Eg5 targeting agents: From new anti-mitotic based inhibitor discovery to cancer therapy and resistance

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Conflict of Interest

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Abstract

Eg5, the gene product of *Kif11* gene, also known as kinesin spindle protein, is a motor protein involved in the proper establishment of a bipolar mitotic spindle. Eg5 is one of the 45 different kinesins coded in the human genome of the kinesin motor protein superfamily. Over the last three decades Eg5 has attracted great interest as a promising new mitotic target. The identification of monastrol as specific inhibitor of the ATPase activity of the motor domain of Eg5 inhibiting the Eg5 microtubule motility *in vitro* and *in cellulo* sparked an intense interest in academia and industry to pursue the identification of novel small molecules that target Eg5 in order to be used in cancer chemotherapy based on the anti-mitotic strategy. Several Eg5 inhibitors entered clinical trials. Currently the field is faced with the problem that most of the inhibitors tested exhibited only limited efficacy. However, one Eg5 inhibitor, Arry-520 (clinical name filanesib), has demonstrated clinical efficacy in patients with multiple myeloma and is scheduled to enter phase III clinical trials. At the same time, new trends in Eg5 inhibitor research are emerging, including an increased interest in novel inhibitor binding sites and a focus on drug synergy with established antitumor agents to improve chemotherapeutic efficacy. This review presents an updated view of the structure and function of Eg5-inhibitor complexes, traces the possible development of resistance to Eg5 inhibitors and their potential therapeutic applications, and surveys the current challenges and future directions of this active field in drug discovery.

Keywords

Eg5

microtubules

antimitotic drugs

kinesin motors

drug discovery

1. Introduction

1.1 Functions of the kinesin motor Eg5 in mitotic spindle formation and maintenance

Eg5, also known as kinesin spindle protein or *Kif11*, was first identified as a protein coded by an mRNA which was specifically deadenylated and released from polysomes after Xenopus egg feritilization [1]. Based on the predicted amino acid sequence, the Eg5 protein was thought to be a member of the kinesin motor superfamily [2], a prediction which was further supported by the use of antibodies raised against short peptides with conserved amino acid sequences shared between members of the kinesin superfamily of motor proteins [3]. Further biochemical and cellular studies confirmed that indeed Eg5 was a spindle microtubule associated protein with a microtubule plus end directed motor activity [4] whose spindle association is dependent on phosphorylation of its C-terminus by Cdk1 [5]. The mitotic roles of Eg5 are not only limited in cross linking spindle microtubules but also recently extended in the regulation of microtubule-end dynamics due to its microtubule polymerase activity [6]. Additional non-nuclear functions for Eg5 are reported that include the transport of secretory proteins from the Golgi complex to the cell surface in non-mitotic cells [7], as well as the enhancement of translation by serving as a linker between ribosomes and microtubules [8]. Furthermore, in postmitotic neurons, Eg5 is involved in cell migration and in axonal and dendritic transport and morphology[9][10][11].

Eg5, as all members of the kinesin-5 family, self assembles into a homotetrameric minifilament, composed of two dimers arranged in an antiparallel fashion mediated by the assembly of a long central rod (Fig 1A). The two opposing ends of the mini filament are composed by the two globular N-terminal motor domains [12]. The motor domains convert the free energy of nucleoside 5′-triphosphate hydrolysis (typically ATP) into directed mechanical motion during their interaction with a microtubule track. Each Eg5 dimer is formed by a long alpha helical coiled coil that follows the motor domain [13]. The tetramer is assembled by the two dimers that built the central rod formed by four alpha helixes all intertwined with each other in antiparallel fashion [14]. Interestingly, the C-terminal tail domain of each of the Eg5 dimer has been shown to interact with the opposing N-terminal motor domain. Recently it was shown that the tail is capable to regulate the Eg5 motor activity by stabilizing the microtubule binding state by slowing the ATP binding resulting in high force production at both homotetramer ends [15]. This slows the movement of Eg5, allowing Eg5 tetramers to cluster and work together, generating more force on microtubules.

Furthermore, the opposing pairs of motors heads located at either end of the coiled-coil stalk could cooperate to enhance processivity. Based on the average run length for the dimer, and assuming each pair of dimers in the homotetramer moves independently, the tetramer should remain attached to the spindle for ~64 steps, on average [16].

At the G2 to M transition, the tetrameric nature of Eg5, with two motor heads in each side allows Eg5 to engage and crosslink two antiparallel microtubules and move them in opposite directions, causing them to slide past each other, an activity necessary for the establishment of a bipolar spindle [4][5][17]. Lack of Eg5 activity either through motor activity inhibition [18] or depletion of the motor by RNAi [19][20] leads to failure of the separation of the duplicated centrosomes and the construction of a monoastral spindle leading to a mitotic block (Fig 1B).

In previous work, a targeted null allele [21] and gene trap allele in *Kif11* (the gene coding for Eg5) produced no apparent phenotype when present in the heterozygotes state with a wild type (WT) allele, but caused pre-implantation embryonic lethality in the homozygous state [22]. Interestingly, through a number of exome sequencing studies of individuals diagnosed with Microcephaly Lymphedema Chorioretinoma Dysplasia (MLCRD) it became apparent that *Kif11* mutations are linked to this human syndrome [23]. The list of mutations in *Kif11* linked not only to MLCRD but also to the related syndrome microcephaly with or without chorioretinopathy, lymphedema, or mental retardation (MCLMR) (OMIM 152950), and to Familial Exudative Vitreoretinopathy (FEVR) [24] as well as to Molecular and cone/cone-rod dystrophies (MD/CCRD) [25]. In most cases, genetic analysis concluded that the mutations were inherited in an autosomal dominant manner. Expression analyses in chick embryos upon stimulation of *in vivo* angiogenesis with vascular endothelial growth factor-A (VEGF-A) indicated up-regulation of *Kif11* kinesin-encoding gene predominantly in lymphoblasts and endothelial cells [26]. In the same study, chemical inhibition of Eg5 activity blocked endothelial cell proliferation and migration *in vitro* and mitosis-independent vascular outgrowth in aortic ring cultures was strongly impaired. *In vivo*, interfering with *Kif11*/Eg5 function caused developmental and vascular defects in zebrafish and chick embryos and potent inhibition of tumor angiogenesis in experimental tumor models [26]. More recent studies aiming to explore the pathogenic basis of *Kif11*-associated retinal vascular disease, led to the generation of a conditional *Kif11* knockout mouse. Postnatal endothelial cells-specific knockout of *Kif11* was associated with a marked hypoplasia of the retinal vasculature and a milder hypoplasia of the cerebellar vasculature, implying that *Kif11* loss causes retarded retinal vascular development

[27]. The most rational explanation for the *Kif11* phenotype was that it reflects *Kif11*'s role in cell proliferation and, more specifically, in mitotic spindle dynamics during mitosis.

1.2 Eg5 as a valid target for the development of inhibitors for anti-mitotic drug therapy.

Eg5 is expressed in $CD34^+$ cell, testis, cardiac muscle cell and some other human normal proliferation tissues. However, the expression level is significantly lower than that in malignant tissues. Overexpression of Eg5 leads to significant instability of the genome in mouse models and carcinogenesis. Studies have evaluated the Eg5 expression and its correlation with clinicopathological characteristics in various malignant tumors. For example, activation of Eg5 expression contributes to the onset of B-cell leukemia [28], Eg5 over-expression has been reported in solid tumors such as lung cancer [29], renal cell carcinoma [30], metastatic castrate-resistant prostate cancer (mCRPC) [31], non-muscle invasive bladder urothelial carcinoma [32], laryngeal squamous cell carcinoma [33], high grade astrocytic neoplasm [34]; in addition, five human pancreatic cancer cell lines [35] and Estrogen Receptor (ER)-positive human breast cancer MCF-7 cell line were also observed higher Eg5 expression [36]. Eg5 overexpression is linked to unfavorable prognosis [37]. Eg5 has been reported to contribute to cancer cell migration, and angiogenesis impairment [26]. However, it is unclear if Eg5 overexpression reflects higher proliferation rate of a cell population or true protein excess in the cell population. Furthermore, in interphase cells, the intracellular localization of Eg5 was identified as a prognostic factor in hormone-naive prostate cancer patients; patients whose tumor expressed nuclear Eg5 had a decreased median overall survival and progressed more rapidly to metastatic castrate-resistant prostate cancer [31].

The cumulative data on Eg5 motor protein described above suggest that due to its important role in spindle formation during cell division, inhibition of Eg5 could indeed have both an anti-proliferative effect as well as an anti-angiogenesis effect. Together with the fact of the low expression of the protein in neurons, using inhibitors of Eg5 in the clinic could have the potential to overcome side-effects associated with classical microtubule targeting agents such as taxol, which include neutropenia, hair loss and peripheral neuropathy [38] and can overcome taxol resistance [39].

This perspectives article focuses on small-molecule inhibitors that target Eg5, and their performance in clinical applications in cancer. Current and future challenges facing the Eg5 inhibition field are also discussed.

1.3 Methods for identifying novel Eg5 inhibitors (phenotype, enzymatic and silicon based HTS methods)

In a high throughput screening (HTS) assays, cell cycle arrest in mitosis is an easily detectable phenotype. A combination of use of a dot blotting with mitotic specific markers, like nucleophosmin, combined with indirect immunofluorescence microscopy using antibodies to spindle proteins, such as tubulin, to evaluate the state of spindle microtubules (Fig 1B) can be used. Indeed, one of the first successful identification of a small molecule by HTS was the identification of a compound that blocked cells in mitosis with the characteristic mono astral spindle phenotype, named monastrol [18]. The discovery of monastrol raised the possibility that Eg5 may be a clinically important mitotic target and therefore a number of chemically distinct small molecules (dihydropyridines, S-Trityl-L-cysteine, β-carbolines and carbazoles, quanizoles, thiazoles, biphenyls and others reviewed in [40]) and their derivatives were further identified as potential antineoplastic agents with potential cancer chemotherapeutic properties (Fig 2).

