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Abstract

Bone cancers are characterised by the development of tumour cells in bone sites, associated with a dysregulation of their environment. In the last two decades, numerous therapeutic strategies have been developped to target the cancer cells or tumour niche. As the crosstalk between these two entities is thightly controlled by the release of polypeptide mediators activating signaling pathways through several receptor tyrosine kinases (RTKs), RTK inhibitors have been designed. These inhibitors have shown exciting clinical impacts, such as imatinib mesylate which has become a reference treatment for chronic myeloid leukaemia and gastrointestinal tumours. The present review gives an overview of the main molecular and functional characteristics of RTKs, and focuses on the clinical applications that are envisaged and already assessed for the treatment of bone sarcomas and bone metastases.

1. Introduction

To be able to play their physiological role (intra- and inter-cellular signal transmission, adaptation to changes in the microenvironment), cells must be able to receive, integrate and respond to numerous extracellular messengers. These communications between cells and their environment are made possible through the attachment of molecules considered as messengers to their receptors, identified as effectors (cytokines, growth factors, etc). As proposed by Ehrlich in 1910, “to act, a substance must be fixed." These receptors are essentially located at the cell membrane, although there are also intra-cytoplasmic receptors such as steroid hormone that can be translocated into the nucleus to regulate expression of numerous genes. Membrane receptors possess: (i) an extracellular hydrophilic domain, often glycosylated, which recognises the ligand; (ii) a hydrophobic trans-membrane domain that makes embedding possible within the lipid bilayer of the plasma membrane; and (iii) an intra-cytoplasmic domain dedicated to signal transduction within the cell. The binding of a ligand to its receptor is specific, reversible and involves a large number of low-energy bonds (hydrogen, ionic, hydrophobic, Van der Waals). Thus, at equilibrium, the dissociation rate is equal to the rate of association. Among the receptors of cytokine/growth factors, six types of receptor have intrinsic enzymatic activity (kinase or phosphatase receptors, and guanylyl cyclase-coupled receptors) or not (the G protein-coupled receptors, the receptor-type "channel", and cytokine receptors).

The ***guanylyl cyclase-coupled receptors*** include natriuretic peptide, nitric oxide, carbon monoxide and enterotoxin receptors. The binding of the ligand to the extracellular domain of its receptor leads to intracellular activation of the guanylate cyclase domain of the receptor chain, and to synthesis of a cyclic GMP for activating the cAMP-dependent protein kinase environment [1]. The ***G protein-coupled receptors*** are characterised by seven transmembrane domains. The trimeric G proteins located on the cytoplasmic side of the cell membrane

transduce and amplify cell signalling through the production of cyclic AMP. The chemokine receptors are included in this family environment [2]. The ***ion channel linked receptors*** are ligand-dependent ion channels and their opening or closing activities are associated with the nature of the ligand. These receptors can be ionotropic or metabotropic. In the first case, the receptor is actually the pore, and opens following a conformational change made possible by the ligand binding. On the contrary, in the case of metabotropic receptors, ligand-stimulated receptors activate a ligand-independent channel through the intracellular effector environment [3]. ***Cytokine receptors*** can be divided into four groups: i) receptors with an immunoglobulin-like ectodomain (IL-1α/β, IL-18); ii) the trimeric members of the TNF receptor superfamily (which include, for instance, RANK, TRAIL receptors, TNF receptors-α/β); iii), class I-cytokine receptors (or haematopoietin receptors) environment [4] and iv) class II-cytokine receptors (or interferon and IL-10 receptors) [5]. Class I/II- cytokine receptors have oligomeric structures, where a specific α-chain warrants specific ligand recognition, while one or two channels (β/γ) are used for signal transduction. For instance, the receptors of interleukins (IL) 2, 4, 7, 9 and 15 consist in a specific chain to the cytokine, and the shared IL-2 γ-receptor chain, IL-2 and IL-34 also share a β-receptor chain environment [6]. Similarly, the IL-6 cytokine family (IL-6, IL-11, CNTF, OSM and LIF) shares the gp130 receptor chain environment [7]. Among the cytokine receptor families, some are characterised by intrinsic kinase activity and consequently by their ability for autophosphorylation. They form the receptor tyrosine kinase (RTK) family.

