. Identifying commonalities between these pathways could present an opportunity to reduce the incidence and impact of drug resistance. Although the incidence of Ras mutations in CSCs is relatively low, Ras-mediated pathways have been implicated (Corces-Zimmerman, Hong, Weissman, Medeiros & Majeti, 2014). In addition to the aforementioned β-catenin-mediated stabilisation of Ras, stabilised OCT4 and SOX2 expression has also been shown in response to AKT activation, by extracellular biochemical and radiation stresses (Maiuthed et al., 2018; Park et al., 2021). Indeed, activation of the MAPK pathway through the Ras-Raf interaction promotes increased expression of SOX2 and NANOG (Chan et al., 2018; Du et al., 2019). Furthermore, correlation has been seen between upregulation of the MAPK, PI3K and BMP pathways in drug-resistant lung cancer (Wang et al., 2015). Thus, not only can these pathways contribute to an increased cellular proliferation rate in bulk cancer, but they can also assist with the self-renewal and stemness properties evident in CSCs. Perhaps unsurprisingly, inhibition of the Hh signalling pathway, when combined with

inhibition of mTOR (downstream of AKT) has been seen to eliminate CSCs in pancreatic cancer (Mueller et al., 2009; Prieur et al., 2017). Furthermore, MEK inhibition has been implicated in reduced pancreatic CSC survival (Walter et al., 2019).

Key examples of drug-resistance in cancer

In recent years, resistance has been documented amongst many cancer therapeutics, including traditional chemotherapeutic agents, and more novel small molecule inhibitors and monoclonal antibodies (Caiola et al., 2015; McMahon et al., 2019; Pietrantonio et al., 2017). The origin of this resistance (intrinsic or acquired) varies considerably between drugs, and so advances have been made in treatment stratification, based on a patient's mutational status, for some therapies. For example, vemurafenib, the BRAF-inhibitor, is restricted to melanoma patients with the BRAF V600E mutation (Hopkins, Van Dyk, Rowland & Sorich, 2019). Likewise, the monoclonal antibody panitumumab is only recommended for patients with wild-type Ras (Amado et al., 2008). However, as mentioned previously, some mutations are only detectable when patients relapse, such as the emergence of FLT3 or NRAS mutations in AML patients (Man et al., 2012; McMahon et al., 2019; Piloto, Wright, Brown, Kim, Levis & Small, 2007; Smith et al., 2017). Many of these mutations occur downstream of the site targeted by the drug. For example, since Ras operates downstream of the epidermal growth factor receptor (EGFR), Ras mutations play a role in rendering cetuximab and panitumumab (which bind and inhibit EGFR) ineffective as the constitutive activation is not inhibited by the drug (De Roock et al., 2010; Li, Liu, Chi, Sun, Cheng & Cheng, 2015; Zhao et al., 2017). However, some of these putative resistance-causing mutations can only be detected at relapse, once they have expanded from only existing in a minor, undetectable subclone (as they did at diagnosis). Thus, it is difficult to predict which patients will develop these resistance mutations, meaning initial treatment stratification is difficult. Thus, combination therapy between these established agents and novel agents targeting these common mutational sites may be a way forward in preventing resistance before it occurs, an example of which is the combination of BRAF and MEK inhibitors, vemurafenib and cobimetinib (Hopkins, Van Dyk, Rowland & Sorich, 2019).

Introduction to Ras

As eluded, Ras mutations underpin resistance to a variety of therapies. KRAS is most commonly mutated of the three Ras isoforms, and is particularly frequently mutated in lung and pancreatic cancer (Moore, Rosenberg, McCormick & Malek, 2020; Prior, Hood & Hartley, 2020). However, patterns of Ras mutation differ between cancers and NRAS is the most frequently mutated Ras gene in AML, and HRAS accounts for most Ras mutations in head and neck squamous cell carcinoma (HNSCC) (Prior, Hood & Hartley, 2020) . Indeed, this variation carries varying prognoses of Ras-mutated cancer, with KRAS mutations conferring lowest overall survival 5 years post diagnosis (43%), and HRAS mutations conferring the highest (63%) (Figure 1) (Cerami et al., 2012; Gao et al., 2013).

Most oncogenic mutations in Ras occur in the residues G12, G13 and Q61, which are located in a region conserved between HRAS, KRAS and NRAS (figure 2A). They occur within the effector lobe, which comprises the first 85 amino acids and is a region of complete sequence identity between the different RAS isoforms. Most key interaction sites occur within the effector lobe, including nucleotide and effector interaction sites, as well as the switch regions that mediate effector interactions (figure 2B). There is 90% similarity in the next 80 amino acids (allosteric lobe). The only considerable sequence differences occur at the C terminal end of the protein, known as the hypervariable region. This is the region in which post-translational modifications occur, and membrane-targeting sequences are found (Hobbs, Der & Rossman, 2016). Initial attempts at direct pharmacological targeting of Ras centred around inhibiting these post-translational modifications, including farnesylation (Cox & Der, 2002; Whyte et al., 1997), however more recent attempts have considered the effector lobe, and structures

within this, to be better therapeutic targets, as discussed later (Canon et al., 2019; Janes et al., 2018; Ostrem, Peters, Sos, Wells & Shokat, 2013).

Ras is activated or inactivated when bound to GTP or GDP, respectively. Oncogenic mutations increase the frequency of GTP-bound (active) Ras, through two key mechanisms. This is through either decreasing the affinity of Ras for GTPase Activating Proteins (GAPs), which stimulate the intrinsic GTPase activity of Ras and therefore facilitate hydrolysis of GTP to GDP (off/inactivating mechanism), or by decreasing the need for Guanine nucleotide Exchange Factors (GEFs), which mediate the on/activating mechanism (Smith, Neel & Ikura, 2013; Wittinghofer & Waldmann, 2000). These differences depend on multiple factors including the specific amino acid mutation and resulting protein conformation (Hunter, Manandhar, Carrasco, Gurbani, Gondi & Westover, 2015; Miller & Miller, 2012; Poulin et al., 2019; Prior, Lewis & Mattos, 2012; Smith, Neel & Ikura, 2013), some mutations (including G12C) promote rapid cycling between the inactive and active states, an area which has been exploited by a range of novel Ras-targeting drugs (Fell et al., 2020; Hunter, Manandhar, Carrasco, Gurbani, Gondi & Westover, 2015; Patricelli et al., 2016).

Ras Signalling – Regular and Oncogenic

Ras is involved in key pathways regulating cell proliferation, differentiation and sensitivity to apoptosis, perhaps the most notable being the MAPK pathway and the PI3K/AKT pathway. KRAS has also been implicated (albeit in a more indirect way) in a range of other pathways, including the Wnt/β-catenin pathway and the NRF2 pathway (DeNicola et al., 2011; Ferino, Rapozzi & Xodo, 2020; Moon, Jeong, Park, Kim, Min do & Choi, 2014; Park et al., 2019; Tao et al., 2014). These pathways are detailed briefly below and are summarised in figure 3.

