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THE SCATTER FACTOR SIGNALING PATHWAYS AS THERAPEUTIC ASSOCIATED TARGET IN CANCER THERAPY

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ABSTRACT

Receptor tyrosine kinases (RTKs) are key regulators of critical cellular processes such as proliferation, differentiation, neo-vascularization, and tissue repair. In addition to their importance in the regulation of normal physiology, aberrant expression of certain RTKs has also been associated to the development and progression of many types of cancer. c-Met and RON are two RTKs with closely related sequences, structural homology, and similar functional properties. Both these receptors, once activated by their respective ligands, the Hepatocyte Growth Factor/Scatter Factor (HGF/SF1) and the Macrophage Stimulating Protein/Scatter Factor 2 (MSP/SF2), can induce cell migration, invasion and proliferation. Soon after its discovery in the mid-1980s, c-Met attracted a great interest because of its role in modulating cell motility. Moreover, the causal role for c-Met activating mutations in human cancer propelled an intensive drug discovery effort throughout academic institutions and pharmaceutical companies. While c-Met is now a well-accepted target for anti-cancer drug design, less is known about the role of RON in cancer and less has been done to target this receptor. In this review we will discuss the biological relevance of c-Met and RON, their deregulation in human cancers and the progress, so far, in identifying c-Met and RON signaling inhibitors. Finally, we will focus on the development of therapeutic strategies and drug efficacy studies based on interfering the scatter factor signaling pathways.

INTRODUCTION

Significant progress has been made towards developing target-based cancer therapies over the past decade. Numerous molecules, designed to block specific signaling pathways important for tumor formation, progression, dissemination and/or angiogenesis, have been approved [1]. Despite the remarkable success, most approved agents do not cure patients, and in addition, pre-existing resistance to these agents is often detectable in many intent-to-treat patients. Furthermore, most patients who initially respond to the treatments nevertheless develop resistance, and the tumors that re-emerge are often more aggressive and difficult to cure. Although the mechanisms for resistance might vary, one major problem remains the extremely complex nature of the tumor cells, characterized by genetic heterogeneity and instability. Recent genomic and biochemical studies indicate that even in tumors originated from the same tissue there are many differences that potentially contribute to the development and spread of the disease [2,3]. Thus, the ultimate success in controlling most if not all cancers, will possibly require the application of multiple agents that effectively inhibit different pro-cancer mechanisms. With this respect proto-oncogenic kinases receptors represent today a class of biologically relevant targets for cancer intervention [4].

C-MET AND ITS LIGAND HGF: THE STRUCTURE

The RTK c-Met is the cell surface receptor for HGF, also known as scatter factor 1 (SF1) [5,6]. HGF is a 90 kDa multidomain glycoprotein, member of the plasminogen-related growth factor family and contains 728 aminoacidic residues. It is secreted by mesenchymal cells [7] as an inactive single-chain precursor, which is proteolytically processed to form the biologically active disulfide-linked α/β -heterodimer [8]. The α -chain folds into a N-terminal domain (N-domain), followed by four Kringle domains and contains the high-affinity c-Met receptor-binding domain, NK1 (Fig.1). The β -chain, required to interact with the c-Met receptor for its activation, is homologous to the chymotrypsin family of serine proteases [9] but lacks any proteolytic activity [10], consistent with the absence of the key serine and histidine residues that comprise the 'catalytic triad' Asp[c102]–His[c57]–Ser[c195] (chymotrypsinogen numbering) of these enzymes. Comparisons of the biologically active, two-chains HGF, and the inactive single-chain HGF precursor, have shown that both forms of HGF bind to c-Met with similar affinity, but only the cleaved, mature form of HGF is able to activate c-Met [10]. In addition, various C-terminally truncated fragments of the α -chain (termed NK1, NK2, or NK4 depending on the number of Kringle domains retained) bind c-Met and in some cases, like NK4, ~~they~~ act as potent antagonists of c-Met functions [11,12].

The c-Met receptor, like its ligand, is a disulfide-linked heterodimer consisting of an extracellular α -chain and a transmembrane β -chain (Fig. 1). The α -chain is heterodimerized to the amino-terminal portion of the β -chain, forming the major ligand-binding site consisting of the Sema domain. The extracellular portion of the β -chain is composed by a

cysteine-rich domain (PSI motifs), and four immunoglobulin-like domains (IPT domains). After the transmembrane domain, the juxtamembrane region contains the receptor down-modulation Cbl-binding domain followed by the kinase domain and the carboxy-terminal tail, both essential for receptor activation and downstream signaling [13]. HGF binding induces c-Met receptor homodimerization and phosphorylation of three tyrosine residues (Y1230, Y1234 and Y1235) within the activation loop of the receptor leading to full kinase activity [14]. The carboxy-terminal tail includes tyrosines Y1349 and Y1356, which, when phosphorylated, serve as docking sites for multiple intracellular adaptor proteins, that promote downstream signaling [15,16].

THE FUNCTIONS OF C-MET

c-Met and HGF are expressed in many tissues in the developing embryo and in the adult. HGF is generally produced by mesenchymal cells and activates c-Met, via a paracrine mode of action, in cells of epithelial and endothelial origin [17]. Studies from knock-out mice show that, during development, HGF and c-Met provide essential signals for development of the liver, placenta and skeletal muscle [7]. Indeed, one of the most striking abilities evoked by c-Met is its capacity to induce motility in the epithelial cells stimulated by HGF. During development, cells frequently migrate over long distances to their final target, where they form tissues and organs. Some skeletal-muscle-progenitor cells migrate to the limbs, the tongue and the diaphragm, where they differentiate to form skeletal muscle. Ablation of HGF or c-Met results in the complete absence of all muscle groups that derive from migrating progenitor cells [18].