All of these receptors tightly control tissue homeostasis, and any dysregulation of these ligand-receptor systems (mutations, overexpression, etc) disturbs cell communication and leads to pathological situations. Bone formation and bone remodelling are then controlled by a large panel of cytokines and growth factors regulating the dialogue between osteoblasts, osteoclasts and their environment [8]. It has been recognised that cancer cells (bone sarcomas,

metastatic cells originating from carcinomas) dysregulate the balance between osteoblasts and osteoclasts, activate osteoclastogenesis and then stimulate bone resorption. Consequently, activated osteoclasts resorb the extracellular bone matrix and release numerous growth factors entrapped in the organic matrix, which stimulate in turn the proliferation of cancer cells. Based on these observations, numerous chemical drugs have been developed to specifically target the various receptor tyrosine kinases activated by mutations, or by the ligands present in the tumour microenvironment. The present review summarises the classification, structure and mechanism, and focuses on the targeting of action of the receptor tyrosine kinases. Their use in the treatment of bone cancers (bone sarcomas and bone metastases) is described and discussed.

2. The receptor tyrosine kinase (RTK) family

2.1. Classification and structure of RTKs

Protein kinases are key enzymes in the regulation of various cellular processes that catalyse the transfer of a phosphate group from ATP to a hydroxyl group of a serine or a threonine. Among the 90 identified genes encoding proteins with tyrosine kinase activity, 58 encode receptors divided into 20 subfamilies [9, 10] (Table I). Of these subfamilies, EGFR / ErbB (class I), the receptor for insulin (class II), for PDGF (Class III), for FGF (class IV), for VEGF (class V) and HGF (MET, Class VI) are strongly associated with oncological diseases. These RTKs are characterised by a single trans-membrane domain and a glycosylated N-terminal extracellular domain with a high number of disulfide bonds. This extracellular domain is involved in the dimerisation process of the receptors, and consequently in ligand recognition (Figure 1). The composition of these domains (immunoglobulin domains, rich in leucine, lysine and cystein, fibronectin type III domain, etc.) depends on the classes of RTKs and then defines the specificity of the ligands. The RTKs are inserted into the cell membrane

thanks to an α-helix trans-membrane domain composed of 20 amino acids. The trans-membrane domain plays a key role in the formation and stabilisation of the dimer of the receptor chains. In the lipid environment of the cell membrane, the α-helix are non-covalently oligomerised [11] (Figure 1). This type of process makes it possible to pre-dimerise the RTKs in the cell membrane capable of interacting with the corresponding ligand [12].

The cytoplasmic domain harbours a specific domain with tyrosine kinase activity that is involved in the catalysis of the ATP-dependent phosphorylation of receptor chains. It includes two domains: a juxtamembrane region composed of 40 to 80 amino acids corresponding to the tyrosine kinase domain and a carboxy-terminal region. The tyrosine kinase domain is composed of 12 subdomains organised into two lobes, connected by the kinase insert domain (subdomain V) (Figure 1). The tyrosine kinase domain includes an activation loop, whose orientation (and phosphorylation) determines the active or inactive state of the kinase domain. The ATP required for kinase activity is housed between the two lobes. The small lobe (named lobe N, for N-terminal, subdomains I to IV), composed of β-sheets and one α helice, binds, stabilises and orients the ATP previously complexed with $Mg2^{+}$ ions. The large lobe (named C, for C-terminal, subdomains VI to IX) is mainly composed of α helices, and plays a part in the chelation of ATP by $Mg2^{+}$ ATP. It then binds the protein substrate containing the tyrosine target and catalyses the transfer of the phosphate group from the ATP to the receptor chains [13]. The size of the tyrosine kinase domain is relatively constant between the different RTKs. On the contrary, the size and content of the juxta- and C-terminal domains, vary considerably between the RTK families, conferring the specificity of intracellular signals. For instance, the intracellular domain of PDGFRβ has 552 amino acids, the intracellular domain of EGFR has 542 amino acids, while the FGFR1 shows 425 and TrkA only 356 amino acid residues. The number of tyrosine residues (phosphorylable or not) and their distribution vary significantly between the RTKs. Thus, 27 tyrosine residues are detected for the PDGFRβ (of which 19 can

be phosphorylated) and only 11 tyrosines can be detected in TrkA (with 6 phoshorylable tyrosines) (Bradshaw *et al*. 2013). However, a pair of tyrosine residues phosphorylated after RTK activation is found in the activation loop and is required for the functionality of the receptor. The activation of these tyrosine residues stabilises the "open" conformation of the activation loop and both lobes, and also allows the ATP and peptidic substrate environment to bind [13]. An additional, third tyrosine amino acid (located in a close upstream domain) participates in the conformational change of the activation loop. All the mutations on these tyrosine residues result in inactivation of the receptor chains. EGFR is an exception in the RTK families and it has only one tyrosine residue at this position, which is not essential for receptor chain activation and function.