One of the disadvantages of the phenotype screening assay is the subsequent work required to identify the intracellular target of the identified inhibitory small molecules. In the case of monastrol the resulting accumulation of mitotic cells with a striking monoastral spindles phenotype led the investigators to note the similarity of the mono asters in cells expressing a temperature sensitive allele of kinesin BimC in *Aspergillus* nidulans [41] as well as after microinjection of antibodies in cells [5]. Quickly after that, it became evident that monastrol could interfere *in vitro* with the ability of Eg5 to move microtubules. Loss of the Eg5 driven microtubule motility by monastrol was further attributed by the ability of monastrol to allosterically inhibit the microtubule stimulated ATPase activity of Eg5 [18].

An alternative to phenotype based screening is biochemical enzymatic assays in a high trough put setting. Eg5, like all kinesins, possesses an intrinsic ATPase activity, which is stimulated in the presence of microtubules by a factor varying from several hundred up to 10,000 times [42]. A fundamental question is how ATP binding, hydrolysis, and release of hydrolysis products, which are localized events, are harnessed to obtain unidirectional motion. A cascade of allosteric conformational changes within the motor domain is instigated following microtubule binding that lead to MgATP hydrolysis that provokes stepping of the motor along microtubules.

This is most likely achieved through the power stroke which is associated by a large, rapid structural change in the motor following ATP hydrolysis that can be used to do mechanical work. The release of the hydrolyzed nucleotide is the rate limiting step in the motor-ATP hydrolysis reaction. MgADP release from the motor is very rapid once the motor encounters MTs. Release of the nucleotide from the motor results in the high affinity state of the motor for microtubules (apo state). Subsequent binding of ATP to the apo-motor reduces the affinity of the motor for the MTs although it still remains bound to MTs. Hydrolysis of the MgATP to MgADP and Pi and the subsequent Pi release from the motor leads to the complete release of the motor from the MTs [43]. Since the MT binding site of the motor is in site distant to the nucleotide binding site the different nucleotide states of the motor must be communicated to the MT binding site in an allosteric manner.

Crystallography elucidated a lot of the biochemical steps associated with the ATP hydrolysis and power stroke associated with chemomechanical step of kinesins [44][45] and particularly Eg5 [46]. The motor domain of Eg5 as well as all other kinesins fits the canonical kinesin motor domain fold with an eight-stranded β-sheet sandwiched between three major α-helices on each side (Fig 3A)[47]. Each motor domain has one molecule of Mg^{2+}ADP bound in the nucleotide-binding pocket on the rear-left side of the motor consisting of the phosphate loop (P-loop), switch-I (sw-I), and switch-II (sw-II). Binding of the motor to MTs triggers ATP hydrolysis leading to the power stroke resulting in the stepping of the motor forwards. A key mechanical amplifier for motion is the neck linker (NL), a flexible 12–18 amino acid sequence that connects each motor head to the α-helical coiled-coil stalk. The neck linker undergoes a transformation from being flexible in both the ADP-bound and nucleotide-free states to being docked along the core motor domain in the ATP-bound state (Fig 3C). NL docking of the leading motor domain positions the lagging motor domain forward along the microtubule track, thereby is specifying the direction of motility. Therefore, small molecules that could bind to the motor domain of Eg5 could potentially interfere with any of the steps involved in the motor activity.

These characteristic enzymatic and structural features were exploited over the years to isolate and characterize molecule inhibitors of Eg5 activity [48][49]. The resulting plethora of small molecule inhibitors of Eg5 along with the elucidation of their corresponding Eg5-ligand crystal structures led also to the virtual screening for the identification of novel Eg5 inhibitors (Fig 4 and 5).

2. Enzymatic and structural characterization of Eg5 inhibitors

Over the last 20 years a number of inhibitors with different chemical scaffolds have been identified either by phenotype or enzymatic motor ATPase based HTS assays (Fig 2). Most of the molecules identified bind to the motor domain of Eg5 in stoichiometric manner inhibiting its microtubule activated ATPase activity and the Eg5 based microtubule motility *in vitro* assays and by doing so induce monoastral spindles in cells. Further chemical optimization of the initial leads has led some of the identified small molecules to clinical trials.

2.1 Loop 5 binding allosteric inhibitors

One group of inhibitors target a specific allosteric site in the Eg5 motor domain composed of helix α2/loop L5 and helix α3, which is approximately 10 Å away from the ATP binding site (Fig 3 and 4). This is the case for the inhibitors such as monastrol [50] and its chemical analogues S-enastron, S-dimethylenastron [51] and Mon97 [52], as well as MK-0731 [53], S-Trityl-L-cysteine (STLC) [54], Ispinesib [55], SB743921 [56], K858 [57][58] and Arry-520 [59]. Binding to this pocket induces a rearrangement of loop L5 from an "open" to a "closed" state, which allosterically transmits conformational changes in the ATP binding pocket, slowing down ADP release (Fig 3A and B). As a result, Eg5 is trapped in an ''ATP-like'' conformation and the mechanochemical cycle of the motor is inhibited. Based on structural information of Eg5 bound to STLC, ispinesib and Arry-520 one common theme has emerged is that ligand binding induces major structural changes in the switch II cluster (helix α4/loop L12 and helix α5 region) and the so-called neck-linker region. The binding of the ligands to the loop L5 region induces conformational changes in and around the inhibitor-binding pocket (switch I), which are transmitted to the switch II cluster and the neck-linker region following helix α6. Both intermediate and final bound states for the three inhibitors were evident by their corresponding structures (Fig 3C). In the intermediate bound state, the switch II cluster is in the obstructive conformation forcing the neck linker region to be perpendicular to the motor domain whereas in the "final" inhibitor bound state the neck-linker swings about 32 Å and is trapped in the docked conformation to the motor domain [59].

2.2 Eg5 ATP binding competitive inhibitors

More recently, another group of Eg5 inhibitors has been found to bind to a different allosteric pocket of the Eg5 motor domain [60][61][62]. Structural analysis for three inhibitors bound to the Eg5 motor domain has been elucidated either in complex with microtubules by cryo-

electron microscopy in the case of GSK1 [63] or bound to the motor domain in the case of PVZB1194 [64] and BI8 [62]. GSK-1 and PVZB1194 bind at the junction of helix-α4 and -α6 pocket which is 15 Å from the ATP-binding pocket (Fig 5) which differs from conventional allosteric inhibitors that bind to the allosteric α2/L5/α3 pocket of Eg5, mentioned in the previous section. In the presence of either inhibitor the ATP binding site was empty of either ATP or ADP consistent with biochemical assays showing the both inhibitors act as competitive inhibitors by binding to the α4 and α6 pocket near but not in the ATP binding site. The neck linker in both structures are in the docked conformation. In contrast to the loop5 L5 binding inhibitors, both GSK-1 and PVZB1194 specifically inhibit MT-bound Eg5.

Another ATP competitive inhibitor of Eg5 is BRD9876 [65]. BRD9876 also only inhibits microtubule-bound Eg5. Based on similar biochemical Eg5 inhibition properties and from docking simulations BRD9876 is predicted to bind to the same helix-α4 and -α6 pocket as GSK-1 and PVZB1194 do.

2.3 Natural products

Natural products are a rich source of bioactive compounds with a broad spectrum of biological activities. A number of them have been identified as inhibitors of Eg5 (listed in Fig 2). Terpendole E is a fungal-derived Eg5 inhibitor, which was originally identified as an acyl-CoA: cholesterol acyltransferase inhibitor. Terpendole E inhibits Eg5 ATPase activity the Eg5 driven gliding of MTs *in vitro* and cells exposed to Terpendole E block in mitosis with the characteristic mono astral spindles [66]. Interestingly, studies combining hydrogen exchange mass spectrometry with molecular dynamics of Eg5 motor domain in the presence of Terpendole E, showed that the inhibitor binds to the loop L5, as many of the allosteric Eg5 inhibitors do, but it appear to have a unique mechanism [67]. Terpendole E upon binding to the loop L5 it mediates allosteric changes that destabilize the β sheet core of the motor instead of inducing changes in the switch II region of Eg5 ATP binding site. The difference in the inhibition mechanism of Terpendole E may explain also the observation that Terpendole E is active in cells that are resistant to STLC or to GSK-1 inhibitors [68].

The marine sponge extract Adociasulfate-2 (AS-2) disrupts the ATPase activity of Eg5 and inhibits the activity of kinesin 1 in MT gliding motility assay [69]. However, AS-2 is a nonspecific

kinesin inhibitor as it competes for microtubule binding, targeting several superfamily members including KHC, MPP1, MKLP1, RabK6, KIFC1,KIFC3, CENP-E, and Eg5 [70][71].

Gossypol is a component of cotton seed extracts and is used as a fertility control agent due to its toxicity to sperm. Gossypol was also identified as an inhibitor of Eg5 ATPase activity [48]. Gossypol has been shown to possess anticancer effects against several cancer cell lines, including HCC cells [72]. In fact, Gossypol is now in phase II/III clinical trials for various cancers (www.clinicaltrials.gov). However, Eg5 inhibition many not be its primary intracellular target since numerous studies have revealed that Gossypol and its derivatives target the Bcl-2 family of proteins containing the anti-apoptosis proteins Bcl-2 and Bcl-XL [73].

Curcumin, a polyphenol derived from the rhizome of the plant Curcuma has been used traditionally in traditional systems of medicine because it induces toxicity selectively in tumor cells. The anti-proliferative mechanisms attributed to curcumin involve multiple signaling pathways and cellular targets including several enzymes, growth factors, transcription factors, cytokines, apoptotic regulators, DNA synthesis, and proteins involved in cell division and cell cycle regulation, including Eg5 [74]. Competition experiments for Eg5 binding in the presence of monastrol indicated that curcumin binds to a site different from monastrol in a stoichiometric manner [75]. Circular dichroism coupled to molecular dynamics indicated that curcumin could bind to a novel druggable site located 44 Å away from the microtubule binding site on Eg5, 34 Å away from ATP binding site and 28 Å away from the monastrol binding site. Curcumin in contrast with the L5 loop binding inhibitors stabilized the interactions between Eg5 and microtubules at higher concentrations. The most important conformational change observed with Eg5 was the opening and closing of switch II loop flanking the active site of Eg5.