Ligand-binding to receptor tyrosine kinases (RTKs) such as EGFR or FLT3, causes autophosphorylation of key tyrosine residues on the RTK, after which adaptor proteins such as Grb2 bind to these phosphorylated tyrosines via SH2 domains. SOS, a Ras-GEF, then associates with Grb2 via two SH3 domains, and ultimately facilitates the exchange of GDP for GTP on RAS, thereby activating it (Freeman, 2000).

Following this, Ras stimulates recruitment of its serine-threonine kinase effector, RAF, to the membrane, which binds through its Ras binding domain (RBD) then proceeds to phosphorylate downstream kinases including MEK and ERK (Marais, Light, Paterson & Marshall, 1995; Molina & Adjei, 2006). ERK then translocates from the cytoplasm to the nucleus, causing the activation of many transcription factors, which go on to regulate a host of processes, including proliferation and differentiation (Guo, Pan, Liu, Shen, Xu & Hu, 2020; Zhang & Liu, 2002).

Alternatively, activated Ras can promote the PI3K-AKT pathway. This pathway also plays a key role in controlling survival, division and metabolism, and can be activated in many different ways (Castellano & Downward, 2011; Mendoza, Er & Blenis, 2011). Ras binds and activates the p110α catalytic subunit of PI3K, which in turn promotes transformation of the plasma membrane-bound lipid PIP_2 to PIP_3, and activation of AKT by binding through PH domains (Cantley, 2002; Castellano & Downward, 2011). AKT interacts with many downstream effectors, including MDM2, BAX, BAD and NF-kB. These interactions regulate apoptosis (Cantley, 2002; Castellano & Downward, 2011; Chang et al., 2003; Duronio, 2008). Furthermore, activation of FOXO by AKT promotes cellular metabolism (Engelman, Luo & Cantley, 2006). Given that the MAPK and PI3K pathways are both strongly involved in controlling cell survival and death, it is clear that aberrant signalling within these pathways will lead to cancer, of which key hallmarks include sustaining proliferative signalling, enabling replicative immortality and resisting cell death (Hanahan & Weinberg, 2011).

Aside from these two classic Ras-dependent pathways, increasing evidence suggests the implication of normal and mutant Ras function in alternative mechanisms, such as redox homeostasis and stem cell survival (Mukhopadhyay et al., 2020; Wu, Lu & Bai, 2019). The role of Ras in CSCs is yet to be fully elucidated, however its crosstalk with the Wnt/β-catenin pathway in tumorigenesis may suggest its involvement in the maintenance of CSCs (Moon, Jeong, Park, Kim, Min do & Choi, 2014). It is understood that, in colorectal cancer, there is overactivation of both the Wnt/β-catenin and Ras pathways, through mutations within APC (a tumour suppressor) and KRAS, respectively (Jeong, Ro & Choi, 2018). There is a synergistic effect seen with these mutations (D'Abaco, Whitehead & Burgess, 1996; Janssen et al., 2006; Jeong, Ro & Choi, 2018; Margetis, Kouloukoussa, Pavlou, Vrakas & Mariolis-Sapsakos, 2017). Mutations within APC cause loss of function of the tumour suppressor gene, whilst KRAS mutations lead to phosphorylation of key tyrosine residues within β-catenin, causing its accumulation within the cytoplasm. This ultimately increases activation of downstream Wnt pathway target genes, including REG4, a marker of CSCs (Hwang, Yoon, Cho, Cha, Park & Choi, 2020; Janssen et al., 2006). Upregulation of this pathway is highly associated with the increased survival and plasticity properties seen in CSCs and has been seen in many cancers (Al-Hajj, Wicha, Benito-Hernandez, Morrison & Clarke, 2003; Koury, Zhong & Hao, 2017; Lapidot et al., 1994). Taken together, it seems plausible that these KRAS mutations may play a role in the protection of the minor subset of cells which likely have a key role in relapse.

Furthermore, Ras mutations can also be implicated in reactive oxygen species (ROS) generation/detoxification. ROS are considered a 'double-edged sword', capable of both helping and hindering cancer cells (Hayes & McMahon, 2006; Wu, Lu & Bai, 2019). At physiological levels of ROS, NRF2 binds Keap1 and is ubiquitinated and degraded on a regular basis. However, upon detection of high levels of ROS (which is carcinogenic), NRF2 is unable to bind to Keap1 (due to conformational changes in Keap1) and so translocates to the nucleus, where it acts as a transcription factor for various downstream detoxification genes. Hence, the DNA of the healthy cells remains undamaged by ROS (Basak, Sadhukhan, Sarkar & Sil, 2017; Gorrini, Harris & Mak, 2013). However, in cancer, the NRF2 pathway can be upregulated to constitutively degrade ROS (induced by chemotherapeutics), thereby conferring a protective effect to the cancer cells, resulting in chemoresistance (Basak, Sadhukhan, Sarkar & Sil, 2017; Gorrini, Harris & Mak, 2013). Therefore, the balance of NRF2 activation level is crucial. In the last decade, it has been shown that KRAS G12D mutations can increase NRF2 transcription, through activation of the TPE Response Element via the MAPK pathway (DeNicola et al., 2011; Mukhopadhyay et al., 2020; Shirazi et al., 2020; Tao et al., 2014). Thus, these KRAS mutations can render the cancer cell more capable of coping with chemotherapy-induced ROS, thereby mediating drug resistance.

The Involvement of Ras in Chemotherapy Resistance

Should mutations occur as described above, it follows that one or many of these pathways can be perturbed, leading to increased cell proliferation, decreased cell death and promotion of CSCs, amongst other effects. The following scenarios illustrate some of these key resistance mechanisms, highlighting the necessity for better Ras-targeting.

