C-KIT RECEPTOR ON MAST CELLS AS A TARGET FOR THE TREATMENT OF COLORECTAL CANCER

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ABSTRACT

The role of mast cells (MCs) in the development and progression of colorectal cancer has long been investigated. A high density of these immune cells, which are able to release numerous pro-tumorigenic factors, has been associated with poor prognosis. One of the most important receptors present on mast cells is the c-KIT receptor, whose activation is dependent on stem cell factor (SCF) ligand, a major cytokine that influences mast cells number. This has led to the development of drugs able to inhibit this receptor and thus decrease mast cell density (MCD). One of the main classes of drugs are the tyrosine kinase inhibitors (TKIs) that were first developed in 2001. Despite their important role and efficacy, TKIs used so far for treatment in colorectal cancer are multi-target inhibitors which present some limitations. For instance, the development of resistance and high toxicity due to off-target effects are common. Future studies should, therefore, lead to a resolution of these two major limitations by increasing affinity for the target. One possible solution, presented in this report, could be the development of new drugs belonging to the third generation tyrosine kinase inhibitors characterized by a single target and irreversible receptor binding.

LAYMAN SUMMARY

Colorectal cancer is the third most common cancer in the world, with high chance of developing metastasis, which are the leading cause of death. For this reason, numerous attempts have been made in order to develop drugs for the treatment of this condition. In 2001, tyrosine kinase inhibitors (TKIs), a class of drugs that inhibits the c-KIT receptor, were first developed. This receptor is present on mast cells, which are immune cells with a key role in tumor development and progression, capable of releasing mediators that lead to increased proliferation, development of metastasis, and immunosuppression.

TKIs have had great success in the past 20 years; however, despite their effectiveness, they have two major limitations: the development of resistance and high toxicity. Therefore, more studies are needed in order to solve these problems and develop a more specific class of inhibitors that could be the first choice of treatment for patients with colorectal cancer. To achieve this aim a possible solution could be single target (c-KIT), third generation TKIs, which have shown less adverse events and can also be effective against common receptor mutations.

INTRODUCTION

Mast cells (MCs) are innate cells characterized by the presence of numerous cytoplasmic granules consisting of a proteoglycan matrix (e.g., heparin), proteolytic enzymes (tryptase, chymase, carboxypeptidase A) and histamine. They were firstly identified by Paul Ehrlich in 1878, who defined them *"mastzellen"* based on their well-fed appearance. [1] Since then, these cells have been extensively investigated, however, their functions have not yet been fully understood. [1 2] At present, it is certain that MCs originate from $CD34^+/CD117^+$ pluripotent progenitor cells and subsequently they migrate to peripheral tissues in order to complete their maturation. [2] This process is strongly dependent on the microenvironment of the target tissue and on the various growth factors (IL-10, IL-9, IL-4, IL-33 etc.) which determine the functional heterogeneity of mast cells.[2] In humans, the classification is based especially on their granule content, therefore, mast cells have been classified into three subtypes: MC_T (which contain tryptase), MC_C (which contain chymase) or MC_{TC} (which contain both) which differ in both function and location. [3] It is also important to consider that MCs are highly heterogeneous, in fact, their function is dependent on the microenvironment in which they originate and reside. Their great heterogeneity is also notable by the fact that, even in the same tissue, MCs are different and, in addition, they can also generate other subpopulations. [4] Another important feature of mast cells is their ability to produce cytokines, chemokines and growth factors (IL-1, IL-3, IL-4, IL-5, IL-6, IL-8, IL-10, IL-13, IL-16, MCP-1, TNF-α and NGF) that play an important role in the regulation of inflammatory responses. [4]

The most important cytokine for MCs is the stem cell factor (SCF), which plays a pivotal role in their growth, differentiation and survival. The SCF is the ligand of the tyrosine kinase KIT receptor (c-KIT receptor or CD117) which is a member of the type III receptor tyrosine kinase family and it is expressed by numerous cells in the body including mast cells. [5] Recent studies have shown that c-KIT alterations promote the development of some human cancers, and for this reason, this receptor has been considered a good candidate for tumor therapies. [6] Receptor tyrosine kinase inhibitors (TKIs) have therefore been developed, and among them, one of the most well-known in cancer treatment is Imatinib, which has shown good results by inhibiting the binding of ATP to the receptor. Despite its efficacy, the use of this drug has led to c-KIT mutations and thus the development of resistance, culminating in tumor recurrence and progression in most patients. [7] In addition, Imatinib has also

shown to be cardiotoxic. To this end, there is a great interest in developing new possible therapies capable of targeting c-KIT in a specific manner and therefore avoid toxic features. [5] [6]

In this review, we will mainly focus on the role of mast cells and c-KIT in colorectal cancer (CRC), which is the third most prevalent cancer in the world with a considerably high death rate (35-50%) both in men and women. [8]

1. ROLE OF MAST CELL IN TUMOR DEVELOPMENT AND PROGRESSION

The first person who noticed a connection between mast cells and cancer was Rudolf Virchow in 1863; subsequently, in 1892, Westphal identified that MCs were usually at the periphery of the tumor. [9] The correlation between mast cells and tumor has long been studied, and opinions regarding their role in cancer development and progression are conflicting. In fact, some authors support the idea that an increase in the mast cells density (MCD) is correlated with a better prognosis. However, the majority and more recent studies showed that an increase in MCD causes an enhancement of pro-angiogenic signals and reduces survival. [10] Indeed, a recent meta-analysis of cohort studies assessing the function of MCs in various human solid tumors showed that increased tryptase and MCs infiltration were linked with metastasis in non-small cell lung cancer (NSCLC), hepatocellular, and colorectal cancer. [4]

To date, it is known that mast cells attracted to the tumor microenvironment induced by SCF released by cancer cells, fibroblast and endothelial cells, are able to induce immunosuppression, degradation of extracellular matrix and angiogenesis. In fact, mast cells depletion in mice was correlated with lower angiogenesis and metastasis. [9] Angiogenesis is crucial in tumor development and progression, referring to the growth of new blood vessels from preexisting ones. Mast cells have a fundamental role in this process due to the fact that they are able to synthesize some proangiogenic factors such as vascular endothelial growth factor (VEGF), which is the most important one in this process. Other important factors are fibroblast growth factor-2 (FGF-2), platelet derived growth factor (PDGF), interleukin-6 (IL-6), chymase and tryptase. The latter is a strong angiogenetic factor released by mast cells (following activation of the c-KIT receptor) which is able to activate matrix-metalloproteinases (MMPs). These enzymes are important in the degradation of the extracellular matrix and can additionally induce the release of SCF. [9 11]

1.1 MAST CELLS IL COLORECTAL CANCER

The majority of colorectal cancers initially begin as polyps that became subsequently malignant. Additionally, it is quite common for patients with this cancer to develop metastasis, which are the main cause of death. During the early phases of this tumor, colorectal cancer cells stimulate mast cells in order to produce por-angiogenic factors, which can create a suitable environment for proliferation, angiogenesis and immunosuppression.[8] [10] In fact, mast cell density (MCD) is associated with a worse prognosis, whereas it has been shown that in mice with mast cell depletion, tumor growth is slowed. [8]