2.4 Depletion of Eg5 expression in targeted cells.

RNAi therapeutics represent a powerful, clinically validated approach for the treatment of a wide range of debilitating diseases with high unmet medical need and to date, up to ten oligonucleotide drugs have received regulatory approval from the FDA [76]. The potential to selectively inhibit Eg5 overexpressing tumors using RNAi-nanomedicines may be a highly promising strategy to halt tumor progression and improve overall patient survival. Indeed, in a HTS screening of a library of 2'-methoxyethyl-modified antisense oligonucleotide (2'MOE ASO) molecules, one of the most effective ASO identified was that targeting Eg5 [77]. Similarly, a

number of siRNAs as well as shRNAs were shown effective in disrupting Eg5 expression in cells leading to monoastral spindles and mitotic arrest. However, efficient tissue-specific delivery of siRNA remains the major limitation in the development of RNAi-based cancer therapeutics [76]. ALN-VSP was the first systemically delivered RNAi therapeutic which included two siRNAs enclosed in lipid nanoparticles of 80-100 nm in diameter, intended to target two genes critical for the growth and development of cancer cells: vascular endothelial growth factor (VEGF) and Eg5 [78]. An alternative approach to silence Eg5 expression was recently achieved using PEGylated DC-Chol/DOPE cationic liposomes, containing Eg5 shRNA-expressing plasmid/liposome lipoplexes that induced the long-term Eg5 silencing in the tumor sites of tumor-bearing mice leading to more sustained anticancer effects [79].

3. Status of past and ongoing clinical trials with anti-Eg5 strategies.

3.1 The basic idea of inhibiting Eg5 as an anti-mitotic agent

Since the discovery of monastrol as mitotic inhibitor, and the demonstration that it targets specifically Eg5 and not microtubules, targeting Eg5 was validated as a proof of concept that it is possible to develop novel anti-mitotic inhibitors, other than microtubule targeting agents, in order to block tumor cell proliferation growth. The purpose of this perspective is not to discuss how anti-mitotic agents kill cells in mitosis or after aberrant mitotic exit. Readers can find excellent reviews on how antimitotic agents kill cells in related reviews [80][81][82]. However, it is widely accepted that the ability of anti-mitotic agents to suppress tumor cell proliferation lies in their capacity to trigger cell death, induced either in mitosis during a prolonged mitotic arrest, or post mitotically following a premature exit from mitosis [81]. Nonetheless though, it is worth mentioning that using single-cell assays it has been shown that the response of several cancer cell lines to anti-mitotic drug treatment varied widely between cell lines [83]. In some cases the sensitivity of the cells to anti-mitotic drugs correlated with the loss of the expression of X-linked inhibitor of apoptosis (XIAP) during the drug response [84]. Similar variation in response to Eg5 inhibitors of different cell lines was noticed and interestingly, Eg5 inhibition induced greater cell death in cancer cell lines than in nontransformed cells [85].

Cancer chemotherapeutic strategies based on microtubule targeting agents, such as taxol chemotherapy, are associated with severe side effects, such as neurotoxicity, myelosuppression, fatigue and others [86][38], since microtubule functions are ubiquitously

needed in all cells, healthy and tumor cells, during all phases of the cell cycle. As mentioned earlier Eg5 is minimally expressed in adult tissues and therefore is expected to have minimal neurotoxic side effects. Furthermore, the emergence of taxol resistance [87] in treated tumors raised also the need for new anti-mitotic agents such as those targeting Eg5 as substitutes to taxol treatment [39]. Therefore, a lot of pharmaceutical and biotechnology companies developed Eg5 inhibitors and a certain of them entered clinical trials as anti-neoplastic agents against a variety tumors (Table I).

3.2 Results of the clinical trials

All of the identified Eg5 inhibitors in addition to their ability to inhibit the enzymatic activity of the motor protein in *in vitro assays*, were efficient in inhibiting tumor cell lines in mitosis and stopping tumor cell proliferation in mouse tumor cells in xenograft models. However, many of the tested inhibitors had limited success in clinical trials. In summary, the results for the various phase I trials were encouraging in that the drugs at their maximal tolerated dose (Table I) were well tolerated with the most common side-effects observed were suppression of neutrophil production (neutropenia), anemia and in some cases liver toxicity. Most importantly, and unlike the microtubule inhibitors, such as taxanes and vinca alkaloids, neurotoxicity was not observed, as it was initially expected.

More specifically, in the case of ispinesib (or SB-715992) partial responses were observed in less than 10% of the cases although stable disease was noted in 55% of patients with renal carcinoma [88]. Two studies with ispinesib focused particularly on mCRPC patients, gave ambiguous results. In a phase I study, six out of fourteen mCRPC patients had stable disease for ≥18 weeks and one patient had a prostate-specific antigen (PSA)-decrease of >50% when ispinesib was combined with docetaxel [89]. However, in a phase II study in which ispinesib was administered as monotherapy, no responses were reported [90]. Twenty out of 21 patients had been treated with docetaxel prior to ispinesib. Immunohistochemistry analysis on archival tumor tissue from sixteen patients indicated that only one tumor stained positive for Eg5. It was concluded that ispinesib is not effective in primary prostate cancer (PCa) due to their low mitotic index, resulting in low Eg5 expression. Similarly, no conclusive evidence of benefit was seen with HCC [91], melanoma and head and neck squamous cell carcinoma [92][93].

In a multicenter phase I/II study of SB-743921 [94] with patients with relapsed or refractory lymphoma it was shown that the dose limiting toxicity and the maximum tolerated dose can be markedly increased if the drug is administered together with granulocyte-colony stimulating factor [95]. Out of 56 patients, one patient was drug resistant, four of 55 patients experienced a partial response (three in Hodgkin lymphoma and one in non-Hodgkin lymphoma); 19 patients experienced stable disease and 33 patients developed progression of disease. The results demonstrated that combination regimens in addition to Eg5 inhibitors may have a positive complementary role.

In patients with either urothelial cancer or refractory acute myeloid leukemia AZD-4877 [96] demonstrated no complete or partial responses [97][98][99]. Patients with solid tumors exposed to ARQ-621 [100] only 6/48 patients experienced disease stabilization for >4 months [101] whereas those exposed to MK-0731 [53] exhibited no complete or partial responses [102]. Phase I and II clinical trials with LY 2523355 ([103]; clinical name litrinosib) in one hundred and seventeen patients showed that the therapeutic index for this drug class is narrow; only 2% of the patients achieved partial response, and 20% maintained stable disease for ≥6 cycles [104][105].

ALN-VSP is designed to target the expression of Eg5 and the vascular endothelial growth factor, or VEGF, required for tumor growth by specific siRNAs [78]. In the trial, the patients were treated at doses ranging from 0.1 to 1.5 mg/kg. The results of ALN-VSP Phase I trial demonstrated safety and tolerability of multiple doses of ALN-VSP. ALN-VSP as a single-agent had anti-tumor activity, and showed RNAi-mediated target mRNA cleavage in both hepatic and extra-hepatic tumors. Both siRNAs in ALN-VSP were detected in liver tumor biopsies and in metastases involving the adrenal gland, lymph nodes, and abdominal cavity, across multiple different tumor types. Strikingly it was noted that the ALN-VSP phase I trial included a complete response in a patient with nodal and extensive liver metastases, as well as prolonged disease stabilization for as long as 1 to 1.5 years in patients with hepatic and extrahepatic metastases Disease control was achieved in one of 13 patients (8%) treated at doses less than or equal to 0.4 mg/kg versus 12 of 24 (50%) treated at doses greater than or equal to 0.7 mg/kg, including seven of 11 (64%) treated at the proposed Phase II dose of 1.0 mg/kg. However, further clinical studies on ALN-VSP are on hold, most likely due to the legal disputes between the two companies involved, Alnylam and Tekmira Pharmaceuticals, regarding the intellectual property of lipid nanoparticle delivery technology.

Within this avalanche of negative results though, Arry-520 [106] (clinical name filanesib) turned out to be a promising agent for the treatment of patients diagnosed with refractory and/or relapsed multiple myeloma [107]. For a more detailed account of the results obtained following clinical trials with Arry-520 one can read the recent review by Algarin et al [108]. Briefly, Arry-520 as single agent achieved only partial response in 1 out of 34 patients suffering from AML and no significant clinical responses were observed with patients with solid tumors treated with Arry-520. However, Arry-520 as single agent showed an overall response rate of 16% and 15% when low dose the anti-inflammatory dexamethasone was added in heavily pretreated multiple myeloma patients [109]. The overall survival of 19 months were obtained with Arry-520 as mono therapy and 10,7 months in combination with dexamethasone. These encouraging results prompted the exploration of potential combinations with proteasome inhibitors such as bortezomib [110] and carfilzomib [111][112] in combination with or without dexamethasone. Combining Arry-520 with the proteasome inhibitor bortezomib produced response rates as high as 42 %. Combination of Arry-520 with the proteasome inhibitor carfilzomib gave response rates as high as 37 %, with 5 % of patients achieving a near complete response and 32 % a partial response. Furthermore, the efficacy of Arry-520 in clinical trials was associated with the expression levels of α-1 acidic glycoprotein (AAG); low levels of AAG in the serum of patients were correlated with a better chemotherapeutic index compared to higher AAG levels [113]. Therefore, the levels of AAG in the serum can be used as a useful predictive biomarker to identify patients that will be responsive to Arry-520 treatment. A more targeted selection of patients based on the level of AAG may increase the Arry-520 response rates possibly making Arry-520 based therapy to be equally effective as the recently developed anti-myeloma immunotherapy based on monoclonal antibodies [114]. Based on these encouraging results Arry-520 is scheduled to enter Phase III clinical trials for multiple myeloma.

3.3 Possible reasons for the failure

3.3.1 Pharmacokinetics and the proliferation rate paradox

The obvious question arising from the above described clinical trials is why Arry-520 was relatively successful compared to the rest of the compounds tested. The low therapeutic index of the tested Eg5 inhibitors, such ispinesib, SB-743921, AZD-4877, and MK-0731 is not due to

lack of target engagement since the inhibitors target normal cell proliferation leading to neutropenia. However, taking into account the pharmacokinetic data derived from the phase I clinical trials of the compounds tested, one determining factor of success might be the elimination half-live of each compound [115]. As shown in Table 1, the elimination half-lives of ispinesib, SB-743921, AZD-4877, and MK-0731 are all <30 h, whereas the corresponding half-life of ARRY-520 was found to be >90 h. Therefore, the longer half-life of Arry-520 compared to other Eg5 may be one of the reason for the better therapeutic index of Arry-520.