Platinum-based agents, such as cisplatin, carboplatin and oxaliplatin, are used in the treatment of a variety of cancers, including HNSCC, testicular cancer and non-small cell lung cancer (NSCLC) (de Vries, Rosas-Plaza, van Vugt, Gietema & de Jong, 2020; Silva, Rocha, Kinker, Pelegrini & Menck, 2019; Weykamp et al., 2020). They are DNA intercalating agents that interfere with RNA transcription and DNA replication, through cross-linking of DNA. This results in the formation of DNA adducts, which in turn drive the tumour cell to apoptosis. Cisplatin also induces mitochondrial ROS, which further increase DNA damage and thus increase the cytotoxic properties of the drug (Marullo et al., 2013;

Srinivas, Tan, Vellayappan & Jeyasekharan, 2019). However, there are many resistance mechanisms associated with cisplatin, including the involvement of oncogenic KRAS mutations (Caiola et al., 2015; DeNicola et al., 2011; Feldman et al., 2014; Garassino et al., 2011). KRAS mutations were shown to induce NRF2 pathway upregulation in NSCLC, thereby decreasing cisplatin-induced ROS within the tumour cell, and ultimately leading to decreased cell death (DeNicola et al., 2011). This was supported by further work indicating oncogenic KRAS can induce NRF2 gene transcription via the TPE response element, resulting in the overactivation of the anti-oxidative stress pathway, rendering the tumour cells resistant to cisplatin-induced ROS (Tao et al., 2014). Furthermore, KRAS mutations can lead to hyperactivation of the PI3K-AKT pathway, which is starting to be implicated as a cisplatin resistance mechanism. As mentioned, upregulation of the PI3K-AKT-mTOR pathway can have multiple effects, including inhibition of apoptosis and increased cell proliferation (de Vries, Rosas-Plaza, van Vugt, Gietema & de Jong, 2020). Whilst there are other reasons for cisplatin resistance, Ras pathway mutations are heavily implicated in the key mechanisms. Thus, pharmacologically targeting Ras would provide an opportunity to overcome many causes of this resistance.

Upregulation of Ras-mediated pathways as a means of chemoresistance is by no means restricted to cisplatin resistance, and is a common mechanism of resistance to tyrosine kinase inhibitors (TKIs), such as those targeting RTKs including FLT3 and EGFR (Eberlein et al., 2015; Massarelli et al., 2007; McMahon et al., 2019; Ortiz-Cuaran et al., 2016; Piloto, Wright, Brown, Kim, Levis & Small, 2007; Van Emburgh et al., 2016). TKIs are used in a variety of cancers, including renal cell carcinoma (RCC), colorectal cancer (CRC), AML and NSCLC, to name a few examples. The mechanism of action of TKIs involves inhibition of phosphorylation sites within the protein, thereby preventing it exerting kinase activity on downstream effectors (Ciardiello & Tortora, 2008; Yamaoka, Kusumoto, Ando, Ohba & Ohmori, 2018). However, resistance to these can occur through two predominant mechanisms: mutations within the RTK, or mutations within downstream pathways (McMahon et al., 2019; Piloto, Wright, Brown, Kim, Levis & Small, 2007; Van Emburgh et al., 2016; Yu et al., 2013). Given that Ras occurs downstream of these receptors, any mutations within Ras will render the cell resistant to the TKI. For example, studies have shown KRAS mutations render patients resistant to gefitinib, used to treat NSCLC (Pao et al., 2005; Zhao et al., 2017). In a similar way, the treatment of CRC with anti-EGFR monoclonal antibodies cetuximab or panitumumab is only successful in a subset of patients, with many eventually developing resistance (Pietrantonio et al., 2017). This has been attributed to Ras mutations and variations in the EGFR extracellular domain (ECD), which reduce antibody binding efficiency, ultimately initiating relapse (Van Emburgh et al., 2016). Although cetuximab and panitumumab are only prescribed to Ras wild-type patients, emergence of mutations from undetectable, pre-existing clones can give rise to resistance in this way, as evidenced through analysis of circulating tumour DNA (ctDNA) (Amirouchene-Angelozzi, Swanton & Bardelli, 2017; Diaz et al., 2012; Misale et al., 2012). The order in which these mutations develop/emerge is likely important in understanding (and ultimately targeting) the process of relapse: Ras mutations often develop earlier than EGFR ECD variations, and typically confer poorer prognosis (Van Emburgh et al., 2016). Therefore, combatting these Ras mutations would not only improve prognosis of Ras-mutated patients, but also provide a second therapeutic option for those that go on to develop ECD variations.

Resistance to FLT3-TKIs is also a highly prevalent issue. It is well-documented that 20-30% of AML patients have an internal tandem duplication in the FLT3 receptor tyrosine kinase (FLT3-ITD) causing increased cell proliferation and decreased apoptosis, via the MAPK, STAT5 and PI3K pathways (Hayakawa et al., 2000; Moore et al., 2020; Papaemmanuil et al., 2016; The Cancer Genome Atlas, 2013). Therefore, many different FLT3 inhibitors are at varying stages in development to overcome the effects of this mutation. Examples include gilteritinib, crenolanib and midostaurin (Aikawa et al., 2020; McMahon et al., 2019; Piloto, Wright, Brown, Kim, Levis & Small, 2007; Zhang et al., 2019).

These TKIs bind to the active conformation of FLT3 and are at varying stages of approval: gilteritinib and midostaurin are FDA-approved, crenolanib is in phase II trials (Galanis et al., 2014; Levis, 2017; Levis et al., 2011; Zhang et al., 2019). Whilst results for these drugs have all been promising, subsets of patients exhibit resistance. This has, in part, been attributed to Ras mutations, re-activating the MAPK and PI3K-AKT pathways (McMahon et al., 2019; Zhang et al., 2019). These mutations were detected in over 30% of patients who developed resistance to gilteritinib, with Ras variant allele frequencies also increasing post-drug exposure in patients who responded poorly to crenolanib. Interestingly, not all resistant patients had FLT3 mutations following treatment either, with different mutational signatures present instead (McMahon et al., 2019; Zhang et al., 2019). This implies a clonal selection mechanism of resistance – a minor subpopulation at diagnosis which became dominant following treatment and elimination of the initial tumour burden. Taken together, it seems likely these Ras mutations, either pre-existing or *de novo*, may contribute to resistance to FLT3 inhibitors, through restoration of the original disease phenotype by expansion of an originally minor subclone. This is perhaps unsurprising in AML, which arises as a result of clonal haematopoiesis (Desai et al., 2018).

Options for Targeting the Ras pathway

With improved capability of detecting minor cancer subclones, alongside the greater understanding of the impact of Ras mutations in various cancers, the next considerable challenge is improved pharmacological targeting of Ras. However, drugging Ras has proven exceptionally difficult. A key drawback is the lack of available binding sites for small molecule inhibitors. The nucleotide binding site (where GTP or GDP binds) seems a desirable pocket to target, however the picomolar affinity with which both GDP and GTP bind, as well as their high intracellular concentrations, effectively outcompete the binding of any drug at this site (Cox, Fesik, Kimmelman, Luo & Der, 2014; McCormick, 2018).

Therefore, targeting alternative proteins within Ras-regulated pathways has been strongly investigated, with positive results seen. For example, trametinib, the MEK inhibitor. Currently approved for patients with BRAF V600-mutant metastatic melanoma or BRAF-mutated (V600) NSCLC, trametinib is well-tolerated in patients (Lugowska, Koseła-Paterczyk, Kozak & Rutkowski, 2015; Odogwu et al., 2018), and is also being assessed in Ras-mutant myeloid malignancies (Borthakur et al., 2016). This compound binds to phosphorylated MEK and inhibits its downstream effectors (e.g. ERK), despite the presence of constitutive Ras signalling. Subsequently, aberrant growth signalling and apoptosis inhibition is reduced (Hofmann et al., 2012). Whilst this has shown promising results (Borthakur et al., 2016; Lugowska, Koseła-Paterczyk, Kozak & Rutkowski, 2015; Odogwu et al., 2018), this compound only inhibits the MAPK pathway downstream of MEK, so constitutive activation of other Ras-dependent pathways (e.g. PI3K-AKT) will still occur in the presence of Ras mutations even when treated with this drug. In this way, cancer can persist (Jones et al., 2019; Stinchcombe & Johnson, 2014). However, if Ras were to be targeted directly, signalling of both of these pathways would be inhibited, leading to cell death.