In the adult, data obtained from inducible c-Met knock-out mice indicate a role for this receptor exclusively in damaged tissues in order to repair the injury [17,19]. In fact, c-Met activation evokes, *in vitro* and *in vivo*, multiple biological responses among which the ability to dissociate, destroy the extracellular matrix, migrate and proliferate [20]. The first steps of this complex biological program are also called epithelial-mesenchymal transition (EMT). During EMT, cells lose the junctions that maintain the epithelial monolayer, rearrange their cytoskeleton and lose their epithelial phenotype to acquire a mesenchymal one. EMT is a necessary event in several morphogenic processes during embryogenesis and tissue repair. Thus, in order to coordinate all these events, the signaling cascade downstream c-Met is very complex. However, studies regarding the effect of HGF on EMT are controversial and context-dependent. For instance, it has been reported that HGF has inhibitory effects on TGF- β , a strong inducer of EMT. The interplay between these two pathways leads to the modulation of tubulogenesis during the formation and repair of organs such as the mammary gland, lung and the kidney [21,22]. Several points of crosstalk between TGF- β and HGF signalling during morphogenesis and repair of tissue damage have been proposed among which modulation of expression of HGF, extracellular matrix (ECM)-degrading proteases and Smad (a fundamental downstream effector of TGF- β) transcriptional co-repressors [23,24]. Many tyrosine residues within c-Met (Y1349 and Y1356 in the carboxyl-terminus

or Y1003 in the juxtamembrane region, see Fig.1) provide docking sites for multiple adapter proteins. Grb2 and Shc couple c-Met to the Ras-mitogen-activated protein kinase (MAPK) pathway and provide a direct link to cell proliferation and motility. The p85 subunit of phosphatidylinositol 3 kinase (PI3K) has a fundamental role as anti-apoptotic agent. Gab1 docking protein provides binding sites for many proteins involved in signal transduction, potentiating and diversifying the signals downstream from this receptor. Finally, Cbl ubiquitin ligases, once docked to Met phosphorylated Y1003, are required for ligand dependent ubiquitination and degradation of the Met receptor [25].

All the physiological responses evoked by c-Met that occur during organ formation or tissue repair are also an hallmark in cancer development and progression. Invasive neoplastic cells have to regain the ability to promote EMT, to move through the extracellular environment, to survive in foreign compartments, and to proliferate to settle at distant sites. As an example c-Met is primarily elevated in the basal and a subset of the ErbB2-positive human breast cancers subtypes, moreover c-Met and the EMT marker SNAIL are highly predictive of poor prognosis in lymph node negative patients [26]. Deregulation of the c-Met and HGF/SF signaling axis promotes tumor formation and invasion via multiple mechanisms: a) the establishment of an autocrine loop that make cells independent from growth factors; b) c-Met overexpression that triggers receptor homo-dimerization and activation even in the absence of HGF; c) and the presence of single aminoacidic point mutations that generate a constitutively active receptor [27]. Indeed, c-Met signaling has been documented in a wide range of human malignancies, including bladder, breast, cervical, colorectal, gastric, head and neck, liver, lung, ovarian, pancreatic, prostate, renal, and thyroid cancers, as well as in various sarcomas, hematopoietic malignancies, and melanomas [28].

It still remains to be established which tumor subtypes depend on c-Met as an exclusively driving oncogene for their growth and spread. As of now only a small subset of cancers were found to be c-Met addicted, and in particular some gastric adenocarcinomas [29] and a few lung cancers [30] all of which harbour c-Met amplification. More likely c-Met is part of a complex network in which multiple RTKs are coactivated in many tumors and redundant inputs drive and sustain downstream signalling [31].

RON AND ITS LIGAND MSP: THE STRUCTURE

The RON high affinity ligand is the macrophage-stimulating protein, MSP [32] an heterodimer that belongs, like HGF, to the plasminogen-prothrombin protein family. The plasminogen-prothrombin family is characterized by multiple kringle domains that are identified by triple disulfide loop structures in the N-terminal domain. MSP is synthesized mainly by hepatocytes, circulates in the blood as a biologically inactive single chain precursor pro-MSP [33], and it is cleaved at the Arg483-Val484 site by the members of the kallikrein family or by trypsin-like enzymes located on macrophage surfaces [33-35]. MSP is a 78-kDa heterodimeric protein composed of a disulfide-linked 53-kDa α -chain

and a 25-kDa β -chain. The α -chain contains a N-terminal hairpin loop followed by four kringle domains (Fig.1). The β -chain has a serine protease-like domain but, like HGF, is devoid of enzymatic activity due to amino acid substitutions in the catalytic triad. Several data have shown that MSP β -chain binds to RON with higher affinity than the N-terminal portions. Metabolically labeled free β -chain, but not α -chain, was specifically absorbed by cells expressing RON and MSP β -chain binds RON in intact cells in a specific and saturable manner [35]. These findings suggest that the MSP β -chain is the primary binding site for RON and represents the optimal site for the design of possible antagonist compounds.