2.2. General mechanism of action

It is admitted that the binding of a dimeric ligand to its receptor chains increases the proximity or/and stabilises the receptor chains that will be then auto-phosphorylated through their kinase domains (a process called trans-phosphorylation). This non-covalent dimerisation is associated with conformational changes that lead to the activation of the cytoplasmic kinase domains of the receptors. In most cases, one of the two receptor chains will trans-phosphorylate specific cytoplasmic tyrosines from the other monomeric chain environment [14]. In some cases, the constitutive form of the RTKs is a dimer such as insulin receptors. In addition, some ligands such as EGF are monomeric, and their binding to their receptor induces a conformational change that shifts the intra-molecular loop and exposes a binding domain in the receptor that results in its dimerisation environment [15]. In others, the dimerisation of the ligand is required to activate the receptor chain (*i.e.* the NGF - TrkA system environment 16].

In the absence of the ligand, the activation loop self-regulates activation of the receptor because its “closed” conformation inhibits catalytic activity (*cis*-inhibition). Dimerisation of the RTK chains following ligand binding induces the rotation of the N- and C- lobes, as well as the major axis of the protein. The activation loop, which is masked by its tyrosine residues, the ATP binding site, moves to enable ATP binding and the autophosphorylation of tyrosine residues located on the opposite receptor chain. The *trans* phosphorylation of key tyrosine residues located in the activation loop stabilises the “open” conformation, and breaks the binding between these tyrosines and the binding sites to the protein substrates, making it possible to access the C lobe, then activating its kinase activity. In addition, other tyrosine residues are phosphorylated by protein kinases previously recruited on the phosphorylated tyrosines of the RTK environment [17]. Several molecular “brakes” in kinase activity have been developed to limit phosphorylation levels. These molecular domains are located in the activation loop, in the juxtamembrane domain (KIT, PDGFR) or in the C-terminal domain (*i.e.* Tie2). In the last two cases, these molecular repressions will be removed by *cis*-phosphorylation of the RTKs during the ligand binding-induced conformational changes [18]. Phosphorylation of the catalytic domain of the RTKs activates and increases the activity of the kinase domain, whereas the non-catalytic domains create various anchoring sites for cytoplasmic targets involved in intracellular signal transduction. These tyrosines are mostly located on the juxta-membrane and C-terminal domains, and at the insert kinase domain residues, allowing the binding, activation and phosphorylation of numerous cytoplasmic proteins that will then relay the signal towards various intracellular activation pathways. These proteins have SH2 or PTB domains that recognise tyrosine phosphorylated receptor chains, and have intrinsic enzymatic activity, such as Src or PLCγ, or serve as adapter proteins for recruiting other enzymes, such as Grb2 linked to the MAPK activation pathway. The proteins recruited by their SH2 domains are named "adapter", while those that

bind directly to the receptor chains or to the Grb2 adaptative protein are called "anchoring proteins". Adaptive and anchoring proteins can bind to similar phosphorylated tyrosine residues or to several tyrosine residues from the same receptor chains. Thus, Gab1 binds to tyrosine^{1068} and tyrosine^{1086} of EGFR. Insulin and FGF receptors bind to a protein assembly that can be phosphorylated and used as adaptive proteins [19].

2.3. RTKs and activated signalling pathways

RTKs are considered as protein platforms, or the starting point for many cellular signalling pathways by recruiting enzymatic effectors (PLCγ, PI3K, Src, etc) either directly on to their intra-cytoplasmic domain, or indirectly through adapter proteins (Grb2, Shc, etc.), forming complexes capable of activating intracellular enzymes (Ras, etc.) (Figure 2). RTK downstream signalling pathways are mainly MAPK, PI3K, Src, and other signalling pathways involving PLCγ, JAK / STAT, etc. While the early stages of signal transduction following the activation of RTKs is based mainly on tyrosine phosphorylation, signal propagation associates various phosphorylations on serine / threonine residues in the majority of cellular processes, as well as other processes such as ubiquitination, glycosylation or acetylation [20].