As often seen with many diseases, combination of trametinib with other therapeutics to inhibit multiple pathways together may reduce the likelihood of continued cancer signalling and potential development of resistance to this and other drugs (Infante & Swanton, 2014; Planchard et al., 2016; Zhou, Zhao, Chen, Zhang & Zhou, 2020). However, even with the approved combination regimen of dabrafenib (BRAF inhibitor) with trametinib (Lugowska, Koseła-Paterczyk, Kozak & Rutkowski, 2015), only the MAPK pathway is inhibited, thereby maintaining the potential for aberrant PI3K pathway signalling, which can in itself cause resistance to MEK inhibitors (Jaiswal et al., 2009; Sos et al., 2009; Vitiello et al., 2019).

Alternatively, positive effects have been shown *in vitro* and *in vivo* of co-administering AKT inhibitors with dabrafenib to inhibit two Ras-mediated pathways (Lassen et al., 2014). However, combination of the two classes of drugs in patients did not yield significant clinical activity in reducing resistance seen in trametinib monotherapy, with 25% of patients exhibiting grade 3-4 toxicity (Algazi et al., 2018). Taken together, whilst downstream pathway inhibition has proved successful, this treatment method does have considerable disadvantages and may not be a long-term solution for many patients. Therefore, there is a distinct clinical need for novel means of treating Ras-mutant cancers, which could include the targeting of Ras itself.

Given the difficulties with targeting Ras, inhibition of elements upstream of Ras has been investigated. This includes inhibition of GEFs including SOS1, to reduce the likelihood of Ras maintaining its GTP-bound state and therefore inhibiting constitutive signalling (Evelyn, Duan, Biesiada, Seibel, Meller & Zheng, 2014; Hillig et al., 2019). For example, BAY293 is a first-in-class compound with the ability to bind directly to SOS1 and inhibit the Ras-SOS interaction, and thereby downstream signalling of the PI3K and MAPK pathways (Hillig et al., 2019). Although the *in vivo* bioavailability for this compound was poor, the concept of Ras-SOS inhibition could prove useful in the future, with promising high throughput *in silico* and *in vitro* screening results serving as a proof of concept for inhibition of Ras via this mechanism (Evelyn et al., 2015; Evelyn, Duan, Biesiada, Seibel, Meller & Zheng, 2014; Hillig et al., 2019). More recently, BI-1701963, a SOS1-pan-Ras interaction inhibitor has reached Phase I clinical trials, the first of its kind to do so. Modified from the structure of BI-3406, a quinazoline-derived compound, this novel inhibitor binds to the catalytic site of SOS1, preventing its interaction with inactive KRAS, thus inhibiting activation (Gerlach et al., 2020; Hofmann et al., 2020).

In addition, alternative, more indirect pathways are also being targeted as a means of inhibiting Ras, which may also be able to eliminate the CSC. For example, the small molecule KYA1797K has been shown to be effective against oncogenic Ras in CRC and erlotinib-resistant NSCLC (Park et al., 2019). This compound indirectly targets Ras, through inhibition of the Wnt/β-catenin pathway, which usually stabilises Ras (Jeong et al., 2012; Moon, Jeong, Park, Kim, Min do & Choi, 2014). This pathway is also upregulated in CSCs (Malanchi et al., 2008). KYA1797K has initiated anti-tumour effects in KRAS-mutant cell lines and a KRAS-mutated mouse model. KYA1797K also exhibited synergy with the current first line therapeutic regimen (cisplatin and pemetrexed) in NSCLC *in vitro* models (Park et al., 2019). However, KYA1797K promoted apoptosis of both KRAS wild type and mutant cells, questioning the specificity of the drug for cancer cells whilst sparing healthy cells. This study found both KRAS and β-catenin were overexpressed in tumour regions compared to non-tumour regions (Park et al., 2019). This may explain the limited toxicity seen during KYA1797K studies, with low doses of KYA1797K having a more substantial effect on tumour cells, compared to healthy cells. Indeed, only limited toxicity was seen during *in vivo* studies (Park et al., 2019). However, protein expression varies considerably between tissues (with KRAS particularly highly expressed in the brain), and so targeting based on comparative expression between cancer and non-cancer regions in one tissue may not represent overall toxicity potential (Newlaczyl, Coulson & Prior, 2017). Taken together, there may be a role for KYA1797K as a concomitant therapy in NSCLC (as well as other cancers such as CRC). It presents a means of eliminating both the primary cause of the disease (if KRAS-mutated), and also minor subclones and CSCs that could give rise to resistance (Cho et al., 2020). Nevertheless, better understanding of the drug's effects on other tissues with high Ras expression must be gained.

Options for Targeting Ras – Direct Ras Targeting

Ras post-translational modifications were targeted as a means of preventing Ras trafficking to the membrane and therefore inhibiting downstream signalling. This included generation of farnesyltransferase inhibitors (FTIs), including lonafarnib and tipifarnib (Van Cutsem et al., 2004).

However, the effectiveness of these was questionable, with most patients with KRAS-mutated diseases (such as pancreatic cancer and leukaemia patients) receiving no clinical benefit from these FTIs (Borthakur et al., 2006; Burnett et al., 2012; Harousseau et al., 2009; Van Cutsem et al., 2004). Inefficacy was largely due to the redundancy mechanism of geranylgeranyltransferase, which sufficiently modifies KRAS in the absence of farnesyltransferase to permit its trafficking to the membrane (Basso, Kirschmeier & Bishop, 2006; Whyte et al., 1997). Interest in this strategy has recently been revived with new personalised medicine approaches now capable of identifying patients harbouring HRAS- or NRAS-driven cancers that are more likely to respond (Gilardi et al., 2020; Lee et al., 2020).

In recent years however, more promising steps have been made regarding direct inhibition of oncogenic Ras. A key feature of this has been the discovery of novel potential binding pockets for small molecule inhibitors, to inhibit GEF activity or effector binding (Cruz-Migoni et al., 2019; Maurer et al., 2012; Ostrem, Peters, Sos, Wells & Shokat, 2013). Fragment-based screening identified a previously-undiscovered hydrophobic pocket located between the Switch I and II regions (termed S-IIP), which was successfully targeted by Ostrem et al. (2013) (figure 4a). Binding of peptide fragments was specific to G12C-mutated KRAS since the compounds functioned through irreversible cysteine binding in this particular pocket (and not with other cysteines found in wild type KRAS). Other key residues within this pocket include, but are not limited to, V7, V9, M72, F78, Q99 and I100. *In vitro* models of KRAS G12C-mutated lung cancer treated showed decreased survival upon treatment with these compounds, with inactive Ras (RAS-GDP) levels considerably greater than Ras-GTP. Further analysis showed that the conformational disruption caused by binding of these fragments, reduced interactions with both SOS and effector molecules and pathways, including B-RAF, C-RAF and the PI3K pathway (Gentile et al., 2017; Ostrem, Peters, Sos, Wells & Shokat, 2013).

The binding of these fragments to Ras also reduce SOS-catalysed nucleotide exchange, a method of Ras inhibition which had been previously explored. Compounds acting in this way either inhibited conversion of Ras-GDP to Ras-GTP (Patgiri, Yadav, Arora & Bar-Sagi, 2011), or increased the amount of Ras-GTP to such a level that it inhibited ERK phosphorylation, since overactivation of Ras can be cytotoxic (the Ras 'sweet-spot model') (Li, Balmain & Counter, 2018). Either way, these compounds were shown to inhibit the MAPK pathway, but were largely tested against Ras wild type (in the context of inhibiting the effects of RTK mutations). Thus, the aforementioned compounds identified by Ostrem et al. were revolutionary in their specificity for targeting Ras-mutated disease.

Discovery and characterisation of this SII-P pocket has led to the development of revolutionary KRAS G12C-selective covalent inhibitors, including ARS-853 and latterly AMG-510 (Figure 4B-D) (Canon et al., 2019; Patricelli et al., 2016). ARS-853 binds irreversibly to the inactive form of KRAS G12C, preventing exchange of GDP for GTP and therefore activation. This in turn inhibits downstream MAPK and PI3K—AKT pathway signalling, with KRAS-CRAF interactions significantly reduced. Moreover, *in vitro* evidence showed increased apoptosis and cell cycle arrest in some (but not all) models tested (Lito, Solomon, Li, Hansen & Rosen, 2016; Patricelli et al., 2016). This compound has subsequently been fully characterised *in silico*, and these studies revealed a dynamic nature of the SII-P pocket, a feature which could be utilised in further study (Khrenova, Kulakova & Nemukhin, 2020).

Based on this work, alternative iterations of KRAS G12C inhibitors have been produced. This was required since the probability of ARS-853 locking KRAS in its inactive state *in vivo* was debatable, given a lack of understanding regarding the cycling efficiency of Ras between its inactive and active states. It had been deduced *in vitro* that the G12C mutation permits rapid cycling of KRAS between these states, hence permitting the binding of ARS-853 (figure 4B). However, the possibility of finding the

correct therapeutic window to translate this compound to *in vivo* work proved complex (Janes et al., 2018; Lito, Solomon, Li, Hansen & Rosen, 2016; Patricelli et al., 2016). Thus, alternatives including ARS-1620 were developed to improve *in vivo* capability (figure 4C). Modifications to the ARS-853 structure resulted in favourable pharmacokinetic (PK) properties, permitting a greater understanding of KRAS activation status and dependency *in vivo* (Janes et al., 2018). ARS-1620, is an orally bioavailable quinazoline based compound with limited side effects witnessed in pre-clinical animal models (Janes et al., 2018; Li, Balmain & Counter, 2018). Optimisation from ARS-853 by inclusion of a fluorophenol, hydrophobic binding group permitted stronger covalent (irreversible) binding within the SII-P pocket (more specifically, interaction with H95 in this pocket), thus improving potency (figure 4). The effects of this compound remained mutation-specific, thereby eliminating the risk of binding to KRAS wild-type in non-tumour cells and thus reduced toxicity potential. This compound was well tolerated in patient-derived xenograft mice, where a reduction in tumour burden through decreased Ras-mediated downstream signalling was evident. This was the first example of an *in vivo* trial using a compound targeting the SII-P pocket (Janes et al., 2018).

From this, AMG-510 was developed, following further modifications to the ARS-1620 structure, including addition of more aromatic entities (figure 4D). This enables AMG-510 to bind within a slightly different groove of SII-P, enhancing potency and selectivity (Canon et al., 2019). Promising pre-clinical *in vitro* and *in vivo* experiments indicated arrest of the MAPK pathway and induction of a pro-inflammatory tumour microenvironment. This compound has since progressed into clinical trials, the first KRAS-G12C selective inhibitor to do so. Early data indicates that, out of 29 patients evaluable for response at the time of publication, 5 exhibited partial response, 18 had stable disease and 6 had progressive disease. As with the previous ARS-1620 animal studies, AMG-510 was generally well-tolerated, with no dose limiting toxicities recorded (Govindan, 2019; Romero, 2020). However, larger cohorts and longer trials are imperative in determining the true impact of this compound. Such compounds could help combat the KRAS-mediated resistance to monoclonal antibodies seen in NSCLC and CRC (Lièvre & Laurent-Puig, 2009; Park et al., 2019). However, success of this compound, and indeed this targeting mechanism, is likely restricted to KRAS-G12C mutant cancer since some key residues for binding of this compound are unique to KRAS and not conserved between the different isoforms. Indeed, *in vitro* work completed by Ostrem, Peters, Sos, Wells and Shokat (2013) illustrated that transduction of lung cancer cell lines with KRAS G12V rescued the cancerous phenotype (resistance to cell death, increased proliferation), thereby illustrating how this mutation renders resistance to KRAS G12C inhibitors, as expected.

Alternative KRAS G12C inhibitors are also in development and showing considerable promise, including MRTX849 (Fell et al., 2020; Hallin et al., 2020a). In a similar way to AMG-510 and the ARS compounds discussed above, MRTX849 covalently binds to the inactive form of KRAS, in SII-P (figure 4E). This induces apoptosis through downregulation of the MAPK pathway. Interestingly, the PI3K-AKT pathway remained relatively unaffected by MRTX849 (Hallin et al., 2020b). *In vivo* trials with MRTX849 exhibited favourable pharmacokinetic/pharmacodynamic properties (Fell et al., 2020), and both cell line- and patient-derived xenograft modelling of pancreatic and lung cancers also indicated up to a 30% reduction in tumour burden (Fell et al., 2020; Hallin et al., 2020b). Individual patient case studies from Phase I trials have also shown MRTX849 to be effective in reducing tumour burden in both lung and colorectal cancers, although this data is largely incomplete (Hallin et al., 2020b). Taken together, it is clear that KRAS G12C inhibitors have promise as a means of abrogating Ras-mediated resistance, although there remain drawbacks which need assessing. Most notably, the lack of efficacy against other Ras mutations, which are prevalent across Ras-mutated disease.