The Recepteur d'Origine Nantais, RON, also known as stem cell-derived tyrosine kinase (STK) in mouse [36], is a member of the c-Met proto-oncogene family [37], with a 25% homology in the extracellular region and 63% in the tyrosine kinase domain. RON is a 185 kDa heterodimeric protein composed of a 35 kDa α -chain and a 150 kDa β -chain linked by a disulfide bond. The mature receptor, exposed on the cell surface, originates from the proteolytic cleavage of a single chain precursor [35]. As for c-Met, the α -chain, composed of 284 amino acids, is completely extracellular and contains part of the Sema domain which retains the ligand-binding activity [38]; the β -chain, which traverses the cellular membrane, has 1096 amino acids and comprises an extracellular sequence, a short transmembrane (TM) segment and a large cytoplasmic portion comprising the tyrosine kinase domain (TK) and a C-terminal regulatory tail (Fig. 1). The extracellular portion of the β -chain is composed of part of the Sema domain, a cystein-rich domain (PSI motif), and in four immunoglobulin-like structures (IPT domains). Similarly to c-Met, the TK and the C-terminal regulatory tail contain various tyrosine residues important for RON activities: Y1245 and Y1246 are the phosphorylation sites responsible for kinase upregulation while Y1360 and Y1367 are major signal transducer docking sites [39].

THE FUNCTIONS OF RON

Activation of RON by MSP can initiate a signaling through pathways analogous to those downstream c-Met. Like c-Met, also RON is required during embryo development: RON homozygous knockout mice die very early in utero during the peri-implantation stage [40]; heterozygous mice are vital and mature to adulthood but show altered inflammatory response [41]. Recently, Meyer et al. demonstrated a function for RON in the mammary gland: mice with a targeted deletion of the tyrosine kinase domain of RON had accelerated ductal elongation and significantly increased branching morphogenesis during pubertal mammary development [42]. RON is preferentially expressed in epithelial cell types and macrophage populations where it regulates cellular proliferation, adhesion, and motility. Like c-Met, RON mediates also EMT as well as protection from anoikis. Anoikis is a form of programmed cell death induced in anchorage-dependent cells when they detach from the surrounding extracellular matrix that provides essential signals for growth and survival. Madin–Darby canine

kidney cells engineered to over-express RON were protected from anoikis when stimulated with MSP [43,44]. The ability, mediated by RON, to promote escape from apoptosis has also been investigated in nonmalignant and malignant colon epithelial cells [45]. Further studies have also demonstrated that RON, following MSP treatment, is involved in invasion and cell motility in normal colon epithelial cells, in colon cancer cells and human breast carcinoma cells that overexpress RON. A fundamental role for RON as an oncogene has been assessed in transgenic mouse models over-expressing RON. Transgenic mice engineered to express RON in distal lung epithelium developed multiple pulmonary adenomas at 2 months of age, multiple tumor nodules at 4 months and respiratory failure by 14 months secondary to tumor progression from adenoma to adenocarcinoma [46]. In another transgenic model RON overexpression produced mammary tumors in 100% of the transgenic mice and distant metastatic foci in ~90% of the animals [47].

Regarding human tumours, RON has been found over-expressed and over-activated in cancers of different origin: colon [45], lung [48], breast [49], stomach [50], ovary [51], pancreas [52], bladder [53], liver [54] and kidney [55]. Besides, RON overexpression correlates with a decreased disease free survival in some human cancers like breast and bladder tumors [56]. As of now no somatic mutations in RON coding sequence have been identified in human cancers but a novel mechanism to activate RON receptor both in cancer-derived cell lines and in several human cancers types has been described: the generation of truncated RON receptors produced via alternative splicing or alternative initiation sites. The first RON splice variant, described in the human gastric cancer cell line KATO-III, lacks 49 amino acids in exon 11. This variant (named RON165) lacks 49 amino acids corresponding to exon 11, coding for three cysteines located in the extracellular domain of the β -chain and responsible for the establishment of intramolecular disulphide bridges and of consequent constitutive activation [57]. Other three RON splicing variants have been described, ROND160, ROND155, and ROND55 that show oncogenic potential [58]. Another RON variant known as short form RON originates from an alternative start site in intron 10. This form has been found in human breast and ovarian cancers as well as in many different cancer-derived cell lines where its expression increases proliferation, motility and anchorage independent growth [59].

C-MET AND RON COOPERATION

Another interesting feature of c-Met is that it interacts dynamically with many other cellular receptors. Many molecules have been proved to be c-Met partners: Epidermal Growth Factor Receptor (EGFR), RON, ErbB2, integrin $\alpha 6\beta 4$, the transmembrane receptors for hyaluronic acid CD44 and the group of B plexins. The crosstalk between c-Met and other membrane partners modulates the activation of c-Met and allows for the integration of the signals that drive cellular responses. For example the activation of the RAS-MAPK and PI3K-AKT pathways is reinforced when the c-Met receptor is activated together with EGFR [60]. Recently the interaction between these two receptors has gained a lot of

interest because of its importance in physiological and pathological processes [61-63]. In non-small cell lung cancer (NSCLC) patients, amplification of c-Met has been implicated in the development of acquired resistance to anti-EGFR chemotherapies therefore the therapeutic combination of EGFR inhibitors and c-Met inhibitors will be an attractive alternative to treat this incurable illness [64]. c-Met also interacts with RON [39]. It was confirmed that ligand induced c-Met activation results in RON trans-phosphorylation and vice versa. The trans-phosphorylation occurs in a direct way, as it does not need the C-terminal docking site of either receptors and the RON kinase inactivation is sufficient to block c-Met transforming activity [39]. These data show that, while specific for their ligands, Scatter factor receptors cross-talk and combine forces to generate specific intracellular signaling pathways. Furthermore, concomitant activation of RON and c-Met synergistically increases invasive growth, and inactive RON decreases the signal of the c-Met receptor. While c-Met can regulate invasive growth and transformation [16], RON is able to promote invasive growth but not transformation [65].