The **MAPK pathway** plays a part in controlling cell proliferation, cell death or differentiation, and migration, as well as promoting angiogenesis. The MAPK signalling cascade is divided into four major pathways used by RTKs and leading to ERK1/2 activation (Figure 2). After activation of the RTKs by their ligand, the adaptive protein Grb2 binds by its SH2 domains, the phosphorylated tyrosine residues of the receptor chains and the adaptive protein SOS by their SH3 domain, which is bound to the PIP2 membrane. This binding allows the activation of Ras, a small G protein, via SOS, a GEF protein exchanging the GDP for a GTP. In fact, Ras oscillates between its active and inactive state, thus acting as a "switch" for intracellular effector molecules. Once activated, Ras allows phosphorylated signal transduction through recruitment and phosphorylation of Raf kinases A, B or C (or MAP3K)

[21]. Activated Raf phosphorylates MEK1 and MEK2 (or MAP2K1/2) on serine^{218}/serine^{222} and serine^{222}/serine^{226} residues of their activation loop, and activated MEK1/2 itself catalyses the phosphorylation of Erk1 and Erk2 (or MAPK1/2) on their threonine$^{202/185}$ and tyrosine$^{204/187}$ residues. Phosphorylated Erk1/2 will be then translocated to the nucleus to activate transcription factors that will regulate the transcription of genes involved in the survival and growth of the cells, or activate cytosolic proteins, such as RSK1/2, which target cytoplasmic effectors or will finally be translocated into the nucleus to act as a transcription factor [22].

The targets of these transcription factors are transcriptional regulators such as STAT, Elk-1, CREB or H3 histone that activate transcription of early genes. Of these early genes, *c-Fos*, *c-Jun* or *c-Myc* stimulate the expression of other genes such as cyclin D1 or CDK6, which control progression in the G_1 phase and G_1/S transition. When RTK activation, and therefore that of Erk1/2, is maintained, expression of the previous proteins is stabilised as c-Fos, which is phosphorylated on threonine residues by its RSK1/2 and Erk1/2, and forms the complex AP-1 with c-Jun, which also activates the transcription of target genes (Figure 2). The MAPK pathway also activates three additional pathways: p38, JNK and ERK5. In the first pathway, p38α/β/γ/δ are activated by a MAP2K such as MKK3 or MKK6, previously activated by a MAP3K such as TAK1, and consequently, p38 induces the transcription of various genes involved in cell proliferation, angiogenesis, inflammation and the production of immunomodulatory cytokines. In the JNK pathway, the TAK1-, MEKK1-, or MLK-MAP3Ks activate the MAP2K4 or MAP2K7, which activates JNK1, 2 or 3, for instance, and lead to the control of cell apoptosis or the development of the immune system [23]. In the ERK5 pathway, WNK1 activates MEKK2 and 3, which phosphorylates MEK5, leading to ERK5 activation. The translocation of ERK5 into the nucleus regulates cell proliferation and survival by activating the transcription of cyclin D1 for example, allowing G_1/S transition in

the cell cycle in the same way as Erk1/2. ERK5 also has more specific substrates, such as the MEF2 transcription factor family, the pro-apoptotic protein BAD, connexin 43, etc. [24].

The **PI3K/Akt/mTOR pathway** controls cell cycle progression, the cell survival/cell apoptosis balance. Its activation facilitates cell proliferation and migration, the metabolism of glucose, etc. PI3K is a "lipid" kinase that phosphorylates membrane lipids via its catalytic p110 subunit (α, β or δ) once recruited by its two SH2 domains from the p85 regulatory subunit on activated RTKs. PIP2 then forms PIP3 (phosphatidylinositol 3,4,5-triphosphate) by transferring a phosphate group, and Akt (PKB, for Protein Kinase B) and PDK-1 then bind to the membrane, where the PDK-1 activated by PIP3 phosphorylates Akt (Figure 2). Activated Akt becomes an activation crossroad for many proteins, allowing cells to survive by inhibiting, ubiquitinating and degrading pro-apoptotic proteins such as BAD and p53, and by inducing the expression of anti-apoptotics such as Bcl-2 or Akt. In addition, Akt also induces cell proliferation by activating various cyclins and by inhibiting several cell cycle repressors such as p21 or p27. Akt also allows the transcription of pro-angiogenic genes such as VEGF and HIF-1α, which are involved in numerous oncological processes. In addition, Akt inhibits the glucose metabolism by suppressing GSK3, and regulates the lipid metabolism through mTOR activation [25].