Clearer understanding of the structure of Ras has not only permitted elucidation of the SII-P pocket, but also alternative binding sites, including a pocket between the Switch I and II regions of Ras (termed pocket I). Key residues available for interaction with small molecule compounds include K5, L6, V7, V8, S39, D54, I55, L56, Y71, T74, G75 and E76 (Maurer et al., 2012; Quevedo et al., 2018). Antibody-fragment-directed site exploration can be used to explore and exploit previously unconsidered drug interaction sites. In the case of Ras, this mechanism has been used to analyse potential compound binding sites within the previously-identified pocket I, that could be targeted using small molecule inhibitors to interrupt effector proteins (such as c-RAF and p110α) from binding to Ras via the Ras binding domain (RBD) (Maurer et al., 2012; Quevedo et al., 2018). This would provide an alternative mechanism of abrogating the effects of oncogenic Ras activation to those discussed previously, and early results showed effectiveness of antibody-derived compounds against a range of Ras mutations and isoforms (Quevedo et al., 2018). However, given high affinity binding of certain effectors to Ras (such as PI3K and B-RAF, with 3.2 μM and 0.04 μM affinity respectively) (Erijman & Shifman, 2016), the high EC_{50}s of the compounds identified in these *in vitro* assays mean that many further modifications would be required to convert these putative compounds into usable therapeutics. Nevertheless, such antibody-derived fragments have the potential to be fused with small molecule protein-protein interaction (PPI) inhibitors to improve efficacy (Cruz-Migoni et al., 2019). Whilst many Ras-effector interaction inhibitors have been trialled pre-clinically, none have been implemented in the clinic in the context of Ras-mutant cancer, owing to lack of efficacy, or toxicity potential (Canon et al., 2019; Keeton, Salter & Piazza, 2017). However, crystal structure determination showed that fusion of compounds developed through antibody-derived fragment screening with known small molecule PPI inhibitors results in better binding within pocket I, thus inhibiting Ras-effector interactions with a lower EC_{50}. Nevertheless, the therapeutic use of pocket I may be restricted since such a pocket has also been detected in wild-type Ras (Cruz-Migoni et al., 2019), thus increasing the risk for on-target toxicity.

In recent times, inhibition of the Ras-effector interaction has been seen through competitive binding of rigosertib at the RBD. This compound elicits effects against MAPK, PI3K and RAL pathway activation, in both wild-type and mutant Ras situations (Athuluri-Divakar et al., 2016). Inhibition of multiple Ras-mediated diseases have been seen in response to rigosertib, including pancreatic cancer and leukaemia (Athuluri-Divakar et al., 2016; Baker, Cosenza, Ramana Reddy & Premkumar Reddy, 2019). Rigosertib is moderately to well- tolerated in clinical trials thus far, although is yet to be specifically tested in the context of Ras-mutant cancer (Bowles et al., 2014; Ma et al., 2012; Navada et al., 2020). As with the antibody-derived, small molecule PPI inhibitors described above, toxicity may be as a result of rigosertib's ability to target both wild-type and mutant KRAS, and thus further studies are needed to fully assess the impact such a drug on healthy cells.

Antibody therapy is also currently being explored as means of direct Ras-targeting. However, a major drawback has been the capability of the antibody to cross the cell membrane, as with any protein based therapy (Bolhassani, Jafarzade & Mardani, 2017). Therefore, the development of inRas37, a pan-Ras targeting antibody, is a considerable step forward in targeting Ras. Although the cellular uptake remains low (approximately 4%), *in vitro* and *in vivo* work has shown promise in the potential of inRas37 to inhibit both the MAPK and PI3K-AKT pathways, in a dose-dependent manner (Shin et al., 2020). Briefly, this drug binds to integrins $\alpha V\beta 3$ and $\alpha V\beta 5$ on the cancer cell surface which then undergo endocytosis. The antibody 'escapes' the endosome as a result of pH-determined cleavage (the antibody is cleaved from the integrins better at pH 7, the cytoplasmic pH, compared to pH 6.5, the pH of early endosomes) (Podinovskaia & Spang, 2018; Putnam, 2012; Shin et al., 2020). The antibody then co-localises with Ras to block the effector binding site, in a manner similar to the PPI inhibitors described above. Mutations introduced into the general antibody structure render it specific

for mutant Ras binding, with little activity against Ras wild-type, thereby limiting on-target toxicity. inRas37 has greater effect on cells with a greater dependency on Ras signalling, which can include some tumour cells (Weinstein, 2002; Yi, Nissley, McCormick & Stephens, 2020). Nevertheless, when tested on large tumour models (spheroids and cell-derived xenograft mice), efficacy decreased considerably (Shin et al., 2020). This therefore shows that treatment with this drug may be suitable for patients at earlier stages of Ras-mutated cancer, before tumour burden is too great and reduces drug efficacy. Thus, it could be used to treat Ras-initiated relapse as soon as it occurs, however its utility in eliminating minor subclones prior to relapse is limited, given the low cellular uptake and the need for a high Ras dependency in the cell.

Resistance to Ras Targeting Agents

Of course, there is potential for resistance to any therapeutic, and Ras-targeting drugs are no different. Some of these have been previously discussed here, such as the use of geranylgeranyltransferase to overcome the effects of farnesyltransferase inhibitors (Whyte et al., 1997), or the upregulation of alternative Ras-mediated pathways, as seen when patients are treated with MEK inhibitors (Vitiello et al., 2019). Other mechanisms of resistance are also possible, such as the re-activation of ERK, which may be a potential resistance mechanism in the case of a Ras-targeting agent (Bruner et al., 2017; Ercan et al., 2012; Ochi et al., 2014). This has already been seen in the case of EGFR-inhibitor resistance, whereby negative regulators of ERK are downregulated, so pro-apoptotic BIM is not fully upregulated and so cannot fully induce apoptosis. Alternatively, the gene encoding ERK1, *MAPK1*, is amplified. These scenarios resulted in *in vitro* and *in vivo* resistance to the putative EGFR inhibitor WZ4002 (Ercan et al., 2012). ERK-reactivation has also been found to contribute to gefitinib resistance, but in this case, was found to be mediated by Src (Ochi et al., 2014). Given these kinases are either side of Ras, their co-operation could result in resistance to a Ras inhibitor. However, Src-mediated ERK reactivation is avoidable through treatment with Src inhibitors (Ochi et al., 2014), which may present a means of overcoming this potential Ras-inhibitor resistance mechanism. Taken together, these studies imply that, while resistance to Ras-targeting drugs is possible, there are already means of overcoming this resistance, just as a Ras-targeting drug would provide the means of overcoming Ras-mediated resistance.

