Chapter 3

The Use of Molecular Pathway Inhibitors in the Treatment of Osteosarcoma

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Abstract

Presently, the 5-year survival rate for metastatic osteosarcoma remains low despite advances in chemotherapeutics and neoadjuvant therapy. A majority of the morbidity and nearly all of the mortality in osteosarcoma rely not in the primary disease but in the metastatic disease. The pursuit of novel molecular therapies is attractive due to their targeted ability to combat metastasis. Unlike traditional chemotherapy agents, which work by targeting rapidly dividing cells, targeted therapies may spare normal cells and decrease the adverse effects of chemotherapy by targeting specific pathways. Here, we discuss key molecular pathways in osteosarcoma and their ability to be modulated for the goal of eradication of primary and metastatic disease. We focus specifically on the aldehyde dehydrogenase (ALDH), epidermal growth factor receptor (EGFR), and insulin-like growth factor-1 receptor (IGF-1R) pathways.

Keywords: osteosarcoma, molecular inhibition, metastasis, ALDH, EGFR, IGF-1R

1. Introduction

Prior to the use of chemotherapeutics, the 5-year survival rate of osteosarcoma (OS) was approximately 20% [1]. Despite new surgical techniques and the adoption of neoadjuvant therapy, patients diagnosed with nonmetastatic OS have a 65.8% 10-year survival rate, while those diagnosed with metastatic disease have a 15–30% 5-year survival rate [2]. These statistics have not improved in a generation. This stagnation may reflect recurrent disease as well as the intrinsic resistance of OS to chemotherapy.

The pursuit of targeted molecular therapies to treat OS has increased in popularity over the past decade. The inhibition of specific molecular pathways critical to OS metabolism may decrease its metastatic potential, slow its rate of growth, and potentially eliminate the disease altogether. Unlike chemotherapeutics, which act on all rapidly dividing cells, targeted therapies may be mechanistically independent in their efficacy. By specifically targeting OS cells, we may save normal cells and decrease the risk of adverse clinical side effects [3].

Here, we examine the inhibition of specific molecular targets that are critical to the biologic pathways of OS, but may spare other critical organ systems from damage.

2. Aldehyde dehydrogenase (ALDH)

Aldehyde dehydrogenases (ALDHs) are a superfamily of nicotinamide adenine dinucleotide phosphate ($NADP^+$)-dependent tetrameric enzymes that participate in aldehyde metabolism via catalysis of exogenous and endogenous aldehydes into their corresponding carboxylic acids and the cell's resistance to oxidative stress [4–7]. Inhibition of ALDH can lead to a build-up of aldehydes that can lead to toxic side-effects, which include enzyme inactivation, DNA damage, impairment of cellular homeostasis, and cell death by forming adducts with various cellular targets [4, 8, 9].

Cancer stem cells (CSCs) comprise a small, distinct subpopulation of cancer cells that demonstrate robust self-renewal properties, enhanced differentiation capacity, the ability to propagate tumor growth, and increased resistance to chemotherapeutic drugs. ALDHs have been identified in numerous studies as elevated in highly malignant tumors and in CSCs [4, 10–12]. ALDHs exert their effects through cellular processes such as target gene expression, protein translation, signal transduction, and antioxidative mechanisms. ALDH has, therefore, been implicated as a potential CSC marker. Cells found to be high in ALDH have demonstrated enhanced tumorigenicity in multiple cancers [7].

Elevated ALDH levels have been associated with poor survival in patients with breast and ovarian cancers [13, 14]. ALDH expression also appears to be linked with metastatic potential. Semisolid matrigel matrix invasion assays showed a correlation between ALDH levels and increased invasiveness when comparing two murine OS cell lines [7]. OS cells treated with disulfiram, an ALDH-inhibitor, show reduced ALDH expression and altered cellular morphology, with fewer invadopodia and greater shape uniformity [6, 15, 16].