In addition to the long elimination half-life of the inhibitor, another determinant factor for the efficacy of a given treatment may be depended also on how often the cells in a given tumor type divide. A given mitotic inhibitor to act in mitosis needs to be present when the cells reach cell division. Most of the inhibitors are administered to the patients in intermittent fashion and not in continuous dosing. However, approximately only 1% of the tumor cells solid tumors are in mitosis at any given time [116], a phenomenon referred to as the 'proliferation rate paradox' [117]. Therefore, any given mitotic inhibitor needs to be present long enough into the serum of patients in order to be effective in mitotic cells of the slowly dividing tumors. This means that for an anti-mitotic agent to be effective *in vivo*, it must remain in contact with the tumor for considerably longer than the average cell cycle time—a criterion that Arry-520 appears to satisfy. The longer half-life of Arry-520 compared to other Eg5 inhibitors combined with the high proliferation rate of relapsed multiple myeloma cells (14% to 83%) may be two key factors to explain the better therapeutic index of Arry-520. In support of this argument may be the observed clinical data using the oral Eg5 inhibitor 4SC-205 indicating that a continuous dosing scheme of 20 mg per patient, once daily could overcome this 'proliferation rate paradox'. A clinical response (stable disease) was observed in 67% of patients for more than 100 days undergoing this regimen [118].

3.3.2 Acquired resistance to Eg5 inhibitors

Another explanation for the lack of efficacy of the Eg5 inhibitors in the clinic may due to the characteristic genetic instability of tumors [119]; there might be tumor cells carrying, for example, preexisting mutations within the L5 loop ligand binding site, rendering thus Eg5 resistant to inhibition. To date though, there are no clinical relevant mutations in Eg5 reported that might be involved in resistance to any anti-Eg5 inhibitor. What has been shown though after clonal selection of cells in the presence of Eg5 inhibitors such as ispinesib [94][120], and

BRD9647 [65], is resistant cells that express mutant Eg5 having amino acid substitutions in the inhibitor binding pockets. For example, the mutation D130V which is in the helix α2/loop L5/helix α3 allosteric binding site is associated with ispinesib resistance and the mutation Y104C, located in the distinct Eg5 allosteric binding site between the α4 and α6 helices is associated with BRD9647 resistance. Clonal selection of cells exposed to STLC also resulted to the identification of single point mutations on the *Kif11* gene that are associated with resistance [121][122].

As mentioned previously the most notable conformational change observed upon binding of the Loop L5 inhibitors is the adoption of a "closed" conformation of loop L5. This is in contrast with the "open" conformation observed in the absence of an allosteric ligand. However, the transition from the "open" to "closed" conformation of loop L5 is taking place also in normal motor function [123]. Interestingly the SB743921 Eg5 inhibitor was identified following a screen using the two ispinesib resistant cell line clones expressing either the Eg5(D130V) or Eg5(A133D) alleles [94]. Strikingly, the crystal structures of the WT Eg5 and Eg5(A133D) bound to SB743921 turned out to be very similar [56]. However, extended molecular dynamics simulations revealed that when the WT motor is bound to the inhibitor, Ala133 along with the neighboring Pro137 residue are able to transmit a perturbation pathway through the protein to the nucleotide site resulting to inhibition. In contrast, in the Eg5(A133D)-SB743921 complex, there are local rearrangement of hydrogen bonding and salt bridging that transmit structural changes throughout the whole complex that would block the inhibitor induced allosteric transmission to the nucleotide site rendering thus, the motor resistant to the inhibition [56].

Furthermore, Infrared spectroscopy was used to provide a direct measure of the mechanochemical coupling between the loop L5 and the β-sheet central core of the Eg5 motor domain in a panel of single point mutations with different amino acid substitutions. The data showed that amino acid substitutions at positions E116 and E118 at the N-terminus of the Loop L5 resulted in changes in the secondary structure of Eg5 in solution irrespective of mutation or inhibitor binding [124]. The results pointed that mutations that may induce loss of Loop L5 rigidity may lead to reduced inhibitor sensitivity and that not all the Loop L5 binding have the same inhibitory effects on Eg5 activity. Furthermore, by infrared spectroscopy was possible to show that the secondary structure changes of the Eg5 motor domain bound to monastrol are different than those coupled to the more potent inhibitor STLC, suggesting thus that the impact of binding of different Eg5 Loop L5 inhibitors may be not the same.

Nonetheless, extensive mutagenesis analysis in the helix α2/loop L5/helix α3 allosteric binding site of Eg5 has been shown to confer resistance to a variety of other loop L5 inhibitors (Table II Fig 3D), including monastrol, STLC, ispinesib and others [125][126][127][128]. Notably, mutations to specific residues in the inhibitor-binding pocket (including D130A; D130V; D133A and L214A), although they do not alter appreciably the binding of monastrol or STLC to the motor domain [126], can overcome inhibition by blocking the allosteric communication network to the ATP binding site [56].

When Arry-520 was assessed in two cell lines selected for the expression of either Eg5(D130A) or Eg5(L214A) STLC-resistant alleles, mutations previously shown to convey resistance to loop L class of inhibitors [129], it was inactive in both [59]. However, when the cells were challenged with ispinesib, the Eg5(D130A) expressing cells were resistant, but those expressing Eg5(L214A) were strikingly sensitive. Molecular dynamics simulations suggested that subtle differences in ligand binding and ligand flexibility may alter allosteric transmission from the loop L5 site that do not necessarily result in reduced inhibitory activity in mutated Eg5 structures [59]. Even though the data predict that cells challenged with Arry-520 in the clinical setting are likely to acquire resistance through point mutations in the Eg5 binding site, the data for ispinesib suggest that this resistance mechanism is not scaffold independent as previously thought [130], and new inhibitors can be designed that retain inhibitory activity in these resistant cells.

3.3.3 Functional plasticity of mitotic kinesins substituting for loss of Eg5 function

Although Eg5 plays a central role in spindle formation by contributing the necessary forces for centrosome separation [17], in cells there exist redundant pathways that can achieve centrosome separation leading to spindle bipolarity. As mentioned in the introduction Eg5 is one of the 45 members of the kinesin superfamily of motor proteins from which more than 15 are implicated in mitosis [2]. One of such mitotic motors, Kif15 (member of the *kif12* subfamily of kinesins), can fully reconstitute bipolar spindle assembly in cells in the absence of Eg5 activity [131][58]. Kif15 is not essential for spindle assembly but when ectopically overexpressed in cells, the over expressed Kif15 together with TPX2, a microtubule binding protein reported to be also a binding partner of Eg5, can crosslink and slide two antiparallel microtubules apart, thereby driving centrosome separation [132].

Studies aiming to understand how cells can adapt to the lack of Eg5 activity demonstrated that resistance to STLC Eg5 inhibitor was associated with the expression of rigor mutant of Eg5, G268V [122]. G268 is located into the switch II nucleotide binding area and the G268V traps the motor in a microtubule binding state leading to a strong microtubule crosslinking. Eg5 is converted in these cells from a motile force generator to a static MT crosslinker. Kif15 is normally restricted to kinetochore-MTs because of its preferential binding to MT bundles. Therefore, due to the Eg5(G268V) induced cross linking Kif15 changes its distribution and is enriched also to the rest of spindle microtubules contributing so in the formation of normal bipolar spindle. It is worth noting also that the Kif15-Eg5(G268V) resistance was not associated to Kif15 overexpression but solely in changes in Kif15 localization to the whole spindle instead to only kinetochore microtubule bundles. This observation strongly suggests that a combination of Eg5 inhibitors and Kif15 inhibitors may be an effective anti-proliferative chemotherapeutic regimen for targeting rapidly dividing cancer types. Although the development of Kif15 inhibitors is in its infancy, GW108X [133], an oxindole compound as well as KIF15-IN-1 [134] and benserazide [135] may serve as leads to further develop Kif15 inhibitors into clinically relevant agents. Moreover, in a SB743921-resistant cell model that depends on Kif15 activity it was shown that inhibition of the centrosome associated AURKA kinase could be an effective option to overcome resistance to Eg5 inhibitors [136].

Resistance to STLC in HeLa cells was also attributed to the prophase centrosome separation pathway, which depends on nuclear envelope-associated dynein, a minus end microtubule directed motor protein [137]. While Eg5 acts on centrosome pairs, dynein pulls on individual centrosomes, and in combination with the activity of Kif15 can drive centrosome separation and establishment of bipolar spindle. In naive cells both Eg5 and nuclear envelope-associated dynein centrosome separation pathways are operating.

One additional aspect regarding the response of the cells to Eg5 inhibitors may also be related to the premature centrosome separation which appears to be related to the epidermal growth factor receptor signaling (EGFR) pathway. Different cancer cell lines upon stimulation of the EGFR signaling pathway by EGF induce premature centrosome separation in S phase [138]. A direct consequence of this is that cells with high EGFR levels fail to arrest in mitosis upon Eg5 inhibition, by STLC for example. Therefore, a combinatorial targeting of EGFR and Eg5 may be

a more effective chemotherapy approach for the types of tumors that are rich in EGFR and not responsive to Eg5 inhibition.

Centrosome separation during the G2/M transition is tightly regulated not only by cell cycle signaling pathways that favor separation, as mentioned above, but also by pathways that inhibit premature separation. In prophase, p21-activated kinases (Pak1 and Pak2) counteract centrosome separation by a Cdk1 dependent phosphorylation of their activator Tiam1. Interestingly, depletion of Pak1/2 allows cells to escape monopolar arrest by Eg5 inhibition by monastrol, highlighting the potential importance of this signaling pathway in the development of an additional pathway that could lead to resistance to Eg5 inhibitors [139].