Whilst other elements of pathways contributing to resistance can be targeted relatively easily, acquisition of secondary mutations in Ras present a more pressing problem. For example, at present, the G12C specific inhibitor is perhaps the most developed means of inhibiting Ras, however a subsequent mutation in Ras would most likely render this inhibitor ineffective. This has already been evidenced in studies into KRAS G12C inhibitors, whereby rescue experiments with the G12V mutation restored the cancerous phenotype (Ostrem, Peters, Sos, Wells & Shokat, 2013). Therefore, a pan-mutation targeting drug, such as a derivative of inRas37, may be favoured.

Discussion

Ras mutations in cancer and chemoresistance are important when considering patient prognosis. Whilst it seems inevitable that resistance will be an issue for a long time to come, better targeting of potential causes is imperative. Advances in Ras inhibition could help reduce the risk of resistance and relapse for a wide range of cancers, given its mutational frequency. At present, some success has been seen when multiple drugs are used to target different pathways implicated in Ras-mutated disease. However, other studies have shown lack of long-term efficacy when combining multiple therapies. Therefore, single agent, multi-pathway targeting agents, including direct Ras inhibitors are becoming more heavily researched. It will be interesting to see the effects of these *in vivo,* since this type of therapy may reduce the potential for future development of drug resistance by upregulation of an alternative pathway. Targeting a common factor at the centre of multiple pathways may target more

cancer cell types and thus reduce the heterogeneity of the tumour, eliminating potential resistance causes before they become dominant.

Previous failures of Ras-targeting agents, including farnesyltransferase inhibitors, as well as perceived unfavourable protein dynamics, has resulted in Ras being largely considered undruggable. However, development of structural analysis techniques and a clearer understanding of the key residues in Ras has altered this thinking, with new compounds with novel, allele-specific Ras binding mechanisms showing great promise. Phase I trials of AMG-510, a first-in-class direct Ras-targeting agent, suggest a turning point has been reached in this field of study. Whilst there is much more to be done, these preliminary data indicate a solution for Ras-mediated resistance may be possible.

Nevertheless, a greater understanding of resistance must be gained before relapse risk can be eliminated. Despite evidence supporting the CSC theory, limited standard-of-care detection sensitivities for initial diagnostic samples prevent identification of the minor subclones present at diagnosis (McMahon et al., 2019). It can therefore be difficult to determine likely causes of relapse upon initial diagnosis, and so constant monitoring for changes in expression of genes commonly implicated in drug resistance, such as Ras, may provide a useful tool for predicting disease trajectory. Nevertheless, this is only useful if the effects of the acquired/emergent mutations can be abrogated. In the cases discussed here, improved analytical tools would ideally be combined with Ras-targeting agents to prevent resistance taking hold.

Ultimately, chemoresistance, either intrinsic or acquired, due to Ras mutations, whether primary or secondary, remains a considerable problem. The concepts presented here, amongst many other examples, illustrate the necessity for Ras-targeting drugs. There is a distinct clinical requirement for the improved targeting of Ras in cancer, with Ras implicated in both initial disease presentation and relapse. Although no universal, direct Ras inhibitor has yet been achieved, considerable progress has been made in the last decade with the advent of allele-specific inhibitors. This brings promise to the field, with the potential for better treatment of Ras-initiated resistance a real prospect.

Nomenclature of Targets and Ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding et al., 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2019/20 (Alexander et al., 2019).

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Figure Legends.

Figure 1: The impact of Ras alterations on disease. Data collected from 32 curated, non-redundant studies, comprising 10967 patient samples, as collated by the TCGA pan-Cancer Atlas. **A)** 10 year profession-free survival (PFS) analysis for patients with and without Ras alterations . Ras wild-type (blue) vs. altered (red) overall survival. Ras wild-type median PFS = 65.88 months, Ras-altered median PFS = 44.19 months. P<0.01. **B)** 10 year PFS stratified by altered Ras isoform. KRAS (orange) median PFS = 36.72 months, NRAS (green) median PFS = 45.67 months, HRAS (purple) median PFS = 74.89 months. P<0.01. **C)** Cancer types where KRAS is altered in at least 10% of cases. **D)** Cancer types where NRAS is altered in at least 10% of cases. **E)** Cancer types where HRAS is altered in at least 10% of cases. *Data obtained from cBioPortal, all TCGA PanCancer Atlas Studies.*

Figure 2: 2D and 3D representation of the structure of RAS. A) physiological binding domains. **B)** key structural domains (switch regions and lobes), with mutational hotspots G12, G13 and Q61 indicated. *Redrawn from Prior et al., 2012. 3D structures based on PDB 4DST.*

Figure 3: RAS-mediated pathways and associated inhibitors. Targets of small molecule inhibitors and monoclonal antibodies used across a range of cancers to inhibit proliferative signalling and survival of cancer cells. Figure includes examples of compounds identified *in vitro*, those which have progressed into trials and those which are approved.

● pre-clinical studies, ▲ phase I clinical trials, ◆ phase II clinical trials, ★ FDA approved use. Further detail is provided in table 1.

Figure 4: Evolution of novel direct RAS-targeting agents. Chemical structure and protein structures of RAS direct targeting agents in complex with GDP-bound (pink) KRAS. A**)** Minor modification of initial compound hit 6H05, 6H05 compound 6 (purple) bound covalently to KRAS G12C, PDB accession no. 4LUC. **B)** ARS-853 (purple) bound covalently to KRAS G12C (orange), PDB accession no. 5F2E. **C)** ARS-1620 (purple) bound covalently to KRAS G12C (orange), PDB accession no. 5V9U. **D)** AMG-510 (purple) bound to KRAS G12C (orange), PDB accession no. 6OIM. **E)** MRTX849 (purple) bound covalently to KRAS G12C (orange), PDB accession no. 6UT0. Molecules shown in relation to the switch regions, largely binding in SII-P. All compounds bind covalently near to mutational hotspot.

Table 1: Summary of current Ras pathway targeting drugs, at varying stages of development.