As previously mentioned, there are three kind of mast cells: MC_T, MC_C and MC_{TC}. In colorectal cancer, the majority of MC contain both tryptase and chymase and are therefore classified as $MC_{TC.}$

MCs are a source of various mediators that can stimulate tumor growth, such as VEGF-A, which is important for the angiogenesis; PDGF-A, which increases proliferation; and finally protease, kinase and metalloproteinase (MMP), which are involved in the degradation of the extracellular matrix, leading to tumor aggressiveness and metastatic potential. [12] The importance of these factors in colorectal cancer was demonstrated using HT29 colon cancer cells (colorectal adenocarcinoma cell line). By using this cell line, it was also possible to determine the importance of IL-8. This cytokine is a member of the CXC cytokine family and it is highly upregulated in colon cancer cells. It supports invasion, metastasis, and tumor growth through metalloproteinase-cleavage. [13] Another study in vitro showed that the chemokine CCL15, which is secreted by colon cancer cells, is important to promote MCs migration. [4]

In a further investigation conducted to better understand the interaction between colon cancer cells and mast cells, it was possible to notice that cox-2 cyclooxygenase (COX-2) was upregulated, this suggests an increase in prostaglandin such as PGE2 (associated with the spread of metastasis). This latter has a role in immunosuppression and tumor growth and, moreover, it can also stimulate mast cell to produce VEGF-A. [8] [12] In addition to COX-2, TNFSF14 and ISG15 (IFN-stimulated gene 15) were also upregulated, and the products of these two genes are associated with increased tumorigenic potential of cancer stem cells.[12]

Studies conducted using mice MC-deficient revealed that MCs are fundamental for the development and growth of preneoplastic polyps. [4] Wang et al. observed that mast cells in polyps were less than in colorectal cancer, and also that the number of MCs was higher in poorly differentiated cancer tissues compared to well differentiated ones. [14] In addition, mediators released by MCs increase

tumor growth by stimulating the proliferation of colon cancer cells. Interestingly, this can happen without the need of cell-cell contact. [14]

Finally, MCs have a role in the formation of a microenvironment favorable for the tumor, thanks to their capacity to interfere with the anti-tumor immunity. CD8+ T cells have the ability to attack cancer cells by recognizing antigens expressed by cancer cells, which is one of the body's main mechanisms for preventing tumor growth. In fact, an increase in CD8+ T cells has been characterized as a positive index of increased survival. Indeed, one study showed not only that in colon cancer the number of CD8+ T cells was inversely related to the number of MCs, but also that cytokines such as CXCL9, CXCL19 and Th1 chemokines were upregulated in tumors in which MCs infiltration was low. These factors are critical for recruiting cytotoxic T cells and natural killer (NK) cells. This has demonstrated the importance of MCs in inhibiting the immune system and also the fact that patients with a better prognosis were those with a lower number of mast cells. [4 15]

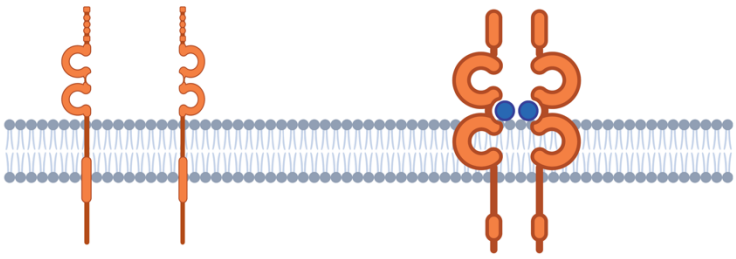
Table 1: factors released by mast cells with a fundamental role in development, growth and progression of colorectal cancer (CRC).

FACTOR	ROLE
VEGF-A, VEGF-B	Angiogenesis
FGF-19	Angiogenesis
Tryptase	Angiogenesis
TGF-β	Metastasis
TNF-α	Metastasis
IL-8	Metastasis
IL-1β	Immunosuppression and angiogenesis
IL-6	Immunosuppression and angiogenesis
IL-13	Immunosuppression and angiogenesis
PDGF-A	Increased proliferation
Protease	Degradation of the extracellular matrix- metastasis
Kinase	Degradation of the extracellular matrix- metastasis
MMP-2, MMP-9	Degradation of the extracellular matrix- metastasis
CCL2	Liver metastasis
CCL5	Tumor progression, immune-suppression
CXCL1	Proliferation and migration
CXCL2	Metastasis
CXCL20	Cell proliferation, metastasis

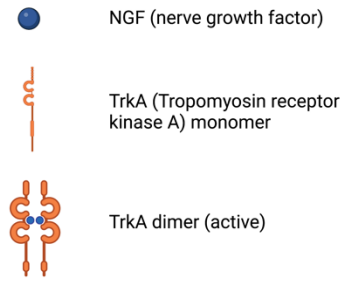
2. C-KIT RECEPTOR

Receptors tyrosine kinases (RTKs) are a class of receptors that play important roles in various physiological functions such as cell growth, metabolism, differentiation, and motility. In total there are 58 RTKs in humans with a similar structure which can be further divided into 20 different families. A common feature of these receptors is their need to combine in the form of dimers in order to be activated. Afterwards, these receptors are generally activated by binding to a specific ligand, which results in the dimerization of the receptor. This can occur in four different ways: in the first case, the activation of the receptor is mediated exclusively by the ligand without any kind of interaction with the extracellular regions of the two receptors (e.g TrkA); in the second case, dimerization is completely mediated by the receptor without any interaction with the two ligands (e.g EGFR); in the third case, the ligands in the form of homodimers bind to two receptors which interact with each other (e.g c-KIT). Finally, in the fourth case, in addition to the two ligands and a direct receptor-receptor interaction, there is the need of accessory molecules that participate in dimerization (e.g FGFR). [16]

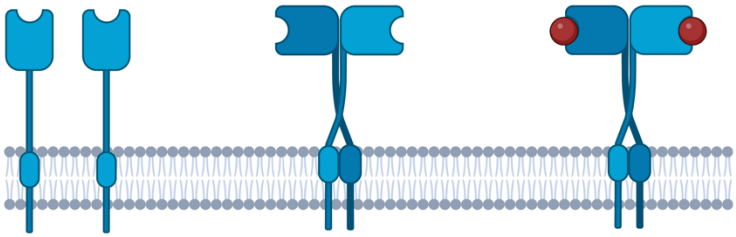
1. TrkA-NGF



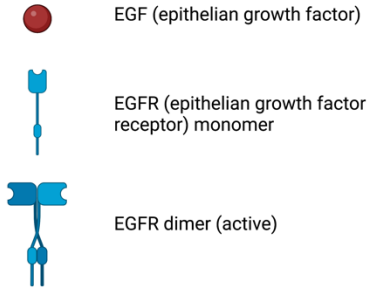
TrkA dimerization involves the binding of two TrkA molecules together, forming a dimer. TrkA dimerization is strongly ligand dependent, meaning that it requires the binding of a ligand to the TrkA receptor in order to activate the TrkA signaling pathway.