C-MET AND RON SIGNALING INHIBITORS

Tumor invasion and metastasis are the main cause of death for all cancer patients. Therefore, there is the need to find therapeutic approaches direct to impair the metastatic spread. c-Met and RON, and their respective ligands HGF and MSP, have been shown to stimulate development, growth and metastatic process in a wide variety of human cancers. Thus, these receptors represent very attractive therapeutic targets for the large amount of cancer patients that would benefit from their pathway inactivation. In fact, in the last few years, several scientists, both in public institutions or private research companies, have tried to develop compounds aimed to interfere and abrogate c-Met and RON signaling pathways.

The strategies adopted to create compounds that neutralize the activities of c-Met and RON can be divided into four different groups. 1) receptor competitors; 2) ligand antagonists; 3) small molecules kinase inhibitors; 4) molecules that silence c-Met and RON expression (Table 1).

Group 1 contains molecules that bind to the receptors, competing with their ligands, but are unable to activate downstream signaling. Usually, these compounds bind to the extracellular portion of the receptor, thus impairing ligand binding and the subsequent receptor dimerization and activation. NK4 was the first identified c-Met receptor competitor. This compound corresponds to an HGF truncated form lacking the serine protease domain (SPD), and is composed of the HGF N-terminal region and four kringle domains (Fig. 2a). NK4 competes with HGF for c-Met binding, but fails to activate it, thereby blocking c-Met downstream pathways and biological functions. In particular,

mouse xenografts experiments demonstrated that this molecule strongly inhibits tumor growth, invasion, metastasis and angiogenesis [9,59].

Group 1 contains also three NK1 mutant molecules. NK1 is an alternative spliced variant of HGF comprising the N-terminal (N) portion and the first kringle (K1) domain. NK1 crystallizes as an head-to-tail dimer with an extensive inter-protomeric interface resulting from contacts between the two short inter-domain linkers and reciprocal contacts between the N and K1 domains of each monomer (Fig. 2b). Despite NK1 retains partial agonistic activity on c-Met, Gherardi and colleagues have engineered new NK1 variant with potent antagonistic capabilities [66]. This engineered NK1 has strong prospects of becoming a therapeutic agent for human cancers because of its simplicity to be produced and its high affinity for the c-Met extracellular domain. A subset of mutants in the NK1 dimer interface, such as the linker mutants Y124A or N127A (Fig. 2c) and the kringle mutant V140A; I142A (Fig.2d), bind c-Met with affinities comparable to the wild-type NK1, but fail to assemble an active dimeric NK1/c-Met complex (Fig.2e). *In vitro* experiments showed that NK1 variants have no detectable agonistic activity and behave as bona fide receptor antagonists blocking cell migration and DNA synthesis [67]. The possible mechanisms by which linker mutants act as receptor competitors could be explained both by the disruption of the dimer interface or by blocking the domain swapping associated to the NK1 dimeric structure. The domain swapping is a mechanism for forming oligomeric proteins starting from the monomer conformation: in the case of a dimer like NK1, the two monomers of the same molecule convert their conformation from a closed one to an open one able to dimerize (Fig.3a, modified from [68]). Each NK1 monomer is composed of two domains (N and K1) connected by a flexible polypeptide linker called hinge loop (Fig. 3b). The conformation of NK1 as a monomer is referred to as closed conformation. The two closed monomers of NK1 can change their conformation from a closed to an open one (Fig. 3c) and dimerize by the mechanism of domain swapping. The result is a dimer in which one domain of each monomer is replaced by the identical domain of the other monomer. The only residues that differs between the open and the closed conformation are the one of the hinge loop (see Fig. 3b), therefore introducing mutations in this region would be optimal to produce a NK1 monomer unable to change its conformation and consequently unable to assemble as a dimeric active structure.

Another c-Met receptor competitor is an uncleavable pro-HGF having a single aminoacid substitution (Arg489Gln) in the proteolytic site (Fig. 3d) [69]. This molecule has been developed by inserting a mutation that prevents the maturation of pro-HGF. The generated new protein is capable of blocking all c-Met induced biological responses. The compound acts in a dual manner: firstly, it competes with the endogenous pro-HGF for the catalytic domain of the enzymes that processes this precursor thus inhibiting endogenous pro-HGF processing and maturation; secondly, it binds to the receptor with high affinity, displacing the mature ligand, thus impairing HGF induced activation. Evidence

has proved that both local and systemic expression of uncleavable HGF inhibits tumor growth, impairs tumor angiogenesis and prevents metastatic colonization [70].