2.1. Pathophysiology

Reactive oxygen species (ROS) are a natural by-product of aerobic metabolism and can lead to DNA damage, protein degeneration, and lipid membrane destruction. Cancer cells often generate abnormally high levels of ROS because of the aberrant metabolism and protein translation typical of diseased cells [17]. ALDHs play a vital role in clearing ROS and reducing the oxidative stress caused by ultraviolet radiation and chemotherapeutic agents. Cells that have high levels of ALDH expression have consistently lower ROS than those incapable of such expression [18–20].

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CSCs have relatively low levels of ROS, which may be because of elevated antioxidant enzyme levels [6, 21, 22]. The protective effects of ALDH for CSCs may also include the inhibition of downstream apoptosis-related pathways [18, 23, 24]. ALDH-positive CSCs have also demonstrated resistance to myriad chemotherapeutic agents such as anthracyclines and taxanes [25, 26], two classes of drugs commonly used in OS treatment. ALDH-positive cancer cells develop this drug resistance in part because of their increased ability to metabolize certain drugs into their nontoxic byproducts [27]. Once tumors are treated with chemotherapy or radiotherapy, the levels of CSCs with high ALDH expression tend to increase, increasing the cells' abilities to become drug-resistant [25, 28].

Retinoic acid (RA) signaling plays a pivotal role both in embryonic [29] and tumor cells [30]. This pathway in fact exerts an antitumor effect. This is due to activation of a series of cellular genetic programs that modulate cell differentiation, apoptosis, and growth involved in the classical RA pathway [4, 31] (**Figure 1**). In this pathway, retinol is absorbed by cells, oxidized to retinal, and then oxidized to RA by ALDH. RA then enters the nucleus and can induce the transcriptional activity of downstream effectors through activation of heterodimers of the RA

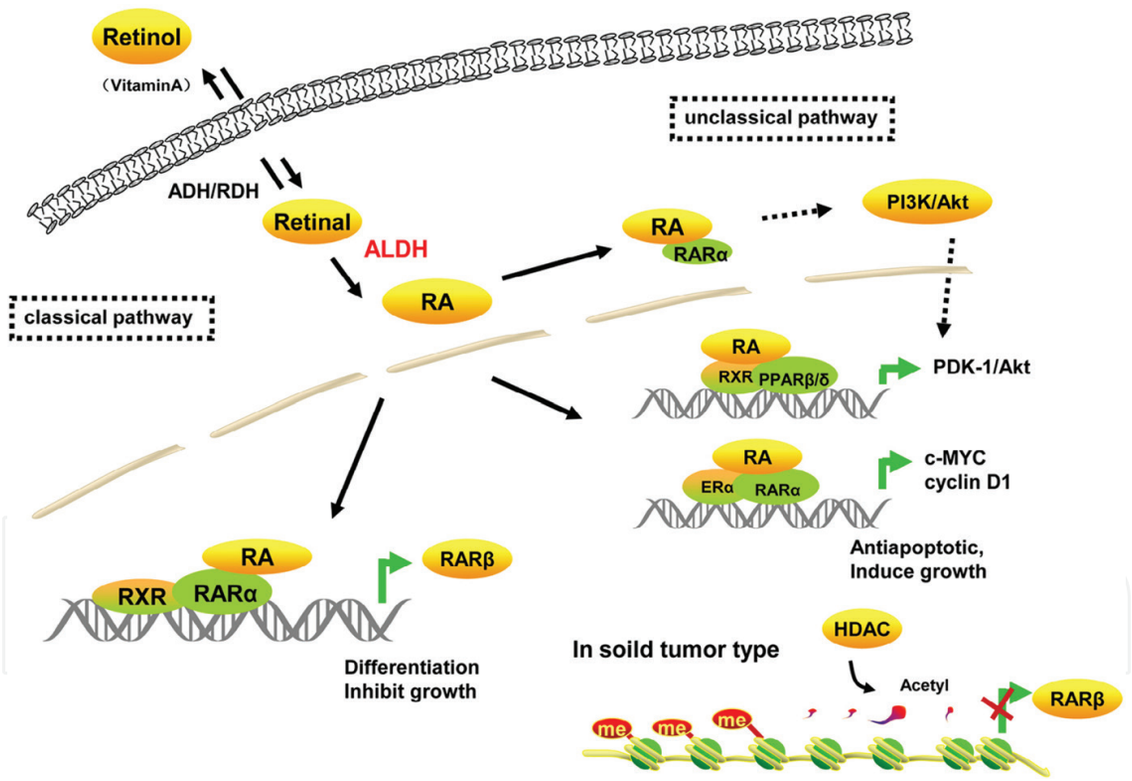

Figure 1. Potential retinoic acid-mediated signaling pathway in CSCs. Retinol (vitamin A) absorbed by cells is oxidized to retinal by retinol dehydrogenases. Retinal is oxidized to retinoic acid by ALDH enzymes. The metabolized product retinoic acid includes ATRA, 9-*cis* retinoic acid, and 13-*cis* retinoic acid, entering the nucleus and associated with RARα. In the classical pathway, retinoic acid binds to dimers of RARα and RXRs to induce the expression of its downstream target genes including RARβ. In the solid tumor type, RARβ promoter is methylated and/or the histones are significantly deacetylated, leading to low expression. In the nonclassical pathway, retinoic acid binds to dimers of RXRs and PPARβ/δ to induce the expression of its downstream target genes including PDK-1/Akt. In cells expressing ERα, retinoic acid can bind to dimers of RXRs and ERα as well as induce the expression of c-MYC and cyclin D1. Retinoic acid which extranuclearly binds with RARα can also induce the expression of c-MYC and cyclin D1 through the PI3K/Akt signaling pathway.

receptor and retinoic X receptors. RA binds its nuclear receptors and activates gene expression that affects loss of CSC markers, differentiation, cell cycle arrest, and morphology [32, 33]. The upregulation of these receptors generates a positive feedback loop for RA signaling. ALDH serves a paradoxical role in the RA pathway, by inducing differentiation of CSCs. The overall effect of this is antitumor, and thus exploiting this pathway is the goal for certain therapeutics.

2.2. Therapeutic applications

Disulfiram (DSF) has been shown to enhance the cytotoxicity of several anticancer drugs, as well as radiotherapy, which early on indicated its potential role as either a novel chemotherapeutic agent or a sensitizer for other treatments [34]. Theories of its mechanism include the induction of oxidative stress and inhibition of proteasome activity through c-Jun N-terminal kinase (JNK), NF-κB, and PI3K pathways [35–38].

In metastatic OS, the phenomenon of CSCs plays a large role in the ability of the disease to withstand a great amount of stress and remain invasive. ALDH is considered not only a surrogate marker for these cells but also a functionally important target [39]. The beauty of ALDH serving both roles is that the effects of DSF can be targeted to tumor cells exclusively due to their high ALDH content and additionally exert its antitumor effects. As described above, ALDH serves a pivotal role in reducing ROS to protect CSCs from oxidative stress and subsequent intracellular destruction. DSF as an inhibitor of this process has been shown to make the cancer cells more susceptible to oxidative stress and subsequently to improve survival in many cancer patients [40, 41].

DSF has also demonstrated efficacy in defeating the invasive nature of cancer by inhibiting matrix metalloproteinases (MMPs). In metastatic cancer physiology, the degradation of the extracellular matrix allows for primary tumor metastasis and distal site invasion. MMPs facilitate this process and are known to be closely associated with tumor growth and metastasis. In one study, nontoxic ranges of DSF successfully suppressed MMP-2 and MMP-9 activity and expression, producing a near complete growth inhibition at a 10 μM concentration of DSF [42].