Name	Drug Type	Target	Development Stage	Mode of inhibition	Selected common adverse effects	References
Gilteritinib	Pyrazinecarboxamide, small molecule inhibitor	FLT3-ITD, FLT3-TKD, AXL	FDA-Approved	Binds active FLT3 (either ITD or TKD), inhibiting constitutive signalling	Acute kidney injury, hypotension, diarrhoea, dizziness	Lee et al. (2017); Perl et al. (2019)
Midostaurin	Indolocarbazole, small molecule inhibitor	FLT3	FDA-Approved	Binds active FLT3, inhibiting constitutive signalling	Nausea, neutropenia, thrombocytopenia, diarrhoea, vomiting	Levis (2017); Stone et al. (2012)
Crenolanib	Benzamidazole-derivative small molecule inhibitor	FLT3-ITD, FLT3-TKD	Phase II (NCT02400255)	Binds active FLT3, inhibiting constitutive signalling	Nausea, vomiting, diarrhoea, stomach pain	Galanis et al. (2014); Marensi, Keeshan and MacEwan (2021); Wu, Li and Zhu (2018)
Cetuximab	Chimeric IgG1 chimeric monoclonal antibody	EGFR	FDA-Approved	Competitively binds extracellular domain of EGFR, preventing ligand-mediated signalling	Oedema, fatigue, anorexia, rash, vomiting	Jonker et al. (2007)
Panitumumab	Human IgG2 Monoclonal antibody	EGFR	FDA-Approved	Competitively binds extracellular domain of EGFR, inhibiting EGFR dimerization and autophosphorylation	Rash, diarrhoea, hypomagnesaemia	Van Cutsem et al. (2007)
BAY293	Quinazoline-based small molecule inhibitor	SOS1	Pre-clinical	Disruption of the KRAS-SOS1 interaction, reducing exchange of GDP for GTP	N/A	Hillig et al. (2019)
SAH-SOS1	Stapled peptide fragment	SOS1	Pre-clinical	Binds KRAS (active or inactive) in the SOS1 binding pocket, reducing GTP binding to KRAS	N/A	Leshchiner et al. (2015)
c	Small molecule inhibitor	SOS1	Phase I (NCT04111458)	Binds inside the catalytic site of SOS1, preventing interaction with (and activation of) KRAS	N/A	Gerlach et al. (2020)

KYA1797K	Thiazolidine-based small molecule inhibitor	Axin	Pre-clinical	Binds to the RGS domain of Axin, causing formation of the β-catenin destruction complex, subsequently destabilising Ras	N/A	Cha et al. (2016); Lee et al. (2018)
Tipifarnib	Quinolinone small molecule inhibitor	Farnesyltransferase	FDA-Approved	Prevents Ras farnesylation and subsequent trafficking to the membrane	Thrombocytopenia, Anorexia, anaemia, nausea, neutropenia	Duffy and Crown (2021); Lee et al. (2020)
ARS-853	Acrylamide-based, small molecule inhibitor	KRAS-G12C	Pre-clinical	Irreversible binding to KRAS-G12C SII-P pocket, inhibiting exchange of GDP for GTP	N/A	Lito, Solomon, Li, Hansen and Rosen (2016); Patricelli et al. (2016)
ARS-1620	Acrylamide-based, small molecule inhibitor (S-atropisomer)	KRAS-G12C	Pre-clinical	Irreversible binding to KRAS-G12C SII-P pocket, inhibiting exchange of GDP for GTP	N/A	Janes et al. (2018)
AMG-510	Acrylamide-based, small molecule inhibitor	KRAS-G12C	Phase I/II (NCT03600883)	Irreversible binding to KRAS-G12C SII-P pocket, inhibiting exchange of GDP for GTP	Anaemia, diarrhoea	Canon et al. (2019); Govindan (2019)
MRTX849	Acrylamide-based, small molecule inhibitor	KRAS-G12C	Phase I (NCT03785249)	Covalent binding in SII-P pocket, inhibiting exchange of GDP for GTP	Nausea, diarrhoea, fatigue, hyponatremia	Fell et al. (2020); Hallin et al. (2020a)
inRas37	Human IgG1 internalising and PPI-interfering monoclonal antibody	pan-Ras	Pre-clinical	Competitive inhibition of RAS-effector interaction	N/A	Shin et al. (2020)
siG12D-LODER	Long-acting siRNA	KRAS-G12D	Phase II (NCT01676259)	siRNA-mediated KRAS G12D silencing	Diarrhoea, abdominal pain, nausea, fatigue	Golan et al. (2015); Zorde Khvalevsky et al. (2013)
Dabrafenib	Sulphonamide-based small molecule inhibitor	BRAF (wild-type and V600-mutated)	FDA-Approved	ATP-competitive inhibitor; binds active BRAF and thus inhibits downstream effector activation	Rash, fever, fatigue, headache, hypertension, arthralgia	Bowyer, Lee, Fusi and Lorigan (2015); King et al. (2013); Rheault et al. (2013)

Vemurafenib	Azaindole-derived small molecule inhibitor	BRAF-V600E	FDA-Approved	ATP-competitive inhibitor, binds active BRAF and thus inhibits downstream receptor activation	Skin lesions (including squamous cell carcinoma), arthralgia, fatigue	Sharma, Shah, Illum and Dowell (2012); Tsai et al. (2008)
Sorafenib	Biaryl-urea-based small molecule inhibitor	RAF, PDGFR, VEGFR amongst others	FDA-Approved	Binds inactive conformation of BRAF, reducing activation. Also sequesters Raf into inactive complexes.	Diarrhoea, weight loss, skin reactions, alopecia, voice changes	Adnane, Trail, Taylor and Wilhelm (2006); Llovet et al. (2008); (Marensi, Keeshan & MacEwan, 2021); Wan et al. (2004); Wilhelm et al. (2006)
Cobimetinib	Carboxamide-based small molecule inhibitor	RAF, MEK	FDA-Approved	Non-ATP-competitive inhibitor, binds active MEK, inhibiting downstream ERK activation	Diarrhoea, rash, fatigue, arthralgia, photosensitivity	Garnock-Jones (2015); Hatzivassiliou et al. (2013); Rice et al. (2012)
Trametinib	Pyridopyrimidine small molecule inhibitor	MEK	FDA-Approved	ATP non-competitive kinase inhibitor; binds the kinase suppressor of RAS-RAS interface, reducing MEK phosphorylation	Diarrhoea, rash, blurred vision	Borthakur et al. (2016); Khan et al. (2020)
LY3214996	Thiazolone-based small molecule inhibitor	ERK	Phase I (NCT02857270)	ATP-competitive inhibitor, reversibly binds ERK1 and ERK2, causes cell cycle arrest in G_1 and initiates apoptosis	Nausea, vomiting, diarrhoea, fatigue, blurred vision	Bhagwat et al. (2020); Pant et al. (2019)
RBC8	Carbonitrile-based small molecule inhibitor	RAL	Pre-clinical	Non-ATP-competitive inhibitor, binds GDP-loaded RAL, preventing activation	N/A	Walsh, Wersäll and Poole (2019); Yan et al. (2014)
Alpelisib	Aminothiazole-based small molecule inhibitor	PI3Kα	FDA-Approved	ATP-competitive inhibitor, binds selectively to PI3Kα	Hyperglycaemia, rash, diarrhoea, nausea, decreased appetite	André et al. (2019); Furet et al. (2013)

Uprosertib	Thiophenecarboxemide-based small molecule inhibitor	AKT	Phase II (NCT01902173)	ATP-competitive inhibitor, binds AKT and reduces downstream signalling	Nausea, vomiting, diarrhoea, rash	Gungor et al. (2015); Nitulescu et al. (2016)
Everolimus	Macrocyclic lactone-based small molecule inhibitor	mTOR	FDA-Approved	Complexes with FKBP12, which binds mTOR and inhibits activation	Leukopenia, hypercholesterolaemia, hyperlipidaemia	Dunn and Croom (2006)