4. Future directions

4.1 Towards single-target selectivity Antibody-drug conjugates

The field of Antibody-Drug Conjugates (ADCs) poses a promising therapeutic option for malignant patients. ADCs are developed for targeted delivery of cytotoxic payloads to tumor cells. ADCs share a common design of antibody, linker, and cytotoxic payload [140]. At present, four ADCs are in clinical use approved by the FDA and European Union (EU) for treating different kinds of cancer; two are conjugated to DNA damaging agents and two to MT disrupting agents. More than 60 ADCs are at different clinical stages and their results have sparked significant interest in the rapidly growing number of ADC candidates. Recently, antibody prodrug conjugates (APDCs) with Eg5 inhibitor payloads represent a versatile novel approach for cancer treatment [141]. A novel pyrrole subclass of Eg5 inhibitor (KSOi SMOL; Fig 2) was linked with a stable peptide linker to tumor recognizing antibodies such as BAY-865 targeting the receptor of TWEAK (tumor necrosis factor [TNF]-like weak inducer of apoptosis) [142]. The peptide linker is a substrate for the Legumain, a lysosomal endopeptidase with optimal activity at an acidic pH of ~4-5 that shows an increased expression and thus higher activity in solid tumors compared to most healthy tissues. In preclinical studies, the Eg5-ADCs targeting TWEAKR demonstrated potent and selective anti proliferative activity in a panel of TWEAKR-positive cancer cell lines in vitro and high antitumor efficacy in patient-derived xenograft models.

More recently BAY-943, a novel ADC consisting of a humanized internalizing anti-IL3RA IgG1 antibody conjugated via lysine residues to the Eg5 motor protein inhibitor was conceived to be

tested in IL3RA-positive tumor cells [143]. IL3RA expression is restricted to myeloid progenitor cells, plasmacytoid dendritic cells (pDCs), basophils and - at low levels - monocytes and B-lymphocyte subsets. BAY-943 showed efficacy in IL3RA-positive AML and HL models and had a favorable safety profile in monkey repeat dose studies. Thus, ADCs with Eg5 inhibitors as payload offer a promising new approach for tumor treatment.

4.2 Virtual screening

Virtual screening strategy is widely applied for identification of novel targeted drugs. This strategy is generally divided into structure based virtual screening and ligand based virtual screening. The increasing number of solved X-ray crystal structures in the PDB of Eg5 bound to structurally diverse inhibitors (Fig 4 and 5) allowed by the combination of pharmacophore modeling and molecular docking the identification of three novel Eg5 inhibitors bearing quinazoline and thioxoimidazolidine scaffolds [144]. In an another virtual screening study based on shape similarity to STLC, molecular docking and phenotype screening allowed the identification of a novel inhibitor, YL001 having a 1,5-disubstituted tetrazole scaffold [145]. YL001 inhibits the ATPase activity of the motor, blocks cells in mitosis and reduced melanoma tumor growth by 60% and significantly prolongs median survival time by more than 50% in a xenograft mouse model. A limitation of the virtual screening strategy is that although it may lead to the identification of small molecules that fulfill all the features of a pharmacophore, resembling those of known inhibitors, they may not display similar inhibitory activities in enzymatic and cell based assays [146]. To date, none of the identified Eg5 inhibitors by virtual screening have entered clinical trials yet. However, since virtual screening methods are relatively economic and efficient in identifying new lead compounds and based on the fact there are over 10^{63} drug-like molecules [147], virtual screening combined with virtual library of compounds may lead to novel Eg5 inhibitors in the future.

4.3 Eg5 as a target for neuronal repair

Based on the role of Eg5 in mitosis in the crosslinking and gliding of anti-parallel microtubules, Eg5 seemed like a good candidate motor to drive microtubule sliding in neurons. Studies on rodents have shown that mouse neurons, in contrast to other somatic cells, express a homolog of Eg5 well past their terminal mitotic division [9]. Eg5 in neurons is thought to normally generate forces that oppose axonal growth, presumably by partially suppressing the forward

advance of microtubules of axons [10]. Eg5 is localized in discrete and functionally important regions of developing neuronal processes and Eg5 plays an essential role in polarizing the MT array in response to cues that cause the growth cone to turn since RNAi knockdown of Eg5 results in a more branched axon [148]. The role of Eg5 in regulating the length of axons and the migration of neurons in culture was linked to its phosphorylation as it was shown for its role in mitosis [149]. Furthermore, in neurons, pharmacological inhibition of Eg5 resulted in longer axons, suggesting a key role for this motor in the development of the nervous system [150][11]. Theses observations raised the interesting possibility that exposure for limited windows of time of Eg5 inhibitors to injury sites could help to nerve regeneration [151].

4.4 Eg5 as a target in glioblastoma.

An ideal glioblastoma (GBM) treatment needs to engage targets that drive proliferation as well as invasion, with brain penetrant therapies. A recent RNA-Seq analysis from biopsies of human glioblastomas revealed that most of the mitotic relevant kinesins including Eg5 are overexpressed [152]. Further analysis showed that expression of Kif11 (the gene coding for Eg5) is up-regulated in tumor initiating cells (TICs) compared to non-TICs, at both the RNA and protein levels. Systemic administration of ispinesib to mice bearing orthotopic, TIC-derived GBMs produced monopolar spindles phenotype, as well as a prolongation in survival, implying that sufficient amounts of drug reach the intracranial tumors to exert a therapeutic effect. However, one major obstacle in effective treatment of glioblastomas in the clinic is the diffuse nature of malignant gliomas. Data obtained by an *in vitro* Transwell assay demonstrated that TIC invasion could be abolished by ispinesib. Since Eg5 is a key player in both proliferation and motility in glioblastoma tumor cells, Eg5 is thought to be a compelling therapeutic target for glioblastoma by administering Eg5 inhibitors intracerebrally with technologies that are already being used clinically, such as convection-enhanced delivery (CED), where prolonged intracerebral CED could be achieved with a subcutaneous implantable infusion pump [152]. More recent data with a mouse model of GBM showed that combining ispinesib treatment with a compound that improves its brain accumulation, such as elacridar, a P-glycoprotein (P-gp) and Breast cancer resistance protein (Bcrp), an efflux inhibitor at the blood-brain barrier, can improve significantly its efficacy [153]. Another putative inhibitor of GBM maybe the orally available Eg5 inhibitor SCH 2047069 [154] which it was shown in rats it could penetrate the blood brain barrier [155].

Furthermore, recent efforts in understanding the role of overexpression of 3 different kinesins, Kif11, Kif12 and Kif23 in malignant peripheral nerve sheath tumor (MPNST) cells led to the observation that MPNST cells are more sensitive to Arry-520 treatment compared to normal fibroblasts; a synergistic effect was also observed when MPNST cells were exposed to Arry-520 and the BRD4 epigenetic inhibitor JQ1, especially in Kif15 depleted cells [156].

4.4 Overcoming resistance

The vast majority of metastatic cancers remain incurable. Treatment with standard approaches may extend survival, but ultimately fails due to the emergence of resistant cells. Resistance to targeted cancer therapy can be considered in terms of Darwinian adaptation. Due to the high levels of intra-tumor heterogeneity (ITH) [119], a huge number of cells in a tumor, due to genetic or epigenetic alterations even before treatment begins, are drug resistant. One approach to overcome single drug resistance is to follow a drug combination approach. Combining different drugs, within a narrow therapeutic window at the same time improves survival by a few months. One limiting factor in drug combination approach is the high toxicity. Nonetheless, the Eg5 inhibitor Arry-520 gave encouraging results in multiple myeloma therapy in phase II clinical trials not only as a mono therapy but also in combination with proteasome inhibitors. Similarly, the presence of elacridar —a potent non-toxic P-gp and Bcrp inhibitor— improves brain accumulation of ispinesib resulting in superior *in vivo* target engagement, reduced tumor growth and extended survival in a mouse model of glioblastoma. It may be possible also to target tumors by targeting Eg5 with different classes of Eg5 inhibitors: one targeting the helix α2/loop L5/helix α3 allosteric site and the other targeting the distinct Eg5 allosteric binding site between the α4 and α6 helices. The double hit approach will significantly limit the chances of selecting resistant clones carrying mutations that could confer resistance to both Eg5 inhibitors with distinct targeting sites.

Another recently proposed method to overcome resistance is to drive by evolutionary steering to collateral drug sensitivity [157]. This approach is based on the prediction that resistance to one drug may make the resistance of cancer clone cells more sensitive to a second drug, a phenomenon known as collateral sensitivity. And indeed, in a recent proof of concept study using a non-small cell lung cancer line treated with trametinib, a MEK1/2 inhibitor, the resistant subclones were very sensitive to Eg5 inhibitors such as, ispinesib and SB-74392, among others. Therefore, there might be a new window of opportunity for already clinically

tested Eg5 inhibitors to be tested as second line treatment following emergence of drug resistance by targeting a different pathway.

4.5 Kinesin-5 as a target to human pathogens

Based on the paradigm of Eg5 as a drug candidate for cancer therapy, kinesin-5 inhibition could have wider applications in disease control, for example in killing eukaryotic pathogens such as fungi. Indeed, screening for inhibitors of the kinesin-5 member of the pathogen *C albicans* led to the identification of an aminobenzothiazole (ABT) compound that turned out to inhibit the fungal kinesin specifically [158]. ABT was 10- to 50-fold less active against Eg5 (human), MmEg5 (mouse) and AnBimC (*A. nidulans*) motor domains. Interestingly, based on biochemical characterization of ABT revealed that the inhibitor does not belong to Loop L5 class of allosteric inhibitors but was an ATP competitive inhibitor resulting in rigor like state leading to tight association of the motor to microtubules.

Recent structural analyses of two kinesin-5 members from two different fungi, Cut7 from *Schizosaccharomyces pombe* [159] and UmKin5 *Ustilago maydi* [160], a fungus that infects corn and causes the disease corn smut , elucidated that the overall structures of the ATP-like state of UmKin5 and Cut7 are similar to that observed for other plus-end directed kinesins, and that the core motor–microtubule interaction are tightly conserved. However, the structures also highlighted non-canonical features in these fungal motors due to divergent inserts in fungal kinesin-5 motor domains. In both fungal kinesins-5 the Loop L5 protrudes away from the motor domain in contrast to the Loop L5 of human Eg5. The identified structural differences in the Loop L5 explain why the Cut7 is not sensitive to STLC inhibition. Based on the structural analysis of the two fungal kinesins-5 as well as on divergent inserts in fungal kinesin-5 motor domains suggest that identification of specific inhibitors to fungal kinesins with a possible fungicide activity may be possible.