The role of the **Src pathway** in signal transmission within the cell was demonstrated for the first time in fibroblasts stimulated with PDGF [26]. Src, Fyn and Yes belong to the Src family, are activated by RTKs, and are associated with numerous other kinases such as Ras, PI3K, PLCγ or FAKs. The members of the Src family therefore have redundant functions in the intracellular signalling pathways described below. Src family members are recruited on RTKs (EGFR, FGFR, IGFR, MCSF-R, HGFR, etc.) after their activation and transmit mitogen signals inducing DNA synthesis, cell survival, cytoskeleton rearrangements, cell adhesion and motility, but also control receptor turnover [27]. Src family members can bind

phosphorylated residues by their SH2 domains, resulting in kinase activity after conformational modifications. This activation is very complex and requires the recruitment of Ras and Ral GTPases. Several studies have shown that SFKs may regulate activation of RTKs directly by phosphorylating tyrosine residues such as tyrosine^{845}, tyrosine^{1101} and EGFR [28]. c-Src can be recruited within membrane complexes formed by integrins, and then phosphorylate these RTKs [29]. Furthermore, the Shp2 protein tyrosine phosphatase also plays a key role in this activation by blocking the activities of negative regulators (Csk for instance) [30].

PLCγ, and JAK / STAT are additional signalling pathways associated with RTK activation. Various RTKs can bind through their phosphorylated tyrosine residue, the SH2 domains of STAT transcription factors, as demonstrated for MET and STAT3. The activation of these trancription factors results in their dimerisation and translocation into the nucleus to activate specific target genes [31].

2.3. Feedback loops controllng RTK activation

RTK activities are tightly controlled by numerous positive or negative molecular feedback loops that prolong the auto-activation of the receptors and signal amplitude, by inducing the production of the ligand for instance. Such feedback loops are essential for stabilising the RTK system [32]. These controls include proteins already present within the cell that are mobilised on activation of RTKs and/or subjected to post-translational modifications for immediately regulating the signal induced (early negative feedback) (Figure 3). They also associate the synthesis of response elements (late negative feedback) such as IEGs early or DEGS late genes that regulate the activity of AP-1, c-Myc, p53 or the MAPKs. Thus, Erk1/2, a downstream protagonist of the MAPK pathway, directly inhibits (early negative feedback) the phosphorylation of the effector proteins by inhibiting the kinase activity of upstream

enzymes (RAF and MEK) [33]. In addition, the translocation of Erk1/2 into the core may also activate the expression of transcriptional repressors, such as phosphatases (*e.g.*: DSPs) to inhibit MAPK activity (negative feedback late) [34].

By decreasing the amplitude of the signals generated and the stimulation of cellular activity, adapter proteins such as kinases, phosphatases and ubiquitin ligases located in the cytoplasm are the first early negative regulators of RTK activities [35]. The signal generated is then attenuated, based on the ubiquitination of RTKs by the E3 ubiquitin ligase c-CBL for instance, which leads to the endocytosis of the receptors and their degradation in the lysosomal compartment [36]. After activation by the ligand, the RTK is effectively clustered in clathrin-rich membrane regions and then internalised in clathrin-dependent endocytic vesicles to reduce the induced signal [37].

3. RTKs in oncology

3.1. RTK mutations and carcinogenesis

RTKs are involved in numerous pathological disorders, especially in oncology. Around 30% of RTKs are mutated or overexpressed in various human cancers (MET, KIT, FLT3, etc.) [38]. Oncogenic mutations or gene duplications in the juxtamembrane region of KIT and FLT3 result in constitutive activation of these receptors in the absence of their ligand, and are consequently directly linked to the carcinogenesis process [39]. Duplications in the juxtamembrane region of FLT3 are responsible, for instance, for the constitutive activation of the receptor in 15-30% of cases of acute myeloid leukaemia [40] and in 65% of gastrointestinal stromal tumours (GISTs) [41]. Autocrine stimulation or overexpression of EGFR were also associated with many solid tumours. Thus, EGFR/ErbB-1 and ErbB-2 are overexpressed in lung [42], breast [43, 44] and prostate [45, 46] cancer, and their expression

is linked to marked aggressiveness and poor prognosis. Such observations have strengthened the therapeutic development of RTK inhibitors in the last three decades.