Monoclonal antibodies directed against c-Met are also considered receptor competitors. Following the binding, these antibodies block receptor activation either by preventing the ligand binding or by promoting receptor down-regulation. Recently, Petrelli and co-workers showed that a monoclonal antibody (DN30) directed against the c-Met extracellular portion induced receptor “shedding”, thus preventing c-Met activation and abrogating its biological activity [71]. The mechanism through which DN30 efficiently downregulates c-Met is via the proteolytic cleavage of the extracellular portion. This results in “shedding” of the ectodomain, and cleavage of the intracellular domain, which is successively degraded by the proteasome machinery. Kong-Beltran’s lab has developed an anti-Met 5D5 antibody that binds c-Met Sema domain and inhibits HGF binding. The Fab fragment of 5D5 (anti-Met 5D5-Fab) has been shown to inhibit HGF-driven c-Met phosphorylation, cell proliferation, and tumor growth [72].

Two different monoclonal antibodies targeting RON have been developed as receptor competitors. IMC-41A10 [73] is a human immunoglobulin G1 (IgG1) antibody that binds RON with high affinity and effectively blocks the interaction with its ligand, MSP. IMC-41A10 is a potent inhibitor of the receptor downstream signaling, cell migration, and tumorigenesis. This antibody antagonizes MSP-induced phosphorylation of RON, MAPK and AKT in several cancer cell lines. In HT-29 colon, NCI-H292 lung, and BXPC-3 pancreatic cancer xenograft tumor models, IMC-41A10 inhibits tumor growth as a single agent, and in BXPC-3 xenografts, it leads to complete tumor regression when combined with Erbitux. This is the first demonstration that a RON specific competitor negatively affects tumorigenesis [73]. Another antibody directed against RON is 2F2 [74]. This antibody binds to a specific epitope on the RON extracellular domain. This binding induces RON internalization and down-regulation of its signaling pathways. 2F2 treatment significantly prevents tumor formation and growth in mice injected with RON Delta160. 2F2 treatment also impairs the growth of human colon or breast tumor xenografts models [73].

The second group of c-Met and RON signaling inhibitors is represented by the ligand antagonists, molecules created to specifically bind the ligand and prevent its interaction with the receptors. The extracellular Sema domains of c-Met and RON are involved in the ligand binding, thus, recombinant Sema proteins produce down-regulation of the c-Met and RON downstream signaling [72,75]. Another ligand inhibitor of c-Met signaling, recently developed by Genentech [76], is a disulfide-constrained 15-mer peptide (VNWVCFRDVGCDWVL), termed HB10 (Fig.3e), selected from a pool of disulfide constrained random peptide phage library. This peptide binds to the recombinant human HGF β -chain and competitively inhibits c-Met binding. In MDA-MB435 cells, HB10 reduces HGF-dependent c-Met, AKT and MAPK phosphorylation as well as HGF-dependent migration. The 2D (1)H-NMR structure of HB10 revealed a β -hairpin loop stabilized by the disulfide bond and cross-strand pairing of Trp3 and Trp13 (Fig. 3e). A mutation in the

HGF β -chain with disrupted c-Met binding shows also reduced HB10 binding, suggesting an overlapping binding site (Fig. 3f).

Another interesting approach to prevent c-Met activation using ligand antagonists is a soluble extracellular c-Met receptor named “decoy Met”. This molecule is capable of preventing both ligand binding and receptor homodimerization with similar results to the recombinant Sema domain [69].

Neutralizing anti-HGF antibodies are also classified as ligand antagonists. Fully human monoclonal antibodies, at subnanomolar concentrations, can bind and neutralize human HGF, inhibiting HGF-mediated receptor phosphorylation, cellular proliferation, survival, and invasion. *In vivo* these antibodies are able to stop HGF-dependent tumor growth and cause tumor regression [77,78].

An alternative strategy to block the action of tyrosine kinase receptors is to use small molecules tyrosine kinase inhibitors (RTKi). These inhibitors are low molecular weight molecules able to compete for the ATP binding site (Fig. 3g) of the receptor preventing receptor transactivation and recruitment of downstream effectors. The kinase domain is composed of a conserved ATP binding site situated between the two N- and C-terminal lobes joined by an hinge region. The C-terminal lobe contains a mobile activation-loop (A-loop) segment, which includes tyrosines that must be phosphorylated in order for the kinase to be activated. The majority of kinase inhibitors form hydrogen bonds to residues in the hinge region thus targeting the ATP binding site of the kinase. These inhibitors are known as type I or type II inhibitors depending on whether they bind to the kinase active conformation (with a phosphorylated A-loop) or an inactive conformation [79,80]. To date more than 70 patent applications on c-Met inhibitors are published. K252a, PHA-665752, SU11274 and PF-2341066 were the first developed RTKi compounds. K252a was the first c-Met inhibitor to be described. This staurosporine-like inhibitor hampers c-Met-induced biological activities if used at sub micromolar concentrations. K252a exhibits its activity against a broad spectrum of kinases and has been the first structure to be solved in complex with the unphosphorylated c-Met kinase domain[80]. PHA-665752 and SU11274, more selective c-Met inhibitors with improved cellular potency and activity in xenograft models, were then identified. *In vitro* and *in vivo* studies showed that PHA-665752 represses both HGF-dependent and constitutive c-Met phosphorylation, resulting in abrogation of the main biological phenotypes elicited by this receptor [81]. These inhibitors were very helpful to validate c-Met as a target in a subset of gastric carcinoma patients [29] but poor pharmacokinetic properties prevented further development of these compounds. PF-2341066 was designed using information from the crystal structure of the c-Met kinase domain in complex with PHA-665752. PF-2341066 is the first orally bioavailable anti-Met RTKi. This RTKi is selective for c-Met compared with a panel of more than 120 diverse tyrosine and serine-threonine kinases and potently inhibits c-Met phosphorylation and c-Met-dependent proliferation, migration and invasion of many human tumor cells *in vitro*. *In vivo* PF-2341066 shows a dose dependent

antitumor efficacy in multiple xenograft tumor models, with reduced tumor cell proliferation, induction of apoptosis, and reduction of microvessel density [82]. Since PF-2341066 release many other patent applications featuring RTKi targeting c-Met have now been filed and multiple orally available molecules have now reached clinical trials (see below).

A ultimate strategy to specifically block RTKs is to reduce the number of receptor molecules exposed on the cellular surface by a recently developed technique: RNA interference (RNAi) or post transcriptional gene silencing (PTGS). RNAi is a procedure that uses short RNA nucleotides (21-23 bases long), called short interfering RNAs (siRNAs), to cleave an homologous target messenger RNA. A well established cell machinery uses the siRNAs, that may be easily transfected into the cells, as a template to find an homologous mRNA and hydrolyze it. An alternative route to activate the degradation of the target mRNA is to infect cells with virus particles carrying an expression cassette that produces short hairpin RNAs (shRNAs), molecules composed of two complementary siRNAs separated by an short hairpin [83,84]. Adenoviral vectors were used to reduce c-Met expression and signaling in cancer cell lines of different origins with impaired cell proliferation, viability, scatter and invasion *in vitro*, and a substantial reduction of tumor growth *in vivo* [85]. c-Met silencing in embrional and alveolar rhabdomyosarcoma cell lines using shRNAs expressed by lentiviral vectors under an inducible promoter affects cell growth, survival, and invasion *in vitro* and promotes a decrease in tumor growth *in vivo* [86]. On the other way Xu et al. [87] have developed siRNAs targeting RON to study the oncogenic capacity of this receptor in colorectal cancer cells. Silencing RON by RNA interference in HT-29, HCT116, and SW620 colorectal cancer cells significantly reduces proliferation, motility, and increases apoptotic cell death as well as impaired tumor growth *in vivo*. These results demonstrate that altered RON expression in colon cancer cells is required to maintain their tumorigenic phenotypes, thus, silencing RON expression could be a potential therapeutic approach to treat colon tumors *in vivo*.

Two major issues remain to be resolved before RNA interference will be useful in clinical applications: first, PTGS cannot achieve a complete knock-out of the targeted protein therefore signal amplification mediated by receptor tyrosine kinases, following ligand stimulation, may still drive cellular responses (i.e. proliferation or protection from apoptosis); second, RNAi (via short interfering RNAs or viral vectors carrying shRNAs) still requires optimization in the delivery procedures and it is still not an accepted therapeutic option in humans.

ADVANCED CLINICAL TRIALS IN CANCER THERAPY

The acceptance of c-Met as a tractable target for cancer therapy has fostered intensive drug discovery efforts across the pharmaceutical industry. The strategies to efficiently block c-Met activity *in vivo* fall mainly in two categories: a) c-Met kinase inhibitors; b) anti-HGF or anti-Met monoclonal antibodies. Clinical trials investigating both small molecules

inhibitors and monoclonal antibodies directed at the HGF/SF-Met axis are currently under way (Table 2). Almost all these molecules have now reached Phase II in clinical trials and 2 selective c-Met inhibitors are now in Phase III (PF-2341066 and XL184).

Small Molecule Inhibitors

Many products of this category have reached clinical trials up to Phase III (see table 2). These molecules can be divided in two major subgroups: highly selective c-Met inhibitors (ARQ197, JNJ-38877605, PF-2341066 and SGX523) and broad spectrum multikinase inhibitors (BMS-777607, GSK1363089, MGCD265, MK-8033 and XL184).

It will be important to assess whether selective c-Met inhibition or more broad spectrum inhibitory activity is to be preferred. It will also be crucial to determine whether single agent therapy or a combination with other systemic treatment options (different targeted agents, radiotherapy, cytotoxic chemotherapy) will result in better antitumor activity.

ARQ197 is an orally administered highly selective small molecule inhibitor that binds to c-Met in a region close to the ATP binding site. Multiple phase I and II trials on this molecule are now active (<http://clinicaltrials.gov/ct2/results?term=ARQ197>). Phase I dose escalation studies demonstrated a good tolerability of the drug with mild anti-tumor responses [88,89]. ARQ197 is also studied in an ongoing Phase 2 clinical trial in MiT (Microphthalmia Transcription Factor)-associated tumors, NSCLC and pancreatic adenocarcinomas. Recently the European Medicines Evaluation Agency (EMA) has designated ARQ197 as an orphan medical product (drugs used to treat illnesses affecting small number of patients) for the treatment of soft tissue sarcomas.

BMS-777607 is a ATP competitor with nanomolar activity against c-Met, but also active on RON and Axl. This molecule demonstrated promising effects on pre-clinical models of human gastric tumors (GTL-16 cells) and prostate tumors (PC-3 and DU145 cell lines) [90] and is now in a Phase I/II trial in patients with advanced or metastatic solid tumors (<http://clinicaltrials.gov/ct2/results?term=BMS-777607>).