Malaria parasites of the genus *Plasmodium*, which infect many vertebrate hosts including humans, and is transmitted by female *Anopheles* mosquitoes [161] code also for a kinesin-5 motor protein. The kinesin 5, of *Plasmodium vivax* and *Plasmodium falciparum* contain Loop-5 sequences that are longer than and divergent from the human ortholog Eg5 [162]. In *Plasmodium berghei* during cell division kinesin-5 associates with the spindle microtubules and is important for efficient production of infectious sporozoites [163]. In a screen for inhibitors selective for the Kinesin-5 ATPase activity of *P.vivax* and *P. falciparum*, identified the lead

compound MMV666693 as an allosteric inhibitor of the motor domain of the two parasites [162]. Interestingly, MMV666693, an oxazine derivative, was a member of the "druglike" compound subset of proliferation inhibitors within the Malaria Venture validated collection known to strongly inhibit erythrocytic-stage *P. falciparum* [164]. However, the binding site of the MMV666693 to the parasite kinesin-5 is not yet known.

5. Concluding remarks

Eg5 has been part of a large group of mitotic targets that are undergoing intensive studies in the pursue of novel cancer therapeutics. We have come long ways since the discovery of monastrol as the first inhibitor of Eg5. The initial burst of excitement and surge of activity in identifying novel Eg5 inhibitors were followed by disappointing clinical results. Further pursue of more potent inhibitors confirmed the rule that going from a promising compound to an approved drug is still a long and uncertain process. Nonetheless, the so far characterized Eg5 inhibitors have been proven to be indispensable as chemical genetics tools for gaining biological insights in basic cell biology issues regarding the roles of Eg5 and other kinesin motors in fundamental cellular functions such as centrosome separation and spindle microtubule organization in cell division. Moreover, the knowledge accumulated from the failures in the clinic led to recent successes giving renewed hopes for the use of Eg5 inhibitors for hematological cancers. Natural product chemists, biochemists, cell and structural biologists in academia and pharmaceutical industry will continue to search for new products with unique chemical structures that may have meaningful medical applications. Having access to a greater portion of the chemical universe [147], the chances of discovering a star molecule targeting Eg5 should be greater too.

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Figure Legends

Figure 1 A) Schematic representation of antiparallel microtubule sliding by Eg5 tetramers. At each end of the Eg5 tetramer there is a dimer of motor domains that interact with microtubules moving towards the + ends of microtubules. The central helical bundle (BASS domain) formed by two antiparallel alpha helical coiled-coils is responsible for the tetramerization of Eg5. The C-terminus of each monomer interacts with the motor domain of the opposing monomer slowing the ATP binding to the motor and favoring the microtubule bound state of the motor.
B) A bipolar spindle of an untreated (left panel) and a monoastral spindle in α-tubulin-GFP expressing HeLa cells treated with an Eg5 inhibitor (right panel), such as 10nM Arry-520 [106].

Figure 2 Eg5 inhibitors. Chemical structures of representative Eg5 motor domain inhibitors, arranged by chemical class or scaffolds. The KSPi-SMOL is chemically linked and used as a payload to an antibody drug conjugate. The thiazole, aminobenzylthiazole, inhibits preferentially the kinesin-5 member of the pathogen *C. albicans,* whereas the MMV666693 was identified as a specific inhibitor of the kinesin-5 of the malaria parasites of the genus *Plasmodium*.

Figure 3 Crystal structure of the Eg5 motor domain bound to Arry-520. **A)** Overall structure of the ternary Mg^{2+}ADP–Eg5–Arry-520 complex showing the front view of the Eg5 motor domain with bound Mg^{2+} (green), ADP (Cαs in white) and Arry-520 (PDB ID 6HKX; violet).
B) Structural superposition of the crystal structure of Eg5 motor domain (PDB ID 1II6; white cartoon) with the structure of the Eg5 motor domain (blue) in complex with Arry-520.
C) Helix α4, helix α5 loop L12 and the neck-linker region undergo conformational changes from the intermediate (cyan) to the final inhibitor-bound state (blue). Helix α4 moves to the permissive position to make space for the neck-linker, which subsequently adopts a docked conformation (blue).
D) Surface representation around the helix α2/ loop L5 / helix α3 allosteric pocket in the "closed" inhibitor bound state (inhibitor is not shown). Amino acid substitutions in the labeled amino acids within the pocket have been shown to confer resistance to various loop L5 inhibitors in biochemical as well as cell based assays without affecting the binding of the inhibitors to the pocket (see Table II).

Figure 4 Structural superposition of the crystal structure of Eg5 motor domain (PDB ID 1II6; white cartoon) with the structures of the Eg5 motor domain (blue) in complex with allosteric inhibitors of the Loop L5 class that all bind to the helix α2/loop L5 / helix α3 pocket (PDB IDs: monastrol 1X88; (S)-enastron 2X7C; R-mon97 2IEH; STLC 2WOG; ispinesib 4AP0; SB 743921 4AS7; K858 6G6Y; EMD 534085 3L9H [165]; MK 0731 3CJO).

Figure 5 Structural superposition of the crystal structure of Eg5 motor domain (PDB ID 1II6; white cartoon) with the structures of the Eg5 motor domain (blue) in complex with ATP competitive inhibitors that bind to the α4 and α6 pocket near but not in the ATP binding site (PDB IDs: GSK1 6TIW; PVZB1194 3WPN; BI8 3ZCW).

Table I

Table I : Eg5 inhibitors that entered clinical trials

Inhibitor	Sponsor	Phase	maximal tolerated dose	Elimination half-lives	Condition	Clinical trial / status	Published Referfences
Arry-520	Array Biopharma	P1; P2	1.50 mg/m^2/day	>90h	solid; MM,AML,MDS	NCT02384083 / unknown; NCT01372540 / completed NCT02092922 / completed ; NCT01989325 / completed NCT00637052 / completed ; NCT00462358 / completed NCT00821249 / completed ; NCT01248923 / completed	[109][110] [111][112] [113]
LY2523355	Eli Lilly	P1; P2	12 mg/m^2 /day	10-31h	solid, leukemia; ovarian,NSLCLC, PC,CRC, gastric, esophageal, HNSCC,BC, SCLC	NCT01358019 / completed ; NCT01416389 / completed NCT01214655 / terminated ; NCT01059643 / completed NCT01214629 / completed ; NCT01214642 / completed NCT01025284 / completed	[104][105]
4SC-205	4SC	P1	20mg/day	10h	solid , lymphoma	NCT01065025	[118]
ALN-VSP02	Alnylam	P1	1.0 mg/kg	10h	HCC	NCT01158079 ; NCT00882180	[78]
ispinesib	GSK	P1; P2	18 mg/m^2//21 days	16h	Burkett Lymphoma; RCC,HNN, PC,ovarian BC, NSCLC,CRC, HCC, melanoma	NCT00354250 / completed; NCT00089973 / completed NCT00119171 / completed ; NCT00095628 / completed NCT00607841 / terminated ; NCT00363272 / completed NCT00097409 / completed ; NCT00098826 / completed NCT00136578 / completed; NCT00101244 / terminated NCT00096499 / completed ; NCT00085813 / completed NCT00095953 / completed ; NCT00095992 / completed NCT00103311 / completed; NCT00169520 / completed	[92][89] [90][91] [93][88]

AZD4877	AstraZeneca	P1; P2	11-18 mg	16h	solid, lymphoma, NHL; bladder, transitional, urethra, RCC	NCT00471367 / terminated ; NCT00661609 / completed NCT00613652 / completed ; NCT00486265 / terminated NCT00389389 / completed ; NCT00460460 / terminated	[97][98] [99]
SB-743921	Cytokinetics	P1; P2	4-6 mg/m^2	29h	solid; NHL HL	NCT00343564/ completed ; NCT00136513 / completed	[95]
ARQ 621	ArQule	P1	280 mg/m^2/week		solid	NCT00825487 / completed	[101]
MK-0731	Merck	P1	17 mg/m^2 /day every 21 days	6h	solid	NCT00104364 / completed	[102]

MM : multiple myeloma; AML : Acute Myeloid Leukemia; MDS : myelodysplastic syndrome; NSLCLC : Non-small-cell lung carcinoma; PC : prostate cancer; HNSCC : Head and Neck squamous cell carcinoma; BC : breast cancer; SCLC : small cell lung cancer; HCC : hepatocellular carcinoma; RCC : Renal cell carcinoma; NHL : Non-Hodgkin lymphoma; HL: Hodgkin lymphoma.

Table II

Table II: Amino acids in the motor domain of Eg5 linked to resistance to Eg5 inhibitors

Eg5 residue	**mutation**	**location**	**tested inhibitor resistance**	**Reference**
D130	A, K, V, G	Loop5	SB-743921, STLC, monastrol, Arry-520	[120][121][59] [129][130]
A133	D, I, V, M, P	Loop5	SB-743921, ispinesib, STLC	[120][127][124]
L214	A	Helix-α3	STLC, monastrol, Arry-520, ispinesib	[125][126][59] [130][129]][130]
Y104	C	β3/ Ploop	BRD9876	[65]
E123	K	Loop5	STLC	[121]
A334	V	β8	STLC	[121]
H354	R	Helix-α6	STLC	[121]
A103	V	P loop	STLC	[121]
G268	V	Switch II helix	STLC	[122]
E116	A, D, R, L, V	Loop5	monastrol, STLC	[124][128]
E118	V, D, L, N	Loop5	monastrol, STLC	[124][128]
R119	A, L, E	Loop5	monastrol, STLC	[126][128][125]
W127	F, L, A	Loop5	monastrol, STLC	[126][128]
P131	A	Loop5	monastrol, STLC	[123]
L132	A	Loop5	monastrol, STLC	[126]][130]
I136	A	Helix-α2	monastrol, STLC	[126]][130]
E215	A	Helix-α3	monastrol, STLC	[126]][130]
R221	A	Helix-α3	monastrol, STLC	[126]
Y211	F, A, M	Loop5	monastrol, STLC	[126][128]
V210	A	Helix-α3	monastrol, STLC	[126]
S120	P	Loop5	ispinesib	[120]

Figure 1

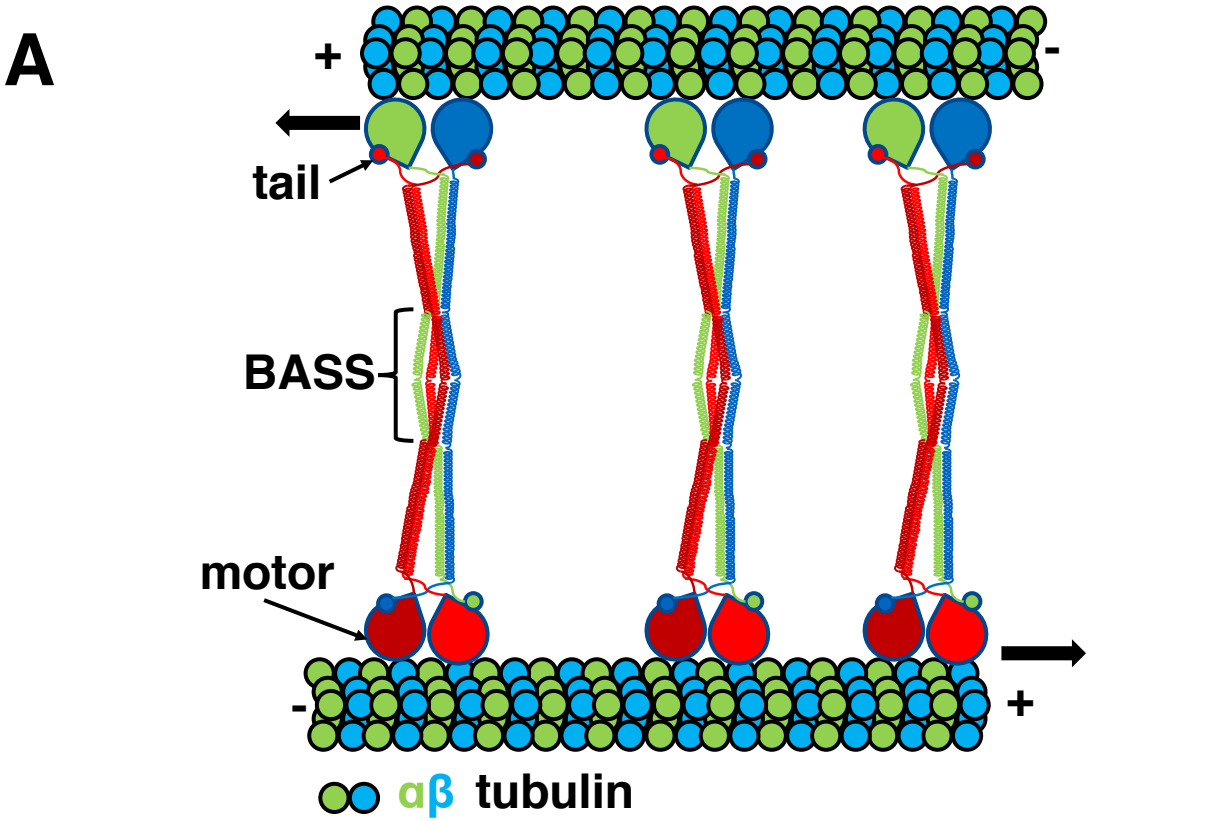


Figure 2

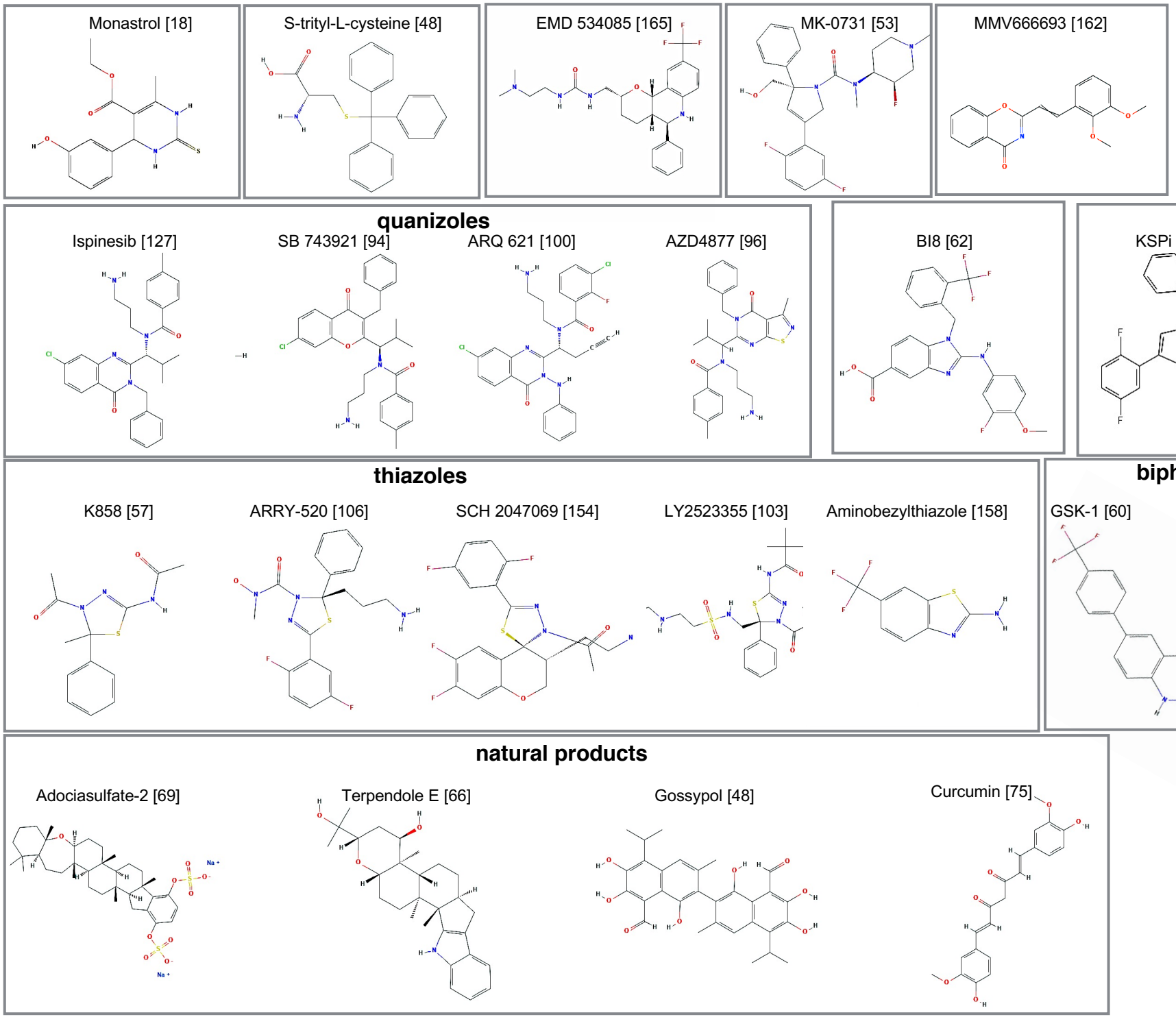

Monastrol [18]
S-trityl-L-cysteine [48]
EMD 534085 [165]
MK-0731 [53]
MMV666693 [162]
quanizoles
Ispinesib [127]
SB 743921 [94]
ARQ 621 [100]
AZD4877 [96]
BI8 [62]
KSPi SMOL[142]
thiazoles
K858 [57]
ARRY-520 [106]
SCH 2047069 [154]
LY2523355 [103]
Aminobezylthiazole [158]
biphenyls
GSK-1 [60]
PVZB1194 [61]
natural products
Adociasulfate-2 [69]
Terpendole E [66]
Gossypol [48]
Curcumin [75]