3.2. RTK inhibitors and bone cancers

3.2.1. RTK inhibitors target the bone tumour niche

Primary malignant bone tumours (bone sarcomas) and bone metastases (from breast, prostate carcinomas, etc.) are characterised by their ability to dysregulate their micro-environment and especially the balance between bone apposition and bone resorption. Osteoblasts [8, 45-51] and osteoclasts [8, 52-54] express numerous RTKs and are then cellular targets of the corresponding ligands released in the cancer micro-environment. Based on these observations, the impact of RTK inhibitors has been assessed in bone remodelling. Recently, Bao *et al*., using broad kinase inhibitor screening applied to the mouse MC3T3-E1 osteoprogenitor cell line, identified two families of inhibitor affecting cell survival differentially [55]. The first family included pro-osteoblastic drugs such as lapatinib (EGFR/HER2 inhibitor), erlotinib (EGFR inhibitor) and sunitinib (FLT3/PDGFR/VEGFR/CSF-1R inhibitor), which stimulated osteoblastic proliferation. In contrast, the second family grouped together seven kinase inhibitors (GSK1838705A, PF-04691502, masitinib targeting KIT or XL880 targeting MET and VEGFR), which inhibited osteoblast viability in a dose- and time-dependent manner. Nilotinib and CEP-751 may be added to the second family. Nilotinib potently inhibited osteoblast proliferation [56]. While nilotinib inhibits numerous RTKs (KIT, EPHA3, EPHA8, DDR1, DDR2, PDGFRB), its effects may be associated with the inhibition of PDGFR [65]. Pinski *et al*. demonstrated that proliferation induced apoptosis, but not quiescent human osteoblasts after treatment with CEP-751, a trk receptor tyrosine kinase inhibitor [57]. Similarly, inhibiting IGF1R also led to the inhibition of proliferation and induction of apoptosis of osteoblasts [58]. Nevertheless, these RTK inhibitors, due to their multiple

targeting, exert very complex effects and can exert dual activities on bone cells. Imatinib mesylate (Gleevec), which targets a broad range of tyrosine kinase proteins, including bcr/abl, c-kit, cFMS and the PDFGR among others, is able to inhibit osteoblast proliferation and also to activate their activities through the inhibition of PDGFRβ activity [59]. Gobin *et al*. confirmed recently this dual activity depending on the doses of inhibitor used. Low doses of imatinib mesylate increased the *in vitro* mineralisation process, and high doses of the drug markedly affected mineral deposits [60].

RTKs are also expressed by osteoclast precursors and mature osteoclasts, and numerous studies have shown that RTK inhibitors strongly affect osteoclastogenesis and bone resorption. Imatinib mesylate decreases osteoclastogenesis, and increases mature osteoclast apoptosis through the inhibition of cFMS signalling [61]. Sorafenib, an RET, and VEGFR inhibitors similarly target osteoclasts [62]. Dasatinib abolishes osteoclast formation *in vitro* by inhibiting cFMS activation, and increases osteoblast activities by repressing PDGFR signalling [63]. In addition, these authors demonstrated that the administration of dasatinib in animals resulted in dysregulated bone remodelling in favour of an increase in bone formation, which may be associated with the inhibition of osteoclast activity [63]. In 2012, Garcia-Gomez *et al*. confirmed the anabolic and anti-catabolic effects of dasatinib [64]. Overall, these works revealed that bone cells are potential targets for RTK inhibitors, and that using RTK inhibitors in an oncological bone context will have an impact on the bone tumour niche.

3.2.2. RTK inhibitors as therapeutic drugs for bone sarcomas

Bone sarcomas derive from the mesoderm, and sarcoma cells originate from mesenchymal stem cells [65]. Osteosarcoma and Ewing's sarcomas are the two main types of bone sarcoma diagnosed in children and young adults. The peak of incidence for both tumours is at puberty, suggesting that there is a strong link with bone growth and the numerous growth factors,

hormones and cytokines released during this period. In this context, RTK inhibitors assessed on bone cells were also assessed in bone sarcomas (Table II) [66, 67]. Recently, Rettew *et al*. identified several RTKs by using a phosphoproteomic approach and demonstrated that Axl, EphB2, FGFR2, IGF-1R and Ret more specifically controlled the behaviour of human osteosarcoma cells *in vitro* from a functional point of view [68]. PDGFR was also identified as a therapeutic target in osteosarcoma, and selective inhibition of PDGFR activation led to apoptosis of osteosarcoma cells *in vitro* [69]. These data were confirmed by a phospho-receptor tyrosine kinase array kit which identified seven receptors (PDFGFRβ, Axl, RYK, EGFR, EphA2 and 10, IGF1R) as molecular targets for imatinib mesylate [60]. In this study, the authors showed that imatinib mesylate induced anti-proliferatives in pre-clinical models of osteosarcoma, and that of the seven modulated RTKs, PDGFRα appeared as the main target of the drug. Similar observations were made in Ewing's sarcoma [70]. Unfortunately, clinical investigations demonstrated only low or no efficacy in children with relapse bone sarcomas, even in patients selected for tumour expression of KIT or PDGFRα [71-73] (Table II). Dasatinib and Sunitinib were used in phase I clinical trials and defined the doses usable in a paediatric context [77, 79]. Although no objective responses were observed, 4 patients with sarcomas were in a stable condition [79]. Complementary investigations are needed to evaluate the therapeutic efficacy of dasatinib and sunitinib in sarcomas. Pazotinib, targeting VEGFR, PDGFR and c-KIT, and sorafenib, targeting RET and VEGFR, had interesting benefits in paediatric sarcomas [71, 54, 85] (Table II).

