Kinases as targets for chemical modulators: Structural aspects and their role in spermatogenesis

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Abbreviations: BTB, Blood Testis Barrier; Dvl, Dishevelled; EMK1, ELKL motif kinase 1; ES, Ectoplasmic Specialization; FAK, Focal Adhesion Kinase; FAT, Focal Adhesion Targeting region; FERM, F for 4.1 protein, E for ezrin, R for radixin and M for moesin; JNK, c-Jun N-terminal Kinase; LIMK2t, LIM domain containing Kinase; MAPK/ERK, Mitogen Activated Protein Kinase/Extracellular signal-regulated Kinase; MARK, Microtubule Affinity Regulating Kinase; MDM2, Mouse double minute 2 homolog; PAK, p21-activated kinase; PKA, Protein Kinase A; POLO, Polo like Kinase Family; Rac/Cdc42, Ras-related C3 botulinum toxin substrate/ cell division cycle 42; SH - SRC Homology; Ste20, Sterile 20 gene; TJ, Tight Junctions; TSSK, Testis Specific Serine/threonine protein Kinase; ZO-1; Zona occludens-1.

Protein phosphorylation and de-phosphorylation events are crucial in deciding the fate of cells. They regulate cellular growth, differentiation and cell death, and kinases are the key players of these events. The members of ser/thr kinases and tyrosine kinases form the majority of protein kinase family, exerting their regulatory mechanism in almost all cells. In testis, they impact signal transduction events, regulate all stages of sperm development from mitosis through fertilization. Understanding the function of these kinases at the structural level and studying their interactions with inhibitors can help in understanding the machinery of spermatogenesis. In view of this, we have reviewed some of the prominent kinases that are known to play a role in spermatogenesis. A better understanding of the impacts of kinase inhibition on spermatogenesis should aid in the interpretation of lesions and hopefully further the development of more efficient and potent drug candidates.

Introduction

Spermatogenesis is an orderly series of carefully regulated events that result in production and maturation of life progenitors, sperms. A cross section of seminiferous epithelium in testis shows the presence of long nursing cells called Sertoli cells and germ cells at different stages of cell division. The germ cells, that are located in between the Sertoli cells undergo spermatogenesis and finally form mature spermatozoa which are released into the lumen of the seminiferous tubules. The seminiferous epithelium is divided as basal and adluminal compartments by the

testis-specific blood testis barrier [BTB]. The BTB is formed by testis specific actin based adheren junctions called basal Ectoplasmic specializations [basal ES], along with tight junctions, gap junctions and desmosomes. BTB is one of the tightest barriers formed by intermixing of varied components that regulate the movements of proteins and other particles from basal to adluminal compartments. Proper organization and restructuring of BTB is essential for transport of preleptotene spermatocytes from the basal into the adluminal compartment of the seminiferous epithelium and also protects the developing spermatids and mature spermatozoa from host immune reactions. Analogous to the basal ES, a specialized actin based adheren junction is formed between the Sertoli cells and spermatids, known as apical ES.¹ Phosphorylation status of integral membrane proteins, adaptors, and regulatory proteins at tight junctions, basal ES, gap junctions and desmosomes are crucial for maintenance and restructuring of BTB as well as apical ES. Several serine/threonine and non-receptor tyrosine kinases play a crucial role in phosphorylation of key proteins in BTB and apical ES, and so disruption of their expression and or kinase activity would disturb the organization of these cell-cell interfaces which would affect spermatogenesis. The role of kinases also extends to other events leading up to fertilization and is vital to cellular signaling machinery and for modulation of other cellular proteins.²⁻⁶ Owing to their functional importance, characterizing their structural and functional regulation helps in identifying the causes for male infertility and also in understanding why drugs that target or inhibit these kinases may lead to spermatogenic disturbances. In view of this, we have made an attempt to review key kinases that are known to exert crucial control on spermatogenesis, and we have discussed the effect of kinase inhibition at various stages of spermatogenesis induced by binding with potent small molecule modulators. This review is intended to provide the reader with the general types of testicular changes and the mechanism of protein interaction

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profile caused by inhibiting a specific kinase. Although there are not enough data to elaborate the effect of most of the inhibitors on spermatogenesis, we have summarized the pathological lesions formed due to absence of these kinase activities which should provide a glimpse of the effects of small molecule inhibitors at various stages of spermatogenesis.

Kinases as Targets for Chemical Modulators

Hampering signal transduction by chemical modulators that target kinases has been known to be the underlying mechanism for understanding various events involved in male reproductive biology. These chemical modulators also help in rectifying the anomalies in molecular events and based on their effect on reproductive function, they can be categorized into drug candidates, environmental toxicants and male contraceptives.

Contraceptives

Small molecule modulators that can prevent the production of sperm, motility of sperm or affect the fertilization of sperm and ova, are termed as male contraceptives.⁷ They can be hormonal or non-hormonal; hormonal contraceptives prevent the secretion of hormones or inhibit their actions, whereas, non-hormonal contraceptives inhibit proteins that are crucial in modulating sperm function.^{8,9} Development of non-hormonal contraceptives confers several advantages over the hormonal contraceptives and recent researches are actively concentrated on developing potent non-hormonal contraceptives. Adjudin, H2-Gamendazole, BMS-189453 and WIN 18,446 are some of the known non-hormonal contraceptive candidates. These molecules target several proteins such as kinases and integrins, and inhibit their function in sperm development. On the other hand, all these molecules are bound with serious disadvantages which emphasizes the continuing search for new targets and new inhibitors that can specifically and reversibly confer male sterility.¹⁰

Environmental toxicants

Exposure to chemicals in the environment including some fungicides, pesticides, heavy metals and industrial chemicals, pose serious threats to male fertility and could have an adverse impact on the overall population. These chemical toxicants can have effects at various sites within the reproductive tract and may result in decreased sperm count, reduced fertility, alterations in sperm morphology and defects in hormonal functions.¹¹ Compounds such as Bisphenol A (BPA) and Cadmium that fall under the category of endocrine disruptors particularly target kinases of signaling pathway such as MAPK/ERK cascade and hinder the synthesis and action of hormones in both males and females.¹² Bisphenol A (BPA), a synthetic molecule used in manufacture of polycarbonate plastics, is known to interact with protein kinases that participate in the signaling cascade in restructuring blood testis barrier (BTB). These molecules by interacting with proteins of Gap Junction (GJ) destabilize the integrity of BTB.¹³ Cadmium, the heavy metal, on other hand, is a well-known causative of male infertility. Similar to BPA, molecular action of cadmium

also involves at least the MAPK pathway. The FAK-occludin-ZO-1 complex is one of the cellular targets of cadmium which upon interaction disrupts the integrity of BTB.¹³ Another common toxicant is 4-tert-octylphenol, which has a weaker estrogenic activity and is also known to affect spermatogenesis and result in decreased sperm count and other irregularities in reproductive ability in rodents. This molecule along with other well-known xenoestrogens targets Protein Kinase A and C, MAPK and activates them which triggers the activation of MAPK signaling pathway and thus result in toxic effects.¹⁴⁻¹⁶

Anti-cancer drugs

Besides known for their role in spermatogenesis, many of the kinases reviewed here including MAPK, ERK, JNK, Pi3K, PKA, PKC, SRC, and FAK are well known cellular targets for development of anti-cancer drugs. PD184352, is a non-ATP-competitive inhibitor specifically bind with and inhibit MEK (Mitogen-activated protein kinase), and therein it inhibits MEK/MAPK activation in tumor cells and hampers tumor growth.¹⁷ Besides, molecules like TAE226, PF-562,271 and Sunitinib are known to inhibit FAK and other tyrosine kinases and they promote cell growth arrest in pancreatic, colon, head and neck cancers.¹⁸ The use of these kinase inhibitors as anti-cancer drugs is well reviewed by various authors and all studies indicate that these kinases play a significant role in cellular functions.

Kinases: Structure, Effect and Mode of Inhibition

Ser/threonine kinases

Members of the Ser/Thr (serine/threonine) family of protein kinases are vital in regulating the signal transduction events, cell growth and differentiation processes. A large number of these kinases are expressed at various stages of sperm development (Table 1). These kinases share a conserved catalytic domain that has N and C lobes with a catalytic cleft placed in between them. They are activated by phosphorylation of conserved Ser/Thr residues in their activation loop and they recognize conserved residues in their targets for phosphorylation. These kinases, especially members like MAPK/ERK, are known to be key players in determining growth, division, differentiation and cell death and so found to be potential targets involved in progression of various cancers. MAPKs also regulate the Sertoli-Sertoli and Sertoli-germ cell interface by modulating the dynamics of ES junctions and BTB. They are also important for sperm motility and fertilization. On other hand, members of JNK family are essential for triggering apoptosis in germ cells hence, identified to be targets for the development of some male contraceptives.²⁻⁵ Of these, MAPK1, MAPK2, JNK, ERK1/2, PKA, POLO-like kinases, TSSK, MARK are well-studied kinase targets in sperm development and function.^{5,19-22} Protein kinase A and C are involved in sperm capacitation and acrosome reaction and acts as key regulators of sperm motility.²²⁻²⁵ LIMK2t, testis specific variant of LIMK2 is known to be important for the progression of spermatogenesis and is specifically expressed in germ cells.²⁶ The members of testis specific serine kinases, TSSKs, are crucial in

Table 1. List of protein kinases involved in spermatogenesis

Protein Name	Synonyms/Protein family	Gene Name	Protein Expression	Role in Spermatogenesis	Reference (PubMed ID)
Serine Threonine Kinases					
PAK6	STE Ser/Thr protein kinase family. STE20 subfamily.	PAK6	Sertoli cells, Leydig cells and germ cells	Phosphorylates nuclear receptors, AR and ER	11950587 ²⁷
MARK4	AMPK Ser/Thr protein kinase family	MARK4	Localized in Sertoli cells, germ cells	Regulation of polarity in germ cells and BTB	22670221 ¹⁹
TSSK1	CAMK Ser/Thr protein kinase family	TSSK1B	TSSK Late spermatids and sperm	Reconstruction of the cytoplasm and flagellum during spermatid development	15044604 ²⁰
TSSK2	CAMK Ser/Thr protein kinase family	TSSK2	Condensed spermatids and mature spermatozoa	Required for the transformation of a ring-shaped structure around the base of the flagellum originating from the chromatoid body	15044604 ²⁰
TSSK3	CAMK Ser/Thr protein kinase family	TSSK3	Round and condensed spermatids	Spermiogenesis, sperm function	15044604 ²⁰
TSSK4	CAMK Ser/Thr protein kinase family	TSSK4	Anterior sperm head and flagellum	Spermiogenesis, sperm function	15044604 ²⁰
TSSK6	CAMK Ser/Thr protein kinase family	TSSK6	Localized in sperm head	Plays a role in DNA condensation during postmeiotic chromatin remodeling	15870294 ⁴³
MAPK					
ERK					
ERK1	MAP kinase subfamily.	MAPK3	Sertoli cells	Regulation of ES dynamics	11700851 ⁷¹
ERK2	MAP kinase subfamily.	MAPK1	Sertoli cells	Regulation of ES dynamics	11700851 ⁷¹
ERK7	MAP kinase subfamily	MAPK15	Rat testis, Sertoli cells	Regulation of ES dynamics	9891064 ⁷²
JNK					
JNK1	MAP kinase	MAPK8	Tetraploid spermatocyte and spermatogonia	Spermatocyte loss through apoptosis	12187281 ⁷³
JNK2	MAP kinase	MAPK9	Tetraploid spermatocyte and spermatogonia	Spermatocyte loss through apoptosis	12187281 ⁷³
JNK3	MAP Kinase	MAPK10	Mammalian testis	Spermatocyte loss through apoptosis	8654373 ⁷⁴
p38					
p38 α	MAP Kinase	MAPK14	Sertoli cells and elongate spermatids	Cell junction dynamics and apoptosis of germ cells	15618353 ⁷⁵
p38 β	MAP Kinase	MAPK11	Sertoli cells and elongate spermatids	Cell junction dynamics and apoptosis of germ cells	15618353 ⁷⁵
p38 β 2	MAP Kinase	MAPK11	Sertoli cells and elongate spermatids	Cell junction dynamics and apoptosis of germ cells	15618353 ⁷⁵
p38 δ	MAP Kinase	MAPK13	Sertoli cells and elongate spermatids	Cell junction dynamics and apoptosis of germ cells	15618353 ⁷⁵
LIMK2t	TKL Ser/Thr protein kinase family	Limk2t-1 Limk2t-2 (Mouse)	Early stages of spermatogenic cells and somatic cells	Regulation of cofilin activity Localization of germ cells	9610354 ⁷⁶
PLK4	Polo-like Kinases	PLK4	Testis	Organization and function of mitotic apparatus	21590086 ⁷⁷
MAK	CDC2/CDKX	MAK	Testis/prostate	Transcriptional co-activation of AR Spermatogenesis	12084720 ⁷⁸
Tyrosine kinases					
c Kit	Receptor protein-tyrosine kinase	KIT	Spermatogonia, acrosomal granules of the round spermatids, and Leydig	Essential for the migration of PGCs to the genital ridges in the embryo and then functions in	19435714 ⁷⁹

(continued on next page)

Table 1. List of protein kinases involved in spermatogenesis (Continued)

Protein Name	Synonyms/Protein family	Gene Name	Protein Expression	Role in Spermatogenesis	Reference (PubMed ID)
c-Ret	Tyrosine-protein kinase receptor	RET	cells in the adult human testis. Expressed in self-renewing SSCs	the maintenance of PGCs Establishment of postnatal spermatogenesis	17597063 ⁸⁰
FAK	Src protein tyrosine kinase family	PTK2	Focal adhesion Complex and in testis it is localized to Blood-Testis –Barrier (BTB)	Key component of sertoli cell junctions; Regulate spermatid transport and spermiation events	24665388 ⁵²
FerT	Src protein tyrosine kinase	FER	Spermatocytes at the pachytene stage of meiotic prophase	Associated with an induction in N-cadherin, b-catenin, and p120ctn at the base of the seminiferous epithelium.	12700184 ⁸¹
Fyn	Src protein tyrosine kinase	FYN	Basal and apical ectoplasmic specialization [ES] of sertoli cells and in sperm head.	Regulates the stability and dynamics of ES and regulates sperm head shaping	11751285 ⁶³
HCK	Src protein tyrosine kinase	HCK	Full-length and truncated forms of Hck kinase express in round and elongating spermatids	Regulates the acrosome formation	17926339 ⁶⁸
Lyn	Src protein tyrosine kinase	LYN	Leydig cells, Sertoli cells, or spermatogonia	Regulates the acrosome formation	20798388 ⁸²
Src	Src protein tyrosine kinase	SRC1	Focal Adhesion Complex Blood-Testis-Barrier (BTB)	Phosphorylates FAK and regulates Adhering Junction Dynamics	12697723 ⁵¹
Other kinases					
GSK-3 α	Serine/threonine-protein kinase	GSK3A	Spermatozoa	Motility initiation and stimulation	15223849 ⁸³

deciding sperm head shape, sperm motility and spermatogenesis events²⁰ and isoform 4 of MARK kinase are also crucial in modulating the dynamics of ES and polarity of sperms.¹⁹ Here, we have reviewed the morphological changes that occur due to loss of function of certain key kinases that are identified as targets (Table 2) and have also discussed the structural aspects of their interactions with inhibitors (Table 3), so that they can be used in future for the development of efficient inhibitors.

p21-activated kinase 6 (PAK6)

PAK6 (p21-activated kinase 6) kinase of serine threonine kinase family, is a novel member of p21-activated kinase family that binds to and modulates the function of androgen receptor [AR] and estrogen receptor [ER]. The effect of PAK6 in spermatogenesis is contradictory in the sense that, stringent regulation of PAK6 is a requisite for normal functioning of spermatogenic events involving Androgen Receptor. PAK6 belongs to a Group II p21-activated kinases [PAK4–6], belonging to the family of Rac/Cdc42-associated Ste20-like Ser/Thr protein kinases.^{27,28} Activated PAKs modulate the downstream events that regulate growth, motility and differentiation of cells. In general, the structure of PAKs have a conserved p21 binding domain, followed by a kinase domain.²⁹ PAK6 is a novel member of the Group II PAK family that possesses nuclear receptor binding ability and is expressed in testis, prostate, kidney, brain

and placenta. PAK6 is also a tumor suppressor, whose expression in prostate cancer cells is found to inhibit translocation of AR into the nucleus as well as binding to nuclear AR and inhibiting its transcriptional activity.^{30,31} In addition, PAK6 modulates the ubiquitination of AR upon androgen stimulation. Hence, PAK6, by inhibiting translocation of AR into the nucleus prevents its transcriptional activity and degrades it, effectively arresting the role of AR in testis.³² Inhibition of PAK6 during spermatogenesis would result in the normal functioning of AR in the stages VII to VIII of the epithelial cycle in seminiferous epithelium, where AR is responsible for prevention of apoptosis of spermatids in stages VII and VIII and for elongation of round spermatids by mediating their adhesion to Sertoli cells.

In view of this, certain kinase inhibitors like Staurosporine, PF-3758309 and Sunitinib have been identified to inhibit the activity of PAK6.^{33,34} Of these drugs, Coburn et al., have found that administration of Sunitinib did not have any toxic effect on male reproduction.³⁵ That study shows that a therapeutically-effective level of Sunitinib could inhibit some PAK6 forms in the body, but leave the male reproductive system unaffected. Gao et al., elucidated the structure of PAK6 with Sunitinib an ATP competitive inhibitor³⁴ which binds in the cleft between the lobes of catalytic domain of active PAK6. By forming hydrogen bonds and hydrophobic interactions with the residues of kinase linker region, it precisely occupies the active site cleft thereby

Table 2. Role of specific kinases in spermatogenesis and probable morphological changes observed when their function is inhibited

Kinase	Functional characteristics	Representative changes observed in the absence of specific kinase
PAK6	Inhibits normal functioning of AR in stages VII to VIII of epithelial cycle	Not yet characterized
MARK4L	Stabilizes actin, tubulin cytoskeleton at apical and basal ES at stages I to early VIII of epithelial cycle.	Loss of spermatid polarity
TSSKs	Chromatin condensation of spermatids and Sperm head shaping	Disorientation of spermatid Premature release of sperm Distal migration of chromatin bodies in sperm head Impairment of mitochondrial sheath in sperm
FAK	Actin polymerization at basal and apical ES	Decreased motility rate and fertilising ability Disrupts BTB and results in haemorrhage Germ cell loss
Fyn	Crucial for development of seminiferous tubule. Early and late stages of epithelial cycle	Premature release of spermatozoa Defective seminiferous tubule
Hck	Late stages of epithelial cycle – spermiogenesis	Underdeveloped lumina Abnormal vesicular structures at apical and basal ES Germ cell necrosis Abnormal sperm head shape Not characterized

preventing the kinase activity of PAK6 (Fig. 1A & B). In addition, Sunitinib exhibits high selectivity for PAK6, making it a specific inhibitor of PAK6 that can be certainly used to treat defects in sperm that arise due to the PAK6 activation.

Microtubule affinity regulating kinase 4 (MARK4)

MARK4 is an isoform of MAP/Microtubule affinity regulating kinases that belong to the family of Ser/Thr kinases and identified to be the key regulators of microtubules.³⁶ MARK4 is unique from its other isoforms in its ability to directly bind to microtubules and affect its dynamics in the cell. Of its 2-splice variant, MARK4L express predominantly in germ cells and Sertoli cells. MARK4L exhibits stage specific expression, wherein it is highly expressed at stages I to early VIII, and its expression is depleted after late stage VIII of the seminiferous epithelial cycle. It is observed surrounding the entire elongating spermatids till stage V, and from VII to early VIII, it is localized to the concave side of spermatid head. It interacts with tubulin and stabilizes the actin, tubulin cytoskeleton at both apical and basal ES. Its interaction with tubulin is essential for conferring polarity to Sertoli cells at basal ES and to spermatids at apical ES. Treatment of cells with adjuvin, a potential male contraceptive, leads to absence of MARK4 at apical ES resulting in loss of spermatid polarity, leading to random orientation of spermatids rather than their normal orientation facing the basement membrane, subsequently leading to premature release of spermatids into the lumen (For lesions, refer to Fig. 6, Tang et al, 2012¹⁹). These observations identify MARK4 as a crucial regulator of tubulin actin cytoskeleton during spermatogenesis.

Structