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Cytoskeleton, Inc.

Custom Services Newsletter

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This Issue Contains

Future Topics

Quarter 4, 2014
Fungal Eg5 inhibitors

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Since the discovery of the first kinesin spindle protein (KSP, also known as Eg5, KIF11) inhibitor, monastrol, in 1999 (1), there have been a plethora of articles, 3D structures, and FDA applications based on the same binding site (review [2]). The binding site is called the allosteric monastrol binding site (AMBS) which functionally plays a significant role in transducing chemical energy to movement of the neck-linker region. Drugs that bind this site are non-competitive inhibitors of ATP and microtubule (MT) binding. To date, the majority of published drugs bind to this site which is coincidental in most cases; the reason for this is not apparent but possibly because the site is one of the most dynamic sub-domains of kinesin (3).

Figure 1: Kinesin's drug binding sites.

Figure 1. Drugable sites within KSP motor domain. The magenta color represents KSP's polypeptide structure in a worm/tube scheme, and the MT is in ghostly grey. Drugs representing the four different known sites in KSP: a) AMBS represented by MK-0731 (12); b) ATP site represented by ADP (13); c) MT site represented by NSC 622124 (10); and d) a second allosteric site (A2) binding near to the MT and ATP sites represented by GSK-1 (9).

More recently, interest has focused on other drugable sites in part because the AMBS compounds are known to select for resistance mutations (4,5). Alternate sites could be ATP or MT binding competitors or other allosteric pockets. These alternatives are represented by Fig. 1, which shows their general location with respect to the MT and coiled-coil tail which binds adjacent KSP molecules in a tetramer design (6). Excellent biochemical work by multiple groups has enabled classification of new binding sites (7-10); their method of classification sets a necessary standard for characterization of future KSP inhibitors (see below).

At present, there are no front runner sites with regard to the optimal site for clinical efficacy. It is interesting to note that very high affinity drugs (1-10 nM range) have been developed for the AMBS but without the expected improvement in clinical efficacy. Although the combination of anti-tumor drugs with KSP inhibitors looks like a promising use of the inhibitors (review [11]), the use of KSP inhibitors alone has not yielded exciting clinical results. One aspect of developing very high affinity drugs is the likelihood that affinity for off-target sites is also improved; these could include binding to serum proteins, detoxification sites, MDR elimination sites, and fat tissue deposits as well as other enzymatic targets in the cell. Two recent reports emphasize these phenomena: 1. the recent finding that monastrol inhibits urease with similar binding affinity (15) and 2. the finding that a new compound shows binding to two sites on KSP alone (8). One way of looking at the phenomena is that there will always be off-target sites for all compounds, and that improving affinity for a particular site will not per se improve efficacy in the clinic. It is interesting to note that even with very high affinity drugs, the dose applied to patients has not been proportionally decreased (Fig. 2); for example, MK-0731 is administered at close to the maximum tolerated dose of 34 mg i.v. (2), whereas a 10 fold excess over the dissociation constant (Kd) would

Figure 2: Two drugs in the AMBS site.

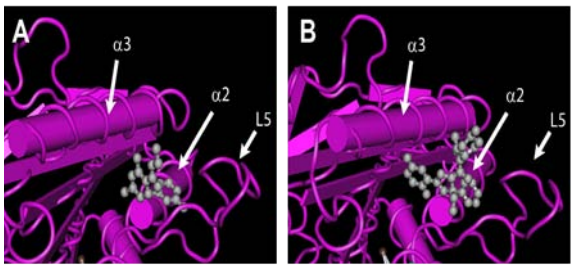

Figure 2. Monastrol and MK-0731 in the AMBS have very different affinities, low and very high, respectively. Monastrol is in panel A (14) and MK-0731 in panel B (12). Note the more complex aromatic structure of the high affinity compound.

imply using only 100 µg per patient. Thus, very high affinity drugs might not be the way forward; instead it may be better to use low micromolar Kd compounds and administer 20-100 mg per dose. In this case, serum binding and elimination mechanisms would be saturated while the target is effectively inhibited.

One key aspect to KSP's drug family future is to develop standard characterization schemes that substantially characterize each compound pre-clinically. These aspects include 1. biochemical: competition with ATP, MTs, and monastrol to determine site of action; 2. cell biological: classification of cell phenotype; 3. cell line resistant mutations and sequence analysis; 4. drug cross-linking and peptide analysis; and 5. 3D protein model representation of the binding site. Two excellent examples of such studies are GSK-1 by Luo et al. (9) and NSC 622124 by Learman et al. (10). At Cytoskeleton we are developing a Pre-Clinical Pharmacological Profile (PCPP) for kinesins (and myosins) which provides a report that characterizes new compounds with these guidelines and provides a reference for documentation purposes (for more information, contact tservice@cytoskeleton.com).

In conclusion, targeting KSP is following the historical drug development trajectory of other important classes of clinical compounds such as GPCR inhibitors and microtubule inhibitors, both of which took 20+ years to reveal their potential.

(see references on the next page)

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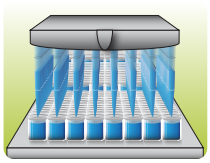
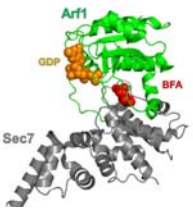
Compound Screening Modules



Type	Format	Deliverable	Module #	Timeline (wks)
Eg5 Kinesin motor assay	Microtubule stimulated ATPase assay, kinetic, absorbance at 360nm	96 assays, consisting of 40 duplicate single concentrations (or 5 x IC50s), plus eight control wells. PDF Report with Executive Summary, Introduction, Methods, Results and Data Analysis.	CDS050 or CDS051	2
Cardiac Myosin motor assay	Ca^{2+}/Sarcomere (thin filament) stimulated ATPase assay, kinetic, absorbance at 360nm	Same as CDS052.	CDS056	2
Dynein motor assay	Microtubule stimulated ATPase assay, kinetic, absorbance at 360nm	Same as CDS052.	CDS065	2
Tubulin polymerization	Tubulin (>99% pure) Polymerization Assay, kinetic, fluorescence at 360nm/410nm	96 assays, with 40 duplicate single concentrations or 5 x IC50s, plus eight control wells (vinblastine, nocodazole or taxol). PDF Report with Executive Summary, Introduction, Methods, Results and Data Analysis.	CDS009 or CDS010	2
GEF/GTPase exchange assay	GTP exchange factor plus Small G-protein (e.g. Rho or Ras) with mant-GTP reporter. Kinetic, fluorescence at 360nm/450nm	60 assays consisting of either 28 duplicate reactions plus 4 controls, or 5 x IC50s plus 1 x control IC50. PDF report with Executive Summary, Introduction, Methods, Results and Data Analysis.	CDS100	2

Gene Cloning and Protein Purification Modules



Type	Name	Deliverable	Module #	Timeline (wks)
Recombinant Small Protein	Small protein or protein domain (<30 kDa) with gene provided by client	Highly purified, His-tagged active protein lyophilized in 10 x 100 µg aliquots (or more depending on yield). Datasheet and assay method. Activity in line with published articles. *E. coli* expression.	REC012	3
Recombinant Small Protein plus cloning	Small protein or protein domain (<30 kDa) including gene synthesis	Same as above with gene synthesis.	REC022	6
Recombinant Kinesin Motor-Protein	Medium to large protein or protein domain (30-100 kDa)	Same as REC012.	REC032	3
Recombinant Kinesin Motor Protein plus gene cloning	edium to large protein or protein domain (30-100 kDa) with gene synthesis	Same as above with gene synthesis.	REC042	8
Native or eukaryotic protein expression & purification	Cited protein purification	Same as above plus using a published procedure.	REC052	4-20

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