Protein assays have identified new RTKs with potential therapeutic benefits. Axl is thus expressed in most osteosarcomas [86] and a correlation was found between its expression and the clinical outcome [87, 88]. In addition, Fleuren *et al*. demonstrated that high Axl expression correlated with worse overall survival compared to Ewing's sarcoma patients with lower expression [89] similar to MET [90]. The MET inhibitor (PF-2341066) then appeared

efficient in a xenograft model of osteosarcoma [91]. EphA2 was the most abundant surface protein on cancer cells and may be involved in the pathogenesis of osteosarcoma by modulating bone remodelling and the communications between tumour cells and their environment [92-94]. Recently, Kuijjer *et al*. provided an *in vitro* rationale for using IGR1R inhibitors in osteosarcoma [95]. However, IGF1R mRNA expression, cell surface expression, copy number, and mutation status were not associated with tumour responsiveness to anti-IGF1R targeting [96]. EGFR are expressed by osteosarcoma cells, but gefitinib and BIBW2992 targeting the receptors were not effective on osteosarcoma cells, so the question of EGFR targeting remains open [97]. Similarly, HER-2 is expressed by osteosarcoma cells but its prognostic relevance is still controversial [98] and the results for the patients treated were limited [99]. A randomised study of patients with HER2-positive osteosarcoma would be of major interest for better understanding the role of HER-2 in the pathogenesis of bone sarcomas, and for evaluating their therapeutic value. EphA10 and RYK are two other RTKs expressed by osteosarcoma cells and represent other therapeutic opportunities [100, 101].

Overall, these data revealed the potential therapeutic interest for targeting RTKs in bone sarcomas. Clinical investigations must nevertheless be adapted to the expression/mutation/activation state of RTKs, which is the prerequisite for patient enrolment.

3.2.3. RTK inhibitors: therapeutic benefits for bone metastases

As with bone sarcomas, bone metastastic cells, from breast or prostate carcinoma for instance, dysregulate local bone remodelling and the associated TRKs/growth factors, which in turn facilitate tumour development [102]. Consequently, numerous TRKs and their ligands have been associated with the pathogenesis of carcinomas and their capacity to form bone metastases. Many investigations at the pre-clinical and clinical levels have thus been developed in the last 10 years (Table III). Unfortunately, whilst most of the drugs developed had interesting anti-cancer effects on the primary tumours or/and the establishment of bone

metastases, the results of the clinical trials were often disappointing. Imatinib mesylate for instance, which is very efficient in soft tissue sarcomas, had no palliative or clinical activity in metastastic castration-resistant prostate cancer [105]. Combining it with bisphosphonates and docetaxel did not improve overall survival and brings into question the value of PDGFR inhibition with taxane chemotherapy in prostate cancer bone metastases [105-107]. Similarly, phase III clinical trials did not confirm the combination of dasatinib (which targets c-KIT, EPHA2, PDGFR) and docetaxel in chemotherapy-naive patients with metastatic castration-resistant prostate cancer (Table III). Sunitinib initially appeared promising in metastatic castration-resistant prostate cancer [115], however the phase III clinical trial did not significantly prolong the overall survival of patients after failure of a docetaxel-based regimen [116]. Sorafenib was developed to target RET and VEGFR [120] and has a moderate activity as a second-line treatment for metastatic castration-resistant prostate cancer [122]. HGFR (c-MET) and its ligand HGF control numerous cellular signalling cascades that direct cell growth, proliferation, survival, and motility, and also regulate the epithelial-mesenchymal transition (EMT) with a stong impact on the developement of metastases. Cabozantinib was specifically developped to inhibit the downstream signalling pathways transduced by c-MET and VGEFR [124-131]. Cabozantinib is currently approved by the U.S. Food and Drug Administration for the treatment of progressive, metastatic medullary thyroid cancer. The clinical evaluation demonstrated in phase II clinical trials that the use of this drug appeared clinically relevant in castration-resistant prostate cancer patients, as it improved bone scans and bone biomarkers, and reduced both soft tissue lesions and the number of circulating tumour cells [133-134]. The phase III COMET-II trials that cabozantinib has not fullfilled the promise reported in the phase II trials (Exelixis announcement: http://www.exelixis.com/investors-media/press-releases). Indeed, 50% of patients in the cabozantinib arm reported a pain response, compared to 17 percent of patients in the control

arm receiving mitoxantrone/ prednisone. This difference in pain response between the arms was not statistically significant. Tivantinib, another c-MET inhibitor, has shown promising therapeutic value in pre-clinical models [136-137]. Erlotinib has moderate clinical effect as a single-agent in chemotherapy-naïve castration-resistant cancer [142] and its combination with docetaxel did not show any added therapeutic value [143]. Genitinib, lapatinib and vandetanib alone or in combination with other drugs failed to show significant therapeutic activity compared to the conventional drugs in breast and prostate cancers (Table III). Dovotinib is a recently developed multi-RTK inhibitor (FGFR, VEGFR) that has shown interesting pre-clinical activity in metastastic castration-resistant prostate cancer: anti-angiogenic activity, anti-tumour activity and clinical activity in 34 patients with bone metastases [159]. However, its combination with histone deacetylase inhibitor did not show any additional value [160]. Clinical trials are required to confirm its therapeutic value.

Although numerous RTK inhibitors initially appeared to be of great interest, based on pre-clinical assessements, most of them have not fulfilled the promise hoped in phase I/II studies. The absence of significant results with their use can be explained by the multiplicity of their targets and the complexity of the mechanisms involved. Indeed, these drugs will affect not only the tumor cells but also its environment. Thus, the Cabozantinib, like dovotinib for instance for which the clinical activity needs to be confirmed, affects the coupling between cancer cells and the bone tumour niche [159, 161, 162]. The bone tumour microenvironment (in bone sarcoma and bone metastases) is then described as a sanctuary that controls at least in part the tumour growth and contributes to the drug resistance acquisition [163, 164]. By modulating the tumour microenvironment, RTK could have a positive and/or a negative impact on the tumour development.

4. Conclusion

In the last 15 years, there have been high expectations in oncology of therapies with RTK inhibitors. Imatinib mesylate was the first to show spectacular clinical success in chronic myeloid leukaemia patients, and has become the first line of treatment. Gastro-intestinal stromal tumour (GIST) is the second success for the use of an RTK inhibitor, and imatinib mesylate is the standard of care in patients who are at high risk for GIST recurrence following resection [165]. Unfortunately, patients develop resistance and relapse due to protein point mutations and/or the introduction of molecular feedback loops. Many other RTK inhibitors have shown disappointing results in clinical applications after encouraging pre-clinical results. In all cases, the efficacy of RTK inhibitors is linked with their ability to disrupt the crosstalk between tumour cells and their environment. A better understanding of both intracellular signal modulating by these RTK inhibitors, and the feedback loops developed during the establishment of resistance, will increase the chances of success for these drugs. In addition, adapted investigational approaches will be needed to define the expression profile of the RTK genuinely activated/mutated/expressed in patients before their inclusion in clinical trials.

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Figure Legends

Figure 1: General organisation of the molecular domains that make up the RTKs. RTKs are characterised by the dimerisation of two receptor chains with an N-terminal (N) extracellular domain (ECM), and a C-terminal (C) intracellular domain (ICD). The extracellular domain is implicated in the recognition of the dimeric ligands and the formation of the receptor chain dimerisation process. The extracellular domain is associated with ligand recognition and is composed of various domains depending on the RTK class. The transmembrane-domain is composed of an a-helix chain which contributes to the stabilisation of the dimeric receptor chains. The binding of a dimeric ligand (in red) to the extracellular domains of the receptor chains strengthens the stabilisation of the receptor chains, which are auto-phosphorylated through their tyrosine kinase domains and then transduced in specific downstream signalling pathways.

Figure 2: Main signalling pathways activated by the ligand-induced RTK auto-phosphorylations. The phosphorylation cascades initiated by the RTK phosphorylations lead to the activation of numerous transcription factors which consequently control the regulation of many physiological processes.

Figure 3: The negative feedback loops regulating RTK activation. The window of time required for inducing mRNA and protein synthesis after RTK activation is between 15 and 90 minutes. These mechanisms are tightly regulated by negative feedback loops. Indeed, the phosphorylation cascade induced by RTK activation leads to the activation of numerous transcription factors and simultaneously of their repressors. The translocation of the various transcription factors can also induce the expression of transcriptional repressors or

phosphases, which in turn can repress the corresponding transcription factors and/or the upstream kinase activites. + : activation; - : repression.