Figure 3

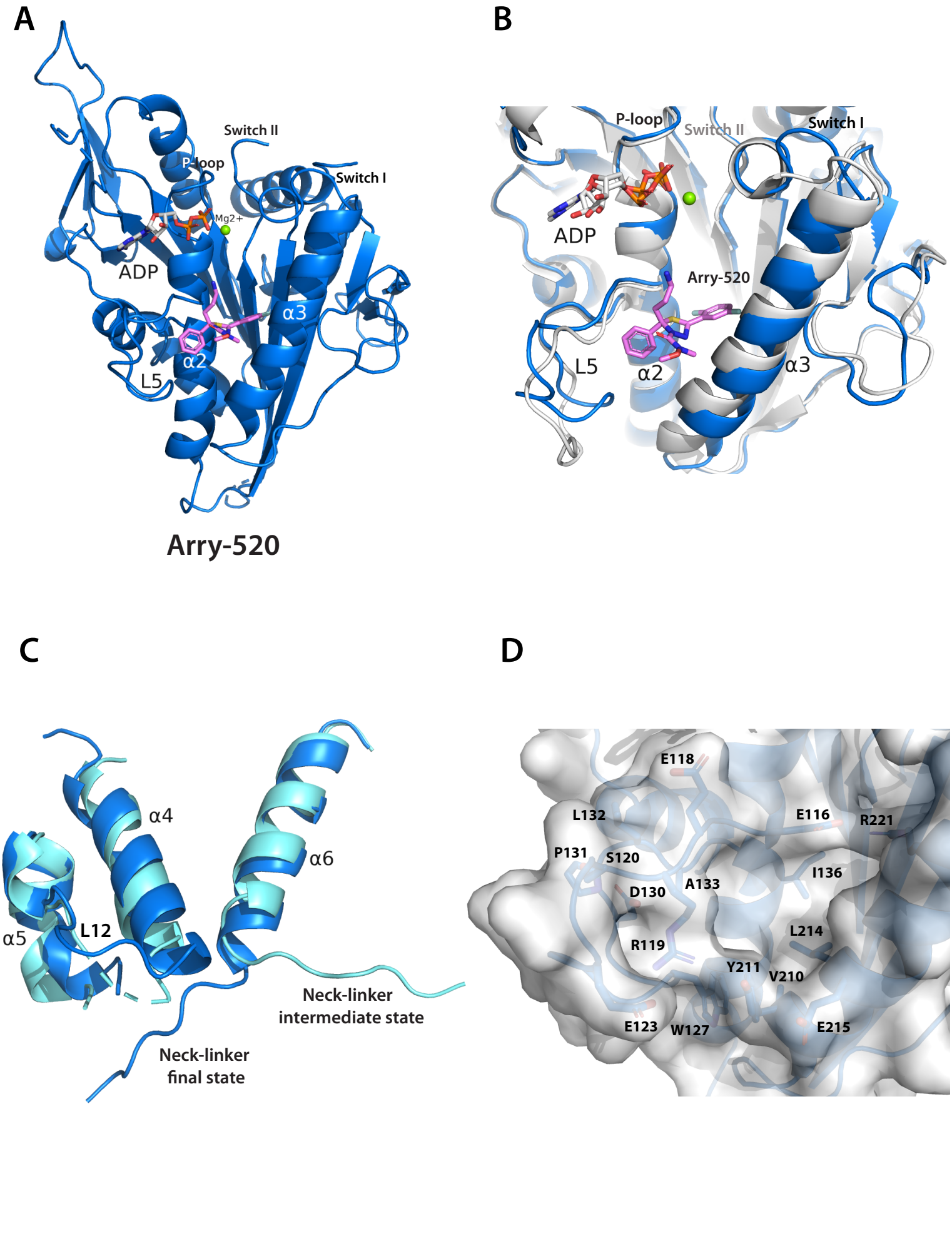
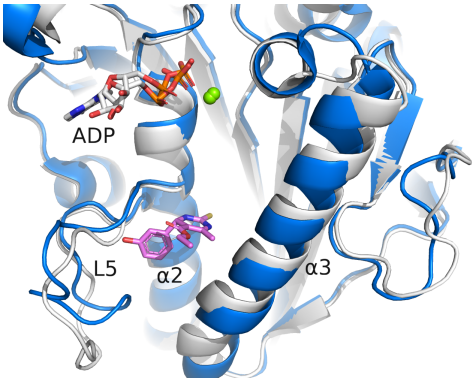
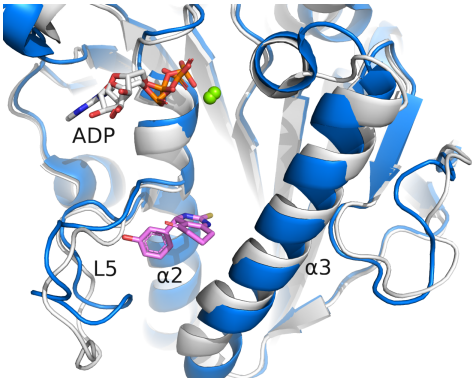

A
Switch II
P-loop
Switch I
Mg2+
ADP
α3
α2
L5
Arry-520
B
P-loop
Switch II
Switch I
ADP
Arry-520
L5
α2
α3
C
α4
α6
α5
L12
Neck-linker
intermediate state
Neck-linker
final state
D
E118
L132
E116
R221
P131
S120
I136
A133
D130
L214
R119
Y211
V210
E123
W127
E215

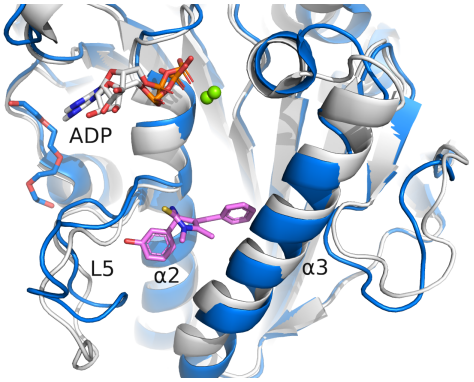
Figure 4



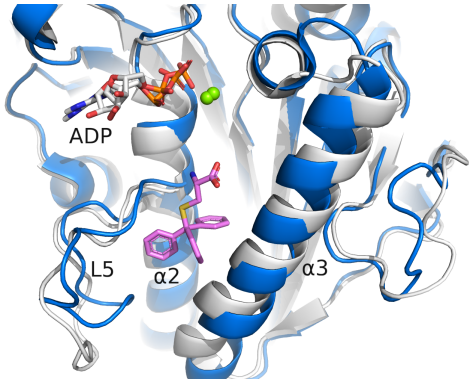
Monastrol



(S)-enastron



(R)-monastrol-97



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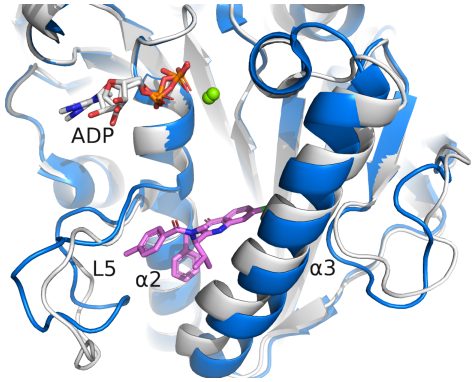
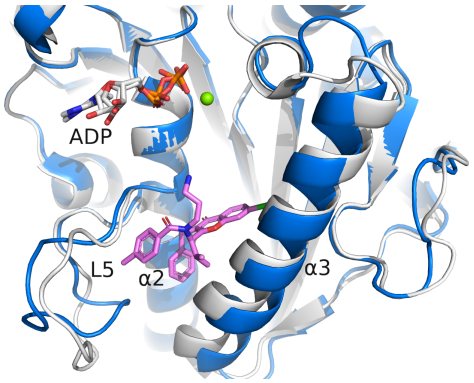
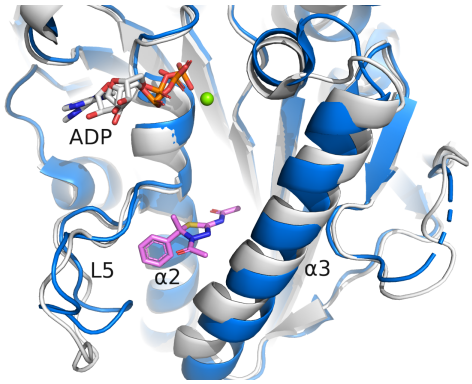


Ispinesib



SB743921



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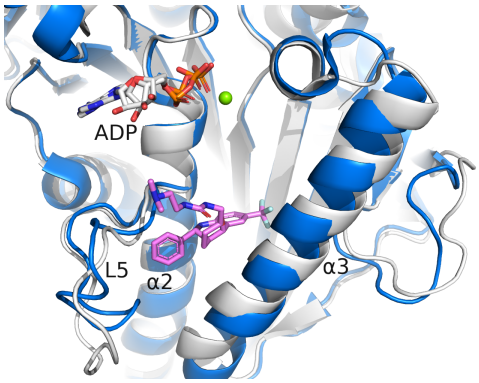

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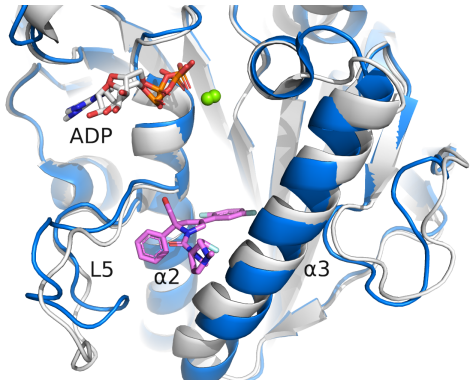
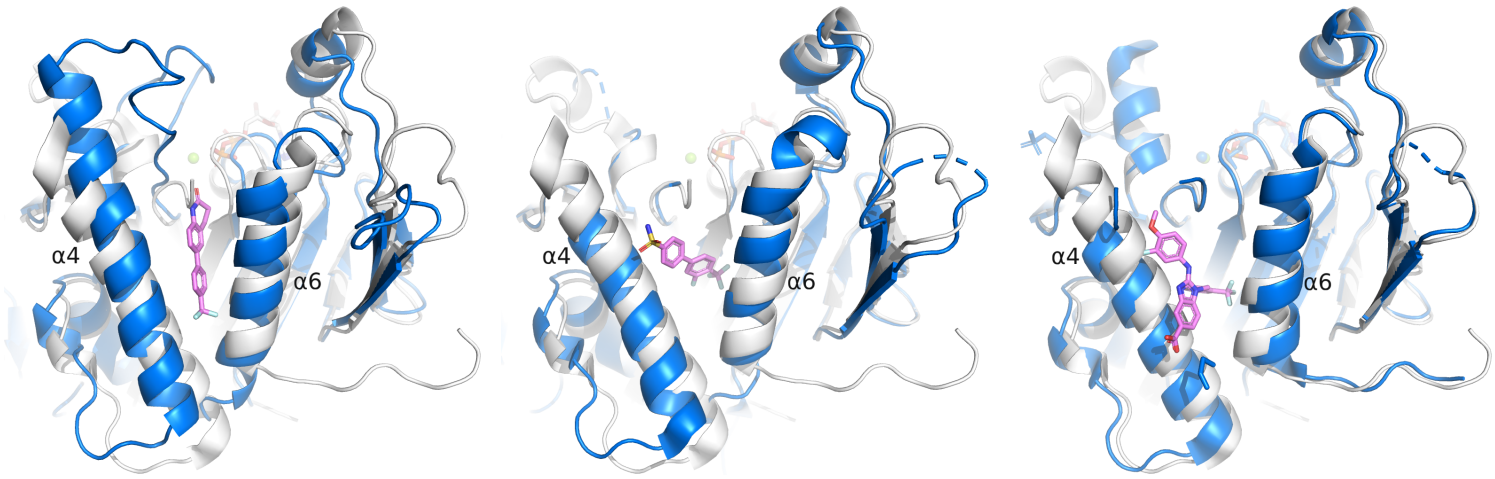


MK-0731

Figure 5



GSK-1 PVZB1194 BI8