Various studies have demonstrated that the cytotoxicity of DSF is copper dependent [38, 43, 44]. Copper plays an essential role in redox reactions and triggers generation of ROS in both normal and tumor cells [37, 44]. As a bivalent metal ion chelator, DSF forms a complex with copper and allows for Ctrl-transporter-independent transport of copper into tumor cells [43, 45]. For this reason, the DSF-copper complex is a much stronger inducer of ROS [46]. Furthermore, the abundance of copper in cancer cells enables DSF to specifically target cancer as opposed to normal tissues [47].

Copper ions promote ROS formation, which has been shown in multiple cancer cell lines [44]. Two forms of intracellular copper (cupric and cuprous) induce the formation of hydroxyl radicals from hydrogen peroxide, which serve to damage a variety of intracellular molecules [48]. Since studies have demonstrated that cytotoxicity of DSF appears to be copper dependent, the high concentration of copper in CSCs allows for an excellent substrate on which DSF can act in the treatment of cancer [43].

RA has been shown to inhibit proliferation of malignant tumors and induce apoptosis and differentiation [32, 49–53]. Most notably, all-*trans*-retinoic acid (ATRA) is an effective treatment

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for acute promyelocytic leukemia (APL) and has been shown to result in complete remission [50, 54]. RA is derived from ATRA by the action of ALDHs. Since ALDH is often specifically upregulated in CSCs, clever design can exploit this pathway for tumor suppression [49].

In mouse model studies, the highly metastatic K7M2 OS cells seem to be preferentially targeted by RA [49]. The role of retinal in decreasing cell proliferation and cell survival was demonstrated by exposing cells to oxidative stress in the form of hydrogen peroxide. ALDH-high K7M2 cells exhibited a greater increase in apoptosis compared to ALDH-low cells. Additionally, RT-PCR demonstrated that retinal treatment resulted in downregulation of various genes involved in cell proliferation and cell survival in a dose-dependent manner [49]. This would suggest that retinal can effectively be used as a cellular "Trojan Horse" of sorts to specifically target OS cells, as the very ALDH-rich nature that is crucial to their metastatic potential leads to their willful acceptance and rapid metabolism of retinal, leading ultimately to their demise.

3. Epidermal growth factor receptor (EGFR)

In order to obtain enough EGFR protein to biochemically purify and sequence, scientists initially used an epidermoid carcinoma cell line which was found to contain 100-fold higher levels of the receptor tyrosine kinase (TK). Since then, aberrant EGFR signaling has been implicated in the development and progression of many types of carcinomas including small cell lung, breast, stomach, prostate, ovarian, and glioblastoma. In the past decade, more attention has been placed on the role of EGFR signaling in OS.

3.1. Pathway physiology

Epidermal growth factor (EGF) was the first growth factor to be discovered and was found to have significant mitogenic effects of multiple cell types. Its receptor EGFR is a receptor tyrosine kinase (TK) which contains an extracellular domain where binding occurs to ligands of the EGF family such as, TGF-α, EGF, β-cellulin, epiregulin, and heparin-binding EGF. EGFR also contains a hydrophobic transmembrane region and a cytoplasmic TK domain [55]. Ligands bind to the cell surface domain and cause a conformational shift in the intracellular domain of the protein, which leads to dimerization and autophosphorylation. This phosphorylation then activates several other proteins downstream such as JNK, Akt, and mitogen-activated protein kinases (MAPK), which are responsible for normal cellular functions such as proliferation, apoptosis, adhesion, DNA synthesis, and migration. Signaling also occurs through other related TKs: HER2, HER3, and HER4. EGFR also has been shown to activate NFκB signaling, as well as being linked to certain G protein-coupled receptor signaling.

3.2. Pathophysiology

EGFR structure and function is closely related to erbB oncogene of avian erythroblastosis virus. The oncogene erbB is a part of a larger family of ErbB TKs including ErbB2 or HER2, HER3, and HER4. In addition, sequence anomalies found in the extracellular domain of EGFR were found to cause constitutive signal transduction independent of binding. Overexpressed

EGFR levels in cancer cells also cause EGFR to undergo ligand-independent firing due to spontaneous activation of TK activity [56].