GSK1363089 (Foretinib) is an orally available, broad spectrum c-Met inhibitor (0.4 nmol/L) with a very good activity on RON activity (3 nmol/L) [91]. Phase I trials indicated that the drug is well tolerated although hypertension was shown as a common side effect. GSK1363089 showed an anti-tumor activity in phase II studies on patients with papillary renal carcinomas [92] and c-Met amplified gastric cancers [93]. Foretinib is now also tested in other solid tumors and advanced hepatocellular carcinomas (<http://clinicaltrials.gov/ct2/results?term=GSK1363089>).

JNJ-38877605 is an orally available selective c-Met inhibitor that exhibited good antitumor activity in preclinical models [94]. This drug is now in a phase I clinical trial in patients with advanced or refractory solid tumors (<http://clinicaltrials.gov/ct2/results?term=JNJ-38877605>).

Developed by Methygene, MGCD265 is a tyrosine kinase inhibitor that targets both c-Met and RON, together with VEGFR-1/2/3 and TIE-2. Its safety, pharmacokinetics and antitumor effects are now studied in Phase I and II clinical trials in advanced malignancies and NSCLC (<http://clinicaltrials.gov/ct2/results?term=MGCD265>) [95].

Merck has produced an anti-Met compound, MK-8033, with an inhibitory activity also on RON. Either alone or in combination with other care agents MK-8033 induced tumor growth regression in gastric cancer and NSCLC cell lines. A single phase I trial is now active (<http://clinicaltrials.gov/ct2/results?term=MK-8033>) [96].

PF-02341066 is one the first orally available selective anti-Met inhibitor. This drug showed good antitumor efficacy in multiple preclinical tumor models [97]. PF-02341066 is one of the two anti-Met drugs that has reached phase III in a clinical trial in patients with advanced NSCLC. Four other phase I and/or II trials that aim to understand the effect of this compound in different tumors (anaplastic large cell lymphoma, lung cancer) are also active (<http://clinicaltrials.gov/ct2/results?term=PF-02341066>).

SGX523, available orally, has demonstrated a potent antitumor activity in xenograft models of glioblastoma, lung cancer and gastric cancer [98]. This molecule is a selective c-Met inhibitor with other 1.000 fold selectivity over a panel of 213 other protein kinases. The safety of this molecule has been examined in two phase I clinical trials (<http://clinicaltrials.gov/ct2/results?term=SGX523>). The Phase I clinical trial was halted due to a reversible acute renal failure in one dose group caused by a metabolite compound crystallizing in the kidney.

XL184 is the only other orally available inhibitor in a phase III clinical trial (in medullary thyroid cancer; <http://clinicaltrials.gov/ct2/results?term=XL184>). This drug is a broad spectrum inhibitor that targets, besides c-Met, also VEGFR-2, KIT, RET, FLT-3 and TIE-2. Data from Phase I trials indicated that XL184 is well tolerated and has a long terminal half life [99]. Six phase I and/or II trials covering different cancer subtypes (NSCLC, glioblastoma, glioblastoma multiforme) are now active.

Monoclonal antibodies

Currently there are only two companies producing monoclonal antibodies that target either HGF or c-Met. AMG102 is a fully human IgG2 monoclonal antibody against HGF that prevents tumorigenesis in preclinical models through blockade of the HGF/c-Met pathway. In a Phase I clinical trials as a single agent showed little adverse effects and no dose limiting effect [100]. AMG102 is now tested in Phase I/II trials in combination with other chemotherapies (platinum, Metoxantrone, ECX and panitumumab) and as a single agent in Phase II trials to treat specific cancers (malignant glioma, renal cell carcinoma, ovarian epithelial cancer, fallopian tube cancer, primary peritoneal cancer; <http://clinicaltrials.gov/ct2/results?term=amg102>).

MetMAB was engineered as a recombinant, humanized monoclonal antibody produced in *E. coli* that acts as an antagonist of HGF-induced c-Met signaling. Preclinical assays indicated that MetMAB was active against different HGF-driven tumor models [101,102]. Phase I clinical trial to test safety and pharmacology of MetMAB in patients with advanced metastatic solid tumors showed that the antibody was well tolerated [103]. MetMAB is now tested in a Phase II clinical trial in patient with advanced NSCLC, in combination with Tarceva (an EGFR inhibitor; <http://clinicaltrials.gov/ct2/results?term=MetMAB>).

SUMMARY

An understanding of the molecular events that occur upon cancer initiation, progression and spread is evolving. c-Met and RON tyrosine kinase receptors have been shown to be over-expressed and over-activated in a large cohort of human cancers and their role in human oncogenesis has been extensively proven. We have focused the attention to the great progress made on the analyses of structural and functional features of these two receptors. We have showed that c-Met and RON are now considered very attractive targets for cancer therapy and how their crosstalk is possibly required for tumorigenesis and is the key to therapeutic resistance.

CONCLUSIONS

Tumor establishment, growth and metastasis in many cancers is likely to be driven by multiple, concomitantly activated, signaling pathways. c-Met and RON might act as redundant oncogenes whereby inhibition of one will allow the cell to “switch” to the other in order to amplify downstream signals and sustain cell survival. Thus, rather than administering single-target anti-cancer drugs, a multi-targeted therapeutic strategy will be required for favourable patient response. The development of therapies that eliminate the potential for compensatory crosstalk between cancer signaling pathways is required to produce treatments that are more effective than single-target therapies.