2. EGFR-EGF



EGFR dimerization is mediated by receptor-receptor interactions. The EGF ligands stabilize the interaction, the presence of the EGF ligand can help to activate the EGFR signaling pathway more quickly and efficiently and to better stabilize the interaction.



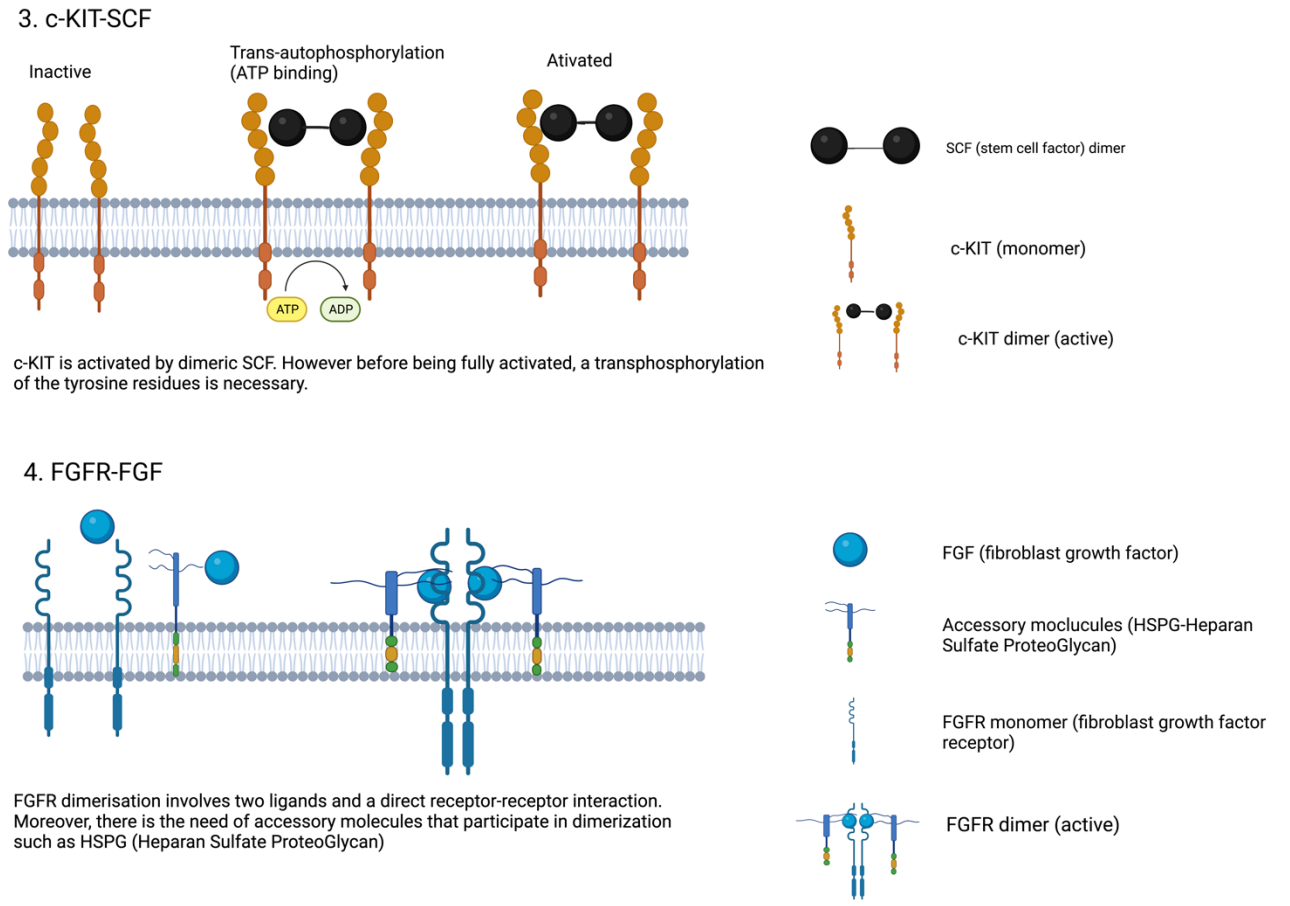


Figure 1: four modes of receptor dimerization. In the first case (TrtkA receptor) the dimerization is completely ligand dependent; in the second case (EGFR) the dimerization is mediated by receptor-receptor interactions; in the third case (c-KIT) the ligand is in form of homodimers and they bind two receptors; in the fourth case (FGFR) there is the need of accessory molecules to activate the receptor (created using BioRender).

Of great interest, a deregulation or malfunction of these receptors has been associated with several human diseases and, in particular, with cancer. Regarding this correlation between receptor tyrosine kinase and cancer, some of the most important RTK families with a pivotal role in this pathology can be mentioned, such as Type I RTKs – EGFR (epidermal growth factor) receptor family, Type II RTKs which is the insulin receptor family, Type III RTKs which include PDGFR, c-KIT, CSFR, FLT3 receptor family and Type IV RTKs such as VEGF (vascular endothelial growth factor) receptor (Figure 2). EGFR is overexpressed in CRC and this overexpression, as with c-KIT, is related to a bad prognosis. In addition, EGFR plays a role in cell division, apoptosis, and cell differentiation by activating different signaling pathway (RAS-RAF-MEK-MAPKs etc). For this reason EGFR has been considered a good target for both tyrosine kinase inhibitors (RTKI) (Afatinib, Brigatinib, Dacomitinib, Dasatinib) and monoclonal antibodies (Panitumumab and Centuximab). [17] [18] VEGF, on the other hand, is fundamental for angiogenesis in different tumors, including colorectal cancer. Hence, VEGFR has

been considered a good target and tyrosine kinase inhibitors such as SU6668 and SU5416 have been developed. [18] Here, we will mainly focus on c-KIT as a target for colorectal cancer, since it has been considered one of the most important targets in order to reduce mast cells density and, therefore, for colorectal cancer therapy. [18]

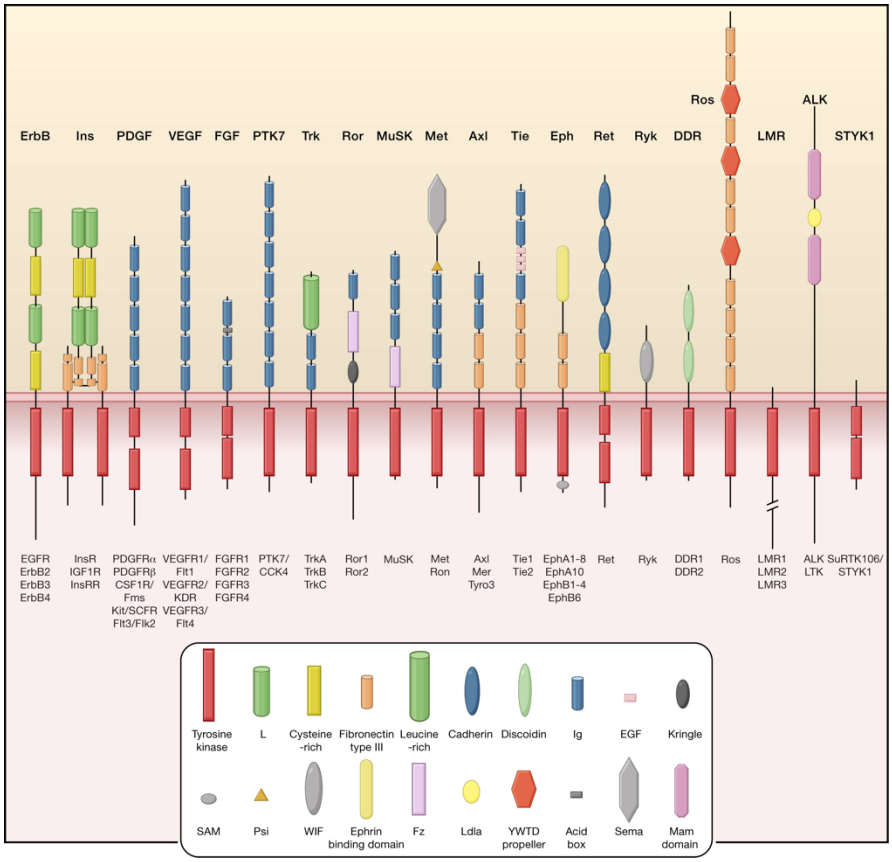


Figure 2: representation of the RTKs divided into 20 subfamilies. The red rectangles represent the intracellular domains and all the other components of these receptors can be found in the legend. [19]

2.1 C-KIT STRUCTURE AND PATHWAYS

c-KIT receptor, also known as stem cell growth factor receptor or cluster of differentiation 117 (CD117), is one of the type III tyrosine kinase receptor with a pivotal role in the development and proliferation of cancer. Its ligand is SCF, which is also known as c-KIT ligand. [20]

This receptor, encoded by the *c-KIT* proto-oncogene located on chromosome 4 in humans and on chromosome 5 in mice, has a mass of 145 kDa and it is 975 amino acids (aa) in length. Like other members of the class III tyrosine kinase receptors, it is composed of three main domains: a hydrophobic transmembrane (TM) domain of 23 aa, an extracellular (EC) ligand binding domain of 519 aa and an intracellular (IC) domain which contains a juxtamembrane (JM) region, a tyrosine

kinase (TK) region and a c-terminal for a total of 433 aa. Moreover, the TK domain can be additionally divided in TK1 (N-lobe) with the TP binding region and a TK2 (C-lobe) with a phosphotransferase domain and an activation loop. [5] [6] The extracellular domain consists of five Ig loops (D1-D5), the first three domains are important to bind the KIT ligand (SCF), while the fourth one has a fundamental role during the receptor dimerization. The TM participates in the attachment of c-KIT to the cytoplasmic membrane, and finally, the IC domain enables signal transduction (figure 3). [21]

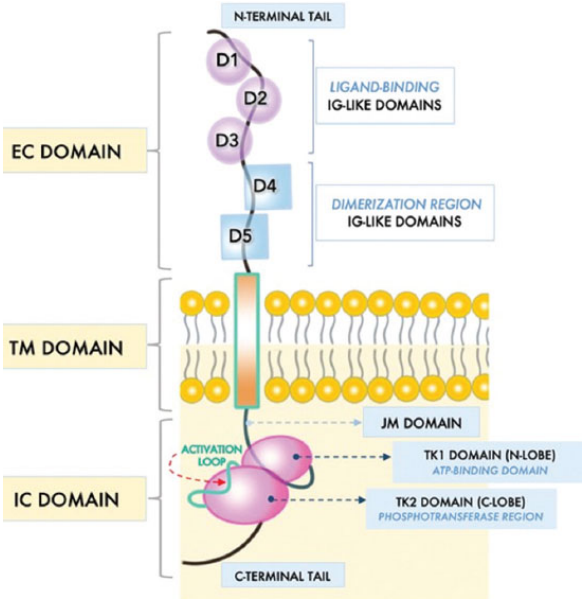


Figure 3: structure of c-KIT receptor. It is composed of three domains: an extracellular domain (EC), a transmembrane domain (TM), and an intracellular domain (IC). The EC is composed of three domains (D1-D3) fundamental for the binding with SCF and two domains (D4-D5) with a role in KIT dimerization. In addition the IC domain consist of a juxtamembrane domain (JM), a tyrosine kinase domain (TK) divided into TK1 (N-lobe) and TK2 (C-lobe), and finally a flexible C-terminal tail [5]

In the inactive form, the c-KIT receptor (monomer) is autoinhibited by the JM domain, however its ligand SCF (as dimer) is able to bind two c-KIT molecules together and this leads to homodimerization of the receptor. This reaction conducts to the initial transphosphorylation of the tyrosine in the JM domain which can dissociate from the TK1 domain. At this point, TK1 and TK2 are therefore separated, allowing the binding with ATP and the release of ADP for another transphosphorylation in the activation loop. The full activation of c-KIT, which happens only after the phosphorylation of the tyrosine residues in the kinase insert and C-terminal tail, leads to signal transduction through different intracellular pathways. The four pathways are: PI3K pathway (proliferation, survival an adhesion), MAPK pathway (gene transcription and proliferation), Src family pathway (cell migration and survival) and JAK/STAT pathway (proliferation and apoptosis). [6]

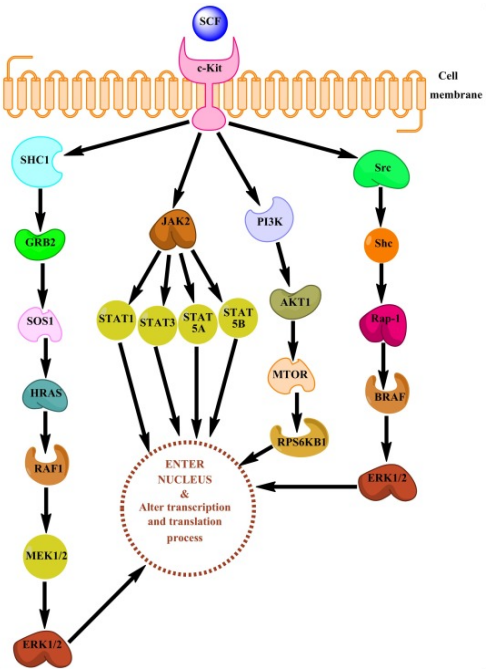


Figure 4: general overview of signaling pathways involving c-KIT. After the binding of SCF to c-KIT and its subsequent, there are four signaling pathways activated: MAPK, JAK/STAT, PI3K, and Src kinase. PI3K pathway plays an important role in cell proliferation, regulation of actin cytoskeleton, cell growth, and tumorigenicity; In MAPK pathway, MEK is phosphorylated and ERK is activated leading to altered gene expression and protein activity; In Src pathway, the activation of this pathway promotes cell migration, chemotaxis, and proliferation; JAK/STAT pathway has a role in proliferation, invasion, survival, inflammation, and immunity. [6]

2.2 PATHWAYS INVOLVED IN COLORECTAL CANCER

There are two pathways among those mentioned above that were shown to be altered in CRC cells: MAPKs and PIK3CA (phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha). The MAPK/ERK pathway is overexpressed in several CRCs and has been associated with carcinogenesis, migration, invasion and metastasis. Overexpression of this pathway is due to many different factors; in addition to an overexpression of c-KIT receptor, an upregulation of EGFR is another major cause. The second key pathway is the PI3K/AKT pathway, which is altered in 10-15% of colon cancers, and 20% of these alterations influence and alter the PI3K gene. [22]

2.3 C-KIT AND COLORECTAL CANCER

The expression of c-KIT decreases during the hematopoietic differentiation, however, it is increased in mast cells, dendritic cells and natural killer, indicating that this receptor has a potential role during inflammation, chronic inflammation, and thus in the promotion of cancer. Moreover, c-KIT, by activating the aforementioned signaling pathways, is involved in cell proliferation. These statements

led to the realization that dysregulations of c-KIT receptor are related to the development of various cancers. Specifically, these alterations can be of different types: gain of function, loss of function, point mutations, and overexpression. The latter has been observed to be the main dysregulation in the development of colorectal cancer. [5]

Several studies have been conducted on colon cancer cell lines, such as HCT-116 or DLD-1, to better understand the role of the proto-oncogene c-KIT in tumor growth and survival. In one study, it was observed that, in colon cancer cells, c-KIT increases ETS Variant Transcription Factor 4 (ETV4) levels, which, by inducing MM7 expression, leads to extracellular matrix degradation and consequently increased metastasis. This was verified using human colorectal cancer HCT-116 cells. [23] Another experiment conducted on DLD-1 cells (colorectal adenocarcinoma *cell line*) revealed that the activation of c-KIT can suppress apoptosis and thus promote tumor survival. Moreover, in the same study, it was observed that, as in the CD34+, in DLD-1 cells the production of MMP-9 was up-regulated, which increases the invasiveness of this tumor. [24]

In colon cancer, there is the presence of stem-like cells, a group of not-yet-differentiated cells with high tumorigenicity which are also known as tumor initiating cells. These cells can be isolated and used to generate a colonosphere, a system which retains the ability to generate "copies" of the tumor and can be used to analyze various aspects of the tumor, such as the ability to differentiate but also the resistance to certain drugs. By using c-KIT(+) (undifferentiated) colonosphere, it was possible to discover that SCF secreted by differentiated tumor cells was an important supporting factor for colonosphere and thus for undifferentiated stem-like cells. This finding reveals that SCF allows cells to maintain their tumorigenic potential, and therefore a lack of SCF or c-KIT can greatly reduce clonogenicity and tumor initiation. [5 25]

3. MAST CELL C-KIT RECEPTOR AS A TARGET

As outlined above, mast cells may have a strong impact on tumor cells and the surrounding environment in order to promote tumor growth. Therefore, they can be considered a good candidate as target for different solid tumors, such as colorectal cancer (CRC). [26] As previously mentioned, SCF is the most important cytokine which regulates the mast cell differentiation, survival, and also the recruitment of new mast cells. This factor can bind the c-KIT receptor expressed by differentiated mast cells, which are one of the only immunity cells to express this receptor even once fully differentiated. Therefore, numerous attempts have been already made to target the c-KIT receptor and decrease growth and metastatic potential of colorectal cancer (CRC). In general, a therapeutic approach should reduce mast cell density (MCS), decrease the mast cells activation, and decrease the mediators released by these cells in order to down-regulate the downstream pathways. [26]

3.1 TYROSINE KINASE INHIBITORS

One of the most important classes of pharmacologic agents targeting the c-KIT receptor are the receptor tyrosine kinase inhibitors (RTKIs), which were first developed In 2001 and have since had great success. By binding the active site, they are able to prevent the phosphorylation and, therefore, decrease proliferation and angiogenesis. [27] There are five main different classes of RTKIs. The first are the type-I inhibitors, which are ATP competitive inhibitors that bind the ATP-binding pocket of the active conformation in a non-covalent way. Type-II inhibitors bind a site close to the ATP site of inactive kinases and retain them in a non-active conformation (usually are non-selective). Type-III have a high selectivity and can bind an allosteric site to inhibit kinases that is distant from the ATP site (reversible inhibitors). Type-IV, similar to type-III, bind an allosteric site outside of the cleft and are reversible. Finally, type-V inhibitors which are able to bind in a covalent way and are, therefore, irreversible. This latter class has the advantage of being stronger and causing less side effects. [28]
At present, there are more than 50 TKIs existent that have been approved by the Food and Drug Administration (FDA) for the treatment of different kind of tumors, and almost 20 of these inhibitors target c-KIT. For the treatment of CRC there are in total nine known potential TKIs (Cabozantinib, Dasatinib, Imatinib, Lenvatinib, Pazopanib, Ponatinib, Sorafenib, Sunitinib, and regorafenib), though only Regorafenib has been approved as monotherapy for the treatment of this tumor. [22] [28] [29]

Imatinib, one of the best-known and most important tyrosine kinase inhibitors, was initially developed for the treatment of chronic myeloid leukemia (CML) in 2001 and later approved for the treatment of other cancers. It competitively binds to the ATP binding site of c-KIT (CD117), Bcr-Abl and PDGF and therefore it inhibits these receptors. Numerous in vitro tests have been conducted to verify the antitumorigenic potential of Imatinib with good results. Moreover, a study with a model of CRC in rats showed the antitumor effect of this drug also in vivo. This tyrosine kinase inhibitor mediates the expression of MIP-1β, INFg, and IL-2, which this leads to an increase in cytotoxic T cells and NK cells and, consequently, to neoplastic cell death. In addition, Imatinib downregulates the MAPK pathway, which is considered a major altered pathway in colon cancer. Finally, Imatinib can inactivate Akt through different mechanisms: by downregulating mTOR expression, by blocking proinflammatory cytokine signaling and consequently reducing NF-kb activation, and lastly by blocking the activation of PI3K and PDPK1. [30]

Sunitinib is a small molecule that is able to inhibit the ATP-binding sites of several targets, such as c-KIT but also VEGFR1/2/3, PDGFR-α and β. In vitro studies showed that Sunitinib inhibit VEGFR and c-KIT, thus decreasing proliferation. Moreover, in vivo studies revealed the pivotal role of this kinase inhibitors in reducing angiogenesis and decreasing the metastatic potential. To verify the effect of Sunitinib on tumor angiogenesis, the authors used a model of HT29 human colon carcinoma in athymic mice. The results showed that this TKI leads to a decrease in the vascular permeability in the tumor. [31]

Apatinib showed effective results in vitro, in vivo (mouse CRC cell lines), and also in a phase II trial. Cabozantinib, similarly to Apatinib, showed positive results by reducing proliferation and increasing apoptosis, leading to tumor growth suppression. Desatinib, Lenvatinib, Pazopanib, Ponatinib, Sorafenib have also been evaluated for CRC, demonstrating positive results as antitumor agents. However all these TKIs except for Regorafenib failed in the clinic. [29]

Regorafenib (Stivarga®) is an oral small-molecule multi-target able to inhibit several angiogenic RTKs, such as c-KIT receptor, as well as VEGFR-1/2/3, PDGFR-α/β, and FGFR-1/2. It has been approved worldwide, including in Europe, USA and Japan. Initially, Regorafenib was tested in CRC clinical models and also in patient-derived tumor organoids (PDTOs). [29]Subsequently, in 2012 it was approved for the treatment of metastatic colorectal cancer (mCRC) following two phase III randomized, double-blind trials that demonstrated its efficacy in patients with this condition. The two

trials were CORRECT and CONCUR. These trials demonstrated the efficacy of this tyrosine kinase inhibitor as monotherapy. Both the CORRECT and the CONCUR trial indicated an increase in median overall survival (OS) in the Regorafenib groups when compared to placebo. [27] [32]

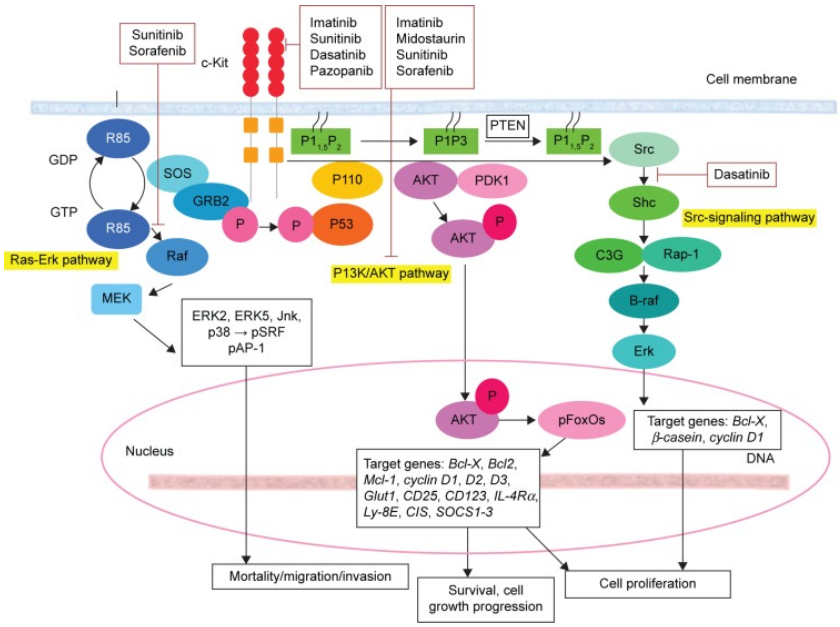


Figure 5: schematic representation of c-KIT inhibition. The pathways activated by the c-KIT receptor (Ras-Erk pathway, PI3K/AKT pathway, and Src-signaling pathway) have different effect on cell function, however the main result is the inhibition of apoptosis which results in increased cell proliferation, growth and migration. In this figure it is also possible to observe, for each c-KIT inhibitor (Tyrosine kinase inhibitor), the pathway they block [33]

3.2 TKIS RESISTANCE

Tyrosine kinase inhibitors have shown positive results in treating different kind of tumors; however, both acquired and intrinsic resistance remains one of the biggest problems within this class of drugs. Intrinsic resistance, also called primary, refers to the tumor's lack of response to treatment, whereas in acquired or secondary resistance, there is an initial response but different mutations lead to resistant tumor cells. This latter is the most common resistance during the treatment with TKIs. Imatinib and regorafenib resistance, for instance, has been observed on numerous occasions. In fact, despite the benefits, a big majority of patients under treatment with Regorafenib develop resistance. The reason why multi-kinase inhibitors usually develop resistance is due to the hypoxic stress caused by the disruption of tumor angiogenesis. [28]

In tumors expressing KIT, it has been demonstrated that mutations in BCR-ABL, EGFR, or mutations of the ATP-binding pocket of the kinase domain are common. Moreover, another typical method, and also the most common one in order to avoid the effect of TKIs, is to activate other kinase that can be downstream or in parallel of the targeted pathway. [28] Therefore, the inhibition of one pathway causes the activation of compensatory pathways. Due to the frequent secondary resistance to this class of drugs, it has been speculated that the combination of different inhibitions would be a good way to inhibit cancer growth. However, one limit is that the administration of these drugs with other treatments may cause further side effects and toxicity. In fact, toxicity is already a feature of multi-target kinase inhibitors caused by off-target effects, and the use of multiple treatments at the same time could further exacerbate this problem. This downside can be partially prevented by determining the affinity of RTKI for other targets and thus choose the most specific one. [28]

3.3 PRECLINICAL MODELS

There are several methods that can be used to test TKIs for colorectal cancer. Models can be cell lines, but also more complex 3D models and finally animals. Cell lines are generally the first choice adopted to evaluate tyrosine kinase inhibitors, however, they have the limitation of not having heterogenicity and also of failing to replicate the in vivo environment. This is due to the fact that there is no interaction with the human immune system. Of key importance during the investigation of kinase inhibitors have been patient derived xenografts (PDXs) and subsequently in vitro tumor organoids (PDTOs). PDXs are animal models, called xenografts, which derived directly from patient tissues that can be subsequently implanted into immunocompromised mice and used to test multiple drugs even simultaneously. Despite their excellent characteristics, the lack of human stromal cells is a problem that needs to be taken into account. PDTOs, on the other hand, are in vitro tumor models that can be used to test and validate tumor drugs in a patient-specific manner. Even in this case, the lack of human stromal cells and vasculature are limitations that can affect the final result and that need to be considered. [29] For these reasons, the use of animal models in vivo allows us to overcome this problem and better understand the pathways involved in tumor development and growth. [29]

3.4 TKIS IN COMBINATION WITH OTHER THERAPIES

In additional to monotherapy, tyrosine kinase inhibitors have been also validated in combinations for the treatment of CRC. This was done mainly because only the 20% of patients with CRC respond to the monotherapy with the available drugs. Another important reason to consider is that the inhibition of one pathway can cause the activation of compensatory pathways to evade the apoptosis, therefore a multiple inhibition might be more effective. [22]

The most important and most studied is the combination between TKIs and chemotherapy/radiotherapy. For instance, Desatinib and Oxaliplatin (an anti-cancer chemotherapy drug) were tested on CRC cells, and the combination of Desatinib, Oxaliplatin, and Capecitabine, showed promising results in a phase I trial. [29] Irinotecan can be combined with tyrosine kinase inhibitors such as Sunitinib, Apatinib, Regorafenib, however, multidrug resistance is quite common while using this chemotherapy. [34]

In general, this combination of chemotherapy with targeted therapy is an important treatment for mCRC; nevertheless, severe side effect are frequent while using these kind of drugs. For this reason the combination with immunotherapy has made progress in the past years, but unfortunately, immunotherapy is effective only in a limited number of patients. [35] One example is the test in vivo of Lenvatinib and anti PD-1 antibody; moreover, Lenvatinib was also tested in a phase II trial with pembrolizumab in patients with mCRC (phase II to test safety is still ongoing- NCT04776148). In addition, also regorafenib has been tested in combination with anti-PD-1 antibody in CRC cells and in a phase I trial (RECOGNIVO) using Nivolumab. This trial showed a manageable safety profile and encouraging antitumor activity; however, phase II trial revealed only manageable safety and therefore more investigations are needed. In contrast, Apatinib with anti-PD-1 antibody showed good results in mice, yet it failed in the clinic. [29] [36]

An additional strategy is the combination of TKIs with antibodies. Cabozantinib, for example, has been tested with Centuximab (an anti-EGFR antibody) in CRC cells, and the results revealed that the use of the antibody can help to reduce resistance. The same tyrosine kinase inhibitor has also been evaluated using Panitumumab (another anti-EGFR antibody) in a phase I trial, but unfortunately, the results were not optimal and toxicity was high. TKIs can also be combined with anti-VEGF antibodies, such as Bevacizumab. This has been done with imatinib in CRC xenograft tumors and subsequently in a phase I and II trials with promising results. [29]

In general, a limitation is the possible development of mutations in the tyrosine kinase receptors which can cause resistance to inhibitors. Therefore, to select the most appropriate treatment, a personal genomic profile can be conducted in order to increase the effectiveness and reduce the possibility of developing resistance. [22]

3.5 MONOCLONAL ANTIBODIES AGAINST C-KIT

As mentioned above, the majority of patients develop off-target effect during the treatment with tyrosine kinase inhibitors, and this cause side effects and toxicity. For these reasons, the use of specific c-KIT monoclonal antibodies has been considered for CRC due to their greater specificity for the target. At the moment, two c-KIT specific monoclonal antibodies under development (CDX-1058 and CDX-0159) are under development, which can suppress MCs and can be therefore used as therapy for solid tumors and CRC. [37]

2G4 is a fully human monoclonal antibody which can inhibit SCF/c-KIT signaling pathway with a very high affinity and, in particular, it binds D2-D3, which means that there is an overlapping with the SCF binding site. In a study using the LAD2 cell line, 2G4 was found to bind the c-KIT receptor with a much higher affinity compared to the normal ligand SCF, and by inhibiting the signaling pathway, proliferation was completely blocked. The monoclonal antibody 4C9 was also tested, but it was not able to completely inhibit the binding of SCF due to the fact that its binding site does not overlap with the SCF binding site, as is the case with 2G4. Therefore, the inhibition of proliferation was limited. [38]

Another monoclonal antibody against c-KIT recently developed is KITMAb which is able to bind the domain of KIT and in particular the 4^{th} and 5^{th} motifs. This monoclonal antibody inhibits the activation of KIT pathway and can also inhibit the cell growth. In vitro studies with cells transfected with c-KIT showed that KITMAb binds the domain of KIT and, by doing this, the dimerization of the receptor is inhibited. In fact, expression of KIT was reduced after the treatment with KITMAb, leading to a decrease in cell proliferation and an increase in cell apoptosis of cells expressing KIT receptor. [38] A positive feature of KITMAb is that it binds the extracellular region and, therefore, it is not necessary for this antibody to enter the tumor cells in order to reach the effective concentration. In addition, it

is also important to mention that this monoclonal antibody can inhibit both the ligand dependent and independent pathway, leading to a better effect. [39]

The antibody CK6 is a c-KIT antagonist antibody which can interfere with the interaction between c-KIT and SCF and decrease tumor growth. However, compared to the previous monoclonal antibody, it is able to block only the ligand dependent pathway. [39]

Table 2: tyrosine kinase inhibitors with c-KIT as a target. In this table is also possible to observe the other targets of these multi target inhibitors, the clinical trial in which they failed, the type of inhibitor, if it is reversible or irreversible and finally possible combinations with other treatments. The name in bold indicates the only tyrosine kinase inhibitor approved for the treatment of colorectal cancer.

Name	cKIT	Other targets	Clinical trials	Inhibitor (Type)	Reversible/Irreversible	Possible combinations tested for CRC
Imatinib	+	ABL, PDGFR	Phase II	Type II	Reversible	Imatinib + Bevacizumab
Cabozantinib	+	MET, VEGFR1, 2 and 3, AXL, RET, ROS1, TYRO3, MER, TRKB, FLT-3, TIE-2	Phase II	Type II	Reversible	Cabozantinib + Cetuximab
Dasatinib	+	BCR-ABL, SRC family, EPHA2, PDGFRα	Phase II	Type I	Reversible	Dasatinib + Oxaliplatin
Lenvatinib	+	VEGFR1,2,3, PDGFRα, FGFR RET	Phase II	Type I	Reversible	Lenvatinib + anti-PD-1 antibody
Pazopanib	+	VEGFR, PDGFRA, PDGFRB	Phase I	Type I	Reversible	Pazopanib + Irinotecan and Centuximab
Ponatinib	+	FGFR, PDGFR, SRC, RET, FLT1	Phase II	Type II	Reversible	Ponatinib + Blinatumomab
Sorafenib	+	PDGFRA, PDGFRB, KDR, FLT3	Phase II	Type II	Reversible	Sorafenib + Cetuximab
Sunitinib	+	PDGFRA, PDGFRB, KDR, FLT3	Phase II	Type I	Reversible	Sunitinib + Irinotecan
Apatinib	+	VEGFR2, s-SRC	Phase II	Type I	Reversible	Apatinib + anti-PD-1 antibody

Regorafenib	+	RET, VEGFR1, 2, 3, PDGFRα and β, FGFR1, FGFR2, TIE2, DDR2, Trk2A, Eph2A, RAF-1, BRAF, BRAFV600E, SAPK2, PTK5, Abl	Approved	Type II	Reversible	Regorafenib + anti-PD-1 antibody

DISCUSSION AND CONCLUSION

Colorectal cancer (CRC) is the third most common cancer in the world and, despite the progress in the existing therapies, the growth rate is expected to increase up to 60% more by 2030. [22]

The discovery of the pivotal role of mast cells in tumor development and growth has led to the study of these immunity cells as possible targets for the development of new therapies. In fact, lower mast cell density (MCD) was associated with better prognosis, as mast cells are able to release numerous pro-tumorigenic factors that promote tumor growth, proliferation, angiogenesis, metastasis (extracellular matrix degradation), and immuno-suppression. [40] Mast cells density can be affected using several methods, among them, one of the main ones is the inhibition the c-KIT receptor in order to decrease the number of mast cells. [26] c-KIT is a receptor belonging to the type III tyrosine kinases receptors, and its ligand, SCF, is the most important cytokine for mast cells. An important feature of this receptor is that its expression decreases during hematopoietic differentiation, while it increases only in mast cells, dendritic cells, and natural killers, indicating its role during chronic inflammation and tumor. [5] These considerations have led to the development of drugs capable of inhibiting c-KIT overexpression on mast cell such as tyrosine kinase inhibitors (TKIs) and later monoclonal antibodies capable of inhibiting c-KIT in a more specific manner. [22]

Over the past two decades, tyrosine kinase inhibitors have played a very important role in the treatment of cancers, both in general and in colorectal cancer. Despite this, there are two major problems associated with this class of drugs: the development of resistance (which is very common) and toxicity due, in particular, to off-target effects. Toxicity is an integral part of TKIs, and one possible solution to reduce it, is to determine more precisely the affinity of the various TKIs for other targets in order to choose the most specific and the best one for each case. [28] All the tyrosine kinase inhibitors available so far with c-KIT as a target, and that can be therefore potentially used for the treatment of colon cancer, are reversible multi-target inhibitors. Consequently, in addition to c-KIT, other receptors such as EGFR, PDGFR, VGFR are inhibited. The only multi-target kinase approved for the treatment of colorectal cancer is Regorafenib, which is indicated as monotherapy only in adults with mCRC who failed previous treatments with chemotherapy, anti-VEGF, and anti-EGFR therapy and therefore with a poor prognosis. This is due to the fact that, although it has shown positive results in the clinic, adverse events (AE) both mild or moderate/severe are quite common (>90%). The majority of patients, in fact, experienced HFSR (hand-foot skin reaction), diarrhea, hypertension, and ore

severe events such as liver injury, gastrointestinal perforation and hemorrhage (more than one in 10 people). All of these toxicities associated with this drug limit the use of Regorafenib. [41]

An initial important subdivision that can be made for TKIs is between those that inhibit only mast cell activation, those that inhibit non-mast cell pathways, and those that inhibit both. While TKIs that can only inhibit mast cell activation/infiltration function by preventing mast cell activation and obstructing the release of inflammatory mediators, TKIs that can inhibit non-mast cell pathways function by targeting pathways involved in the growth and progression of cancer, including angiogenesis, cell cycle inhibition and apoptosis. [42]

In general, TKIs for the treatment of colorectal cancer are capable of inhibiting both pathways. Regorafenib, for instance, is able to inhibit numerous kinases, including those involved in mast cell activation and infiltration. In addition, numerous studies have shown that Regorafenib is also able to inhibit the activity of other non-mast cell pathways, such as MAPK and PI3K on cancer cells. [32] [43]

Another example of TKI that is able to inhibit both is Sorafenib. In fact, it has been found to inhibit both the mitogen-activated protein kinase (MEK) and Raf/MEK pathways. It is thought to work by preventing cells from forming new blood vessels, which limits the nutrient and oxygen supply to tumors. This inhibition of both pathways can help to reduce the risk of tumor progression and metastasis. [44]

Based on this evidence, it is advantageous to have a TKI that can inhibit both mast cells and non-mast cell pathways for the treatment of cancer in general, and in particular for colorectal cancer. This enables a more thorough method of targeting cancer, it can decrease the potential to acquire treatment resistance, increase efficacy and enhance patient outcomes. [45]

In addition to the subdivision mentioned above, TKI can be divided into five types of which only the last one creates an irreversible covalent bond, while the first four classes are reversible inhibitors.

Recently, covalent/irreversible inhibitors have been shown to have several advantages in both reducing resistance and avoiding off-target effect. One important feature for a drug is to obtain strong potency, which is increased in covalent inhibitors whereas, in non-covalent ones, the potency is dependent on intramolecular forces. In addition, an irreversible binding allows a reduction in the doses to be administered because the duration of action of an irreversible inhibitor is longer than that of a reversible inhibitor. Finally, as also previously mentioned, irreversible inhibitors are less likely to develop resistance. In fact, in reversible inhibitors, mutations lead to mutant-active structure and

resistance are directly proportional to the number of cancer cells. For covalent inhibitors, on the other hand, is more difficult to develop resistance. [46] One thing that needs to be taken into consideration while using irreversible inhibitors is that this class in not the best choice when toxicities are due to a prolonged target inhibition. [46]

For the treatment of colon cancer, currently, there are no irreversible tyrosine kinase inhibitors, but only reversible ones. Recently, a second generation tyrosine kinase inhibitors, Afatinib (Giotrif®), has been developed for the treatment of non-small cell lung cancer (NSCLC). This inhibitor targets wild-type EGFR, HER-2 and HER-4, however, it was found to be effective also against common EGFR mutations both in vitro and in vivo. In addition, adverse events of this irreversible tyrosine kinase inhibitor were controllable, manageable, and expected. In fact, patients experienced mainly diarrhea rash or acne, and stomatitis or mucositis. [47]

Based on these evidences, a possible attempt for the treatment of colon cancer could be the development of irreversible tyrosine kinase inhibitors against the c-KIT target. This could decrease the major problem of resistance and potentially also avoid toxicities which are common due to off-target effects during the treatment with reversible first-generation inhibitors such as Regorafenib.

The choice to take c-KIT into analysis as a target for the development of therapies for colorectal cancer is due to the fact that its ligand, SCF, is the principal factor influencing the number of mast cells, their phenotype, and their function. Despite this, should future treatments targeting this receptor prove unsuitable, it is important to keep in mind that other receptors such as EGFR and VEGFR play a key role in the development and growth of this tumor. Therefore, they can also be considered possible good candidates as targets in order to decrease mast cell density (MCD). In fact, Epidermal Growth Factor receptors (EGFR, HER2, HER3, HER4 receptors) expression is a negative prognostic factor for CRC and has become a target for the treatment of metastatic colorectal cancer. Numerous multitarget tyrosine kinase inhibitors and monoclonal antibodies have been developed against this receptor, however, also in this case there are no single-target inhibitors. [22]

To conclude, despite the many steps forward in recent years, further improvements are needed in order to solve the major problems associated with these drugs. The main goal is to develop more selective TKIs and not only multi-kinase inhibitors, which cause high levels of toxicity. Moreover, since this limited selectivity is also the main cause of resistance, which is another unresolved issue, next generation TKIs could lead to an improvement also on that front. This will increase the overall survival (OS) and ensure a better quality of life for patients with this condition. [48]

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