Recently, more attention to the action and therapeutic intervention of aberrant EGFR signaling in OS has been studied. Immunohistochemistry demonstrated high EGFR protein expression in 57% of 37 established bone tumor-derived cell lines [57]. Additionally, 90% of 27 OS biopsy samples showed moderate-to-high EGFR protein levels, as well as in four established OS cell lines HOS, KHOS/NP, MG-63, and U-2 OS . EGFR expression was not found to correlate to response to preoperative chemotherapy or survival [58]. Another group demonstrated that OS cell lines, MG-63 and Saos-2 proliferative abilities, were decreased by natural flavonoid Icariside II. Treatment also inactivated EGFR/mTOR signaling pathway including PI3K, serine/threonine protein kinase (Akt), mitogen-activated protein kinase kinase (MEK), and Extracellular-Signal-Regulated Kinases (ERK) [59].

3.3. Therapeutic applications

3.3.1. Gefitinib (Gef)

This molecular inhibitor of EGFR acts by binding to the cytoplasmic adenosine triphosphate binding site of the TK domain [60]. Signaling dysfunction leads to an inhibition of downstream malignant phenotypes through Akt, MAPK, and Ras signal cascades. Gef is used clinically in non–small-cell lung cancer known to be harboring aberrant EGFR levels, typically used in combination with other chemotherapy regimens.

Researchers have shown under serum starvation, EGFR inhibition in OS cells by Gef was more pronounced compared to normal conditions, suggesting that aberrant EGFR signaling contributes to OS progression but is not the major driver for proliferation. The EGFR inhibitor Gef was found to moderately synergize with doxorubicin and methotrexate in attenuating the proliferative capabilities of OS cell lines U-2 OS, Saos-2, OS-9, and others. Gef EGFR inhibition antagonized the cytotoxic effects of cisplatin [61].

3.3.2. Erlotinib (Erl)

Erl is another molecular inhibitor of EGFR via the ATP binding site of the cytoplasmic domain [62]. Erl is used in treating advanced metastatic non–small-cell lung cancer and pancreatic cancer, usually in combination with other chemotherapies.

Canine OS cell lines treated with another selective EGFR inhibitor Erl did not inhibit downstream protein kinase B (PKB/Akt) activation, and vascular endothelial growth factor (VEGF) levels increased. Conversely, Erl enhanced the effects of radiation therapy on a subset of OS cell lines [63].

3.3.3. Trastuzumab (Tra)

As the name suggests, Tra is a monoclonal antibody which interferes with normal HER2 receptor functioning of EGFR [64]. It has been suggested that Tra does not alter receptor expression but instead causes inhibition of downstream Akt and MAPK proliferation signaling. A phase

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II clinical trial of metastatic OS with EGFR2 overexpression showed that Tra can be safely delivered in combination with anthracycline-based chemotherapy [65].

Targeting one substrate of the receptor TK signaling cascade is likely insufficient to effectively abrogate downstream effects. Incremental improvements for the treatments of OS will depend on the novel chemotherapeutic interactions now being observed in the laboratory. BreAkthroughs will occur by further testing intricate combination therapies including sensitizers like EGFR inhibitors (Erl and Gef) with traditional chemotherapeutics such as doxorubicin, methotrexate, and cisplatin.

4. Insulin-like growth factor-1 receptor (IGFR-1R)

Insulin-like growth factor-1 receptor (IGF-1R) has been shown to play role in various cancers, including pediatric sarcomas. IGF-1R is just one cog in the complicated system of insulin-like growth factor (IGF) and insulin family of growth factors and is located in various tissues including bone. It plays an important role in regulating bone homeostasis, and activation of this unique TK receptor leads to several important downstream signaling cascades that play a crucial role in cell proliferation and protein synthesis. Aberrant signaling in the IGF-1R pathway may be implicated in the development of OS. Studying the basic physiology and pathophysiology in this pathway has been critical to the development of OS-targeted therapy. Here, we examine the basic biology of IGF-1R in relation to OS- and molecular-targeted therapies that exploit this signaling pathway.

4.1. Physiology

IGF-1R signaling is involved in normal osteogenesis and bone homeostasis [66]. IGF-1R is a type II receptor TK consisting of two α- and two β-subunits. The binding of IGF-1 to IGF-1R induces autophosphorylation of tyrosine residues in the kinase domain. This autophosphorylation leads to the downstream activation of insulin receptor substrate (IRS) proteins and Shc, an adapter protein between IGF-IR and the network of their signaling pathways [67, 68] (**Figure 2**). Phosphorylation of Shc and its binding to Grb2 is required for the activation of mitogen-activated protein kinases (MAPK)/extracellular-signal-regulated kinases (ERK), both important regulators of proliferation, invasion, angiogenesis, and inflammatory responses [69, 70].

There are four isomers of IRS, and of these isomers, IRS1 and IRS2 are expressed in osteoblasts. These adaptors are important in normal bone turnover. Furthermore, deficiencies in IRS1/2 impair osteoblast proliferation and differentiation and result in decreased bone mass [71, 72]. IRS1 is one of the many activators of phosphatidylinositol 3 kinase (PI3K). PI3K converts phosphatidylinositol 4,5-biphosphate (PIP2) into phosphatidylinositol 3,4,5-triphosphate (PIP3), which then recruits the signaling proteins PDK1 and Akt to the plasma membrane [73]. The PI3K/Akt pathway is implicated in the proliferation and invasion of malignant OS via multiple pathways, such as increasing the expression of cyclins and cyclin-dependent kinases that act as positive regulators of the cell cycle in OS [74]. The mammalian target of rapamycin (mTOR) is one of the most important downstream effectors of PI3K/Akt and controls cell cycling and protein synthesis by activation of its downstream targets p70S6K and 4E-BP [68].

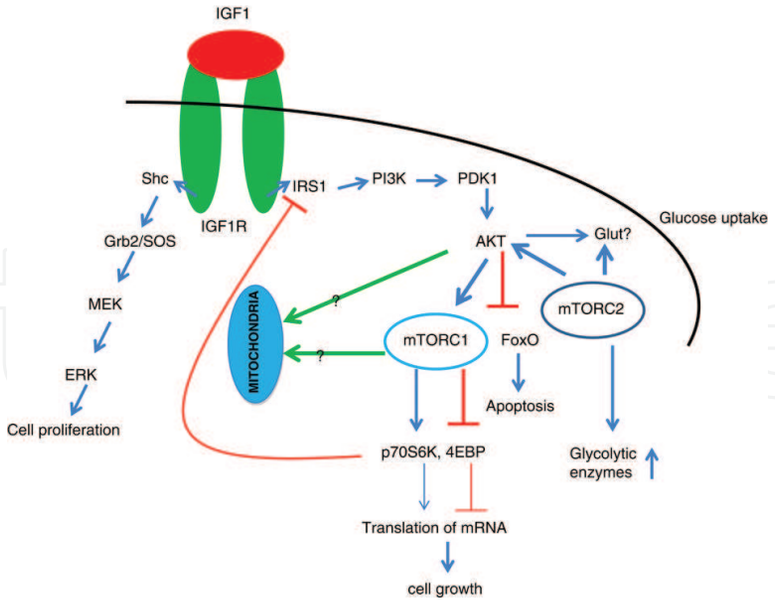

Figure 2. IGF-1 signaling pathway which can activate MAPK and PI3K signaling pathways.