It has to be taken into account that, however, inhibitors that target multiple kinases have the potential risk of offtarget toxicity and non-optimal inhibition of one of the kinases may lead to reduced clinical response and the emergence of drug resistance. Optimisation of these compounds remains a considerable challenge, and, additionally, multi-target inhibitors will not provide validation for individual targets. The development of selective, single-target kinase inhibitors is becoming increasingly feasible as more is understood about the structure and molecular mode of action of kinases. Some researchers propose that the use of single-targeted kinase inhibitors in combination with cytotoxic agents should avoid off-target activity and allow maximal dosing. This approach is not without disadvantages, however, as drug–drug interactions and an accumulation of previously unnoticed sub-clinical toxicities may develop. Additionally, complications arising from intellectual property and liability may arise if the two therapeutic entities are owned by

different organisations. Consequently, there is still considerable debate as to whether single- or multiple-targeted kinase inhibitors are desirable as the next generation cancer therapies.

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LEGENDS

Fig.1 Structural features of c-Met and RON receptors and their ligands. c-Met and RON tyrosine kinases are disulphide-linked heterodimers made of a single-pass transmembrane β -chain and a completely extracellular α -chain. The extracellular region contains a Sema domain, a cysteine-rich domain called c-Met-related sequence (MRS), and four immunoglobulin-like structures (IPT domains). The intracellular portion of the receptor is made of a juxtamembrane portion followed by a catalytic site and a C-terminal regulatory portion. c-Met and RON ligands, HGF and MSP respectively, contain a hairpin loop (N), followed by four kringle domains (K1–K4), flanked by an activation portion and a serine protease domain devoid of proteolytic activity.

Fig. 2 A) NK4 fragment of HGF is composed of the N terminal domain and four kringle domains. B) Crystal structure (1NK1, PDB code) of NK1 fragment of HGF: the head to tail dimeric structure of NK1 is represented in licorice and surface representations viewed from the N- and C-terminal side. Each NK1 monomer is shown in two different colors, green and blue. All the images of this panel show a large interprotomeric interface between the two NK1 monomers. It is also evident how the C-terminal arms of both monomers embrace each other, further stabilizing the dimeric structure of NK1 (graphics processing using Pymol). C) New cartoon representation of the NK1 dimeric structure. The two monomers of the crystal structure are represented by two different colors, red and blue. In CPK are represented the two residues of the linker (Y124 and N127) that have been mutated in order to disrupt the dimer interface (graphics processing using VMD). D) In CPK the two residues of the kringle1 domain are (V140 and I142) that have been mutated in order to disrupt the dimer interface. E) Scheme of the mechanisms by which the disruption of the dimer interface, with the insertion of mutations in the linker or kringle domain at the interprotomeric interface, results in an inactivation of c-Met signaling. The first panel shows a possible mechanism of activation of c-Met by NK1, mediated by heparin molecules. In the second panel a possible mechanism by which NK1 mutants bind to the receptor ~~is shown~~, through the interaction with heparin molecules.

Fig.3: A) Schematic representation of the domain swapping definition (modified picture from [100]): closed monomers are composed of structural domains linked by a polypeptide called hinge loop. The interface between domains in the closed monomers is referred as closed interface. Closed monomers can get an open conformation and dimerize by domain swapping. In the swapped dimer the interprotomeric interface is the same of the interface of the closed monomer besides the fact that each interface is made between a domain from one subunit (black) and a domain from the other ~~subunit~~ (gray). The only residues whose conformations significantly differ between the closed and the open conformation are the one of the hinge loop. B) Graphic representations of the two possible conformations of the hinge loop of NK1: a model of the conformation of the hinge loop of the closed NK1 monomer (blue), the conformation of

the linker in the open NK1 (green) and the superpose of the two possible conformations of the hinge loops. C) Graphic representation of the superpose of the monomers of NK1 in the open conformation (green) and in the model of the closed conformation (blue). In detail, it is clear the perfect superpose of the N-domain and how the Kringle domain could be positioned with respect to the N-domain. It is important to consider that in the case of the closed form of NK1 the interaction between the N and K1 domains are the same that occurs between the N-domain and the K1 domain of two different monomers of NK1, like in the crystal structure. D) Uncleavable pro-HGF carrying the mutation R489Q. E) Solution structure of the HB10 peptide developed by Genentech shown with the side-chains of its constituent aminoacids [70]. F) Graphic representation of the interaction, between the Sema domain of c-Met and the SPD domain of HGF. The first panel represents the crystal structure (1SHY) of the complex between the Sema domain of c-Met and the SPD domain of HGF. The second panel represents the enlargement of the interaction between of Glu221 of the Sema domain of c-Met with the catalytic site of the inactive SPD domain of HGF. It has been shown, by point mutations experiments, that HP10 binds to the SPD domain in the region surrounding the cavity of the catalytic site where the Glu221 of c-Met is inserted when HGF interact with its receptor. In blue and surface representation, the SPD domain of HGF and in CPK green the Glu221 of c-Met and how it is positioned in the cavity of the SPD domain. G) Graphic representation of the crystal structure (2WGJ) of the complex of the novel RTK inhibitor PF-2341066 represented in green with c-Met kinase domain represented as surface in red. This image shows the kinase inhibition by ATP competition: PF-2341066 perfectly occupies the cavity of c-Met kinase that binds to the ATP when the receptor is activated, thus inhibiting its action. All graphics processing using VMD.

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