Aside from regulating insulin's control of carbohydrate metabolism, the ligands IGF-1 and IGF2 may play a role in the neoplasticity of OS [75]. It has been demonstrated that there may be increased local IGF-1 levels in primary OS, which may affect survival, aggressiveness, and chemotherapeutic response [76]. Activation of IGF-1R by IGF-I stimulates OS cell growth *in vitro* and *in vivo* [77]. IGF-1 levels peak during adolescence, also the same age where OS incidences peak [78]. Interestingly, IGF-2 levels are increased in OS after chemotherapy treatment and may increase OS cell survival by inducing an autophagic state of dormancy, protecting OS against chemotherapy [79]. These ligands' influences in the tumorigenicity of OS have made them attractive targets in OS treatment. However, the only IGF-1 neutralizing antibody in clinical trials is MEDI-573 and is still in the early stages of development [80].

It is not completely clear yet whether mutations in IGF-1R contribute to cell growth, differentiation, apoptosis, and so on. Interestingly, mutations in IGF-1R are rare and produce growth retardation rather than neoplasia [81]. The recent discovery of somatic mutations in the IGF-1R kinase catalytic domain showed a small reduction in peptide phosphorylation. However, the mutant kinase domains were active, not hyper-activated relative to the wildtype [82]. Interactions between wildtype and mutant variants of the tumor suppressor gene, p53, and IGF-1R have also been studied. Normally, p53 suppresses the activity of IGF-1R, thus preventing cell proliferation. However, mutant variants of p53 derived from tumor have shown to enhance promotor activity and increase the transcription of IGF-1R, increasing the survivability of malignant cells [81, 83, 84].

4.2. Therapeutic applications

Currently, there are several IGF-1R inhibitors categorized into TK inhibitors, monoclonal antibodies, or microRNA targets of IGF-1R. Monoclonal antibodies against IGF-1R ligands have been studied but may be ineffective because of the redundancy in autocrine and paracrine secretion

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of this growth factor [85]. Several monoclonal antibodies against IGF-1R, such as Ganitumab or Dalotuzumab, are still being tested but tend to have a stronger inhibitory effect when combined with other therapies such as Rapamycin, an mTOR inhibitor [80]. Here, we focus on one small molecular IGF-1R inhibitor, OSI-906, and assess its current status in OS therapy.

The ATP-binding or substrate-binding site in the IGF-1R kinase domain can be targeted by small-molecule inhibitors, thus inhibiting IGF-1R signaling. An example of these inhibitors is OSI-906 (Linsitinib), a highly selective, small-molecule dual IGF-1R/IR kinase inhibitor given in an oral formulation that is in clinical trial. It has been shown that OSI-906 inhibits the downstream effectors of IGF-1R, ERK1/2 and Akt, thus affecting cell survival and proliferation [86]. One of the issues with molecular targeting of IGF-1R is the high degree of homology between the binding sites in IGF-1R and the insulin receptor. Molecular targets that cross-react with the insulin receptor may produce unwanted side effects such as dysregulating glucose metabolism [87]. Fortunately, OSI-906 exhibits a nine-fold selectivity for human IGF-1R over human insulin receptor [88]. The inhibitory effect of OSI-906 was tested on four unique OS cell lines and was found to inhibit phosphorylation of IRS-1 and proliferation in three of the four OS cell lines tested [89]. OSI-906 in combination with the EGFR inhibitor, Erl, has also been tested on human colorectal cancer cell lines and found to exhibit a synergistic inhibition of cell proliferation and survival [88]. Though OSI-906 has been somewhat successful as a single-agent for inhibiting IGF-1R in OS, further studies examining combination therapies with OSI-906 are necessary.

5. Conclusion

There is definitely hope and evidence to apply targeted molecular therapies to treat OS. As our understanding of the different molecular pathways that affect OS improves, we will be better equipped to attack this disease in ways that were not available before. Though numerous molecular pathways have been described here, it is important to understand that there are many more pathways that exist or are under investigation. Clearly, there is still much to learn about the biology of OS and its targeted therapies. The weight of evidence described above suggests that we are steadily moving forward in the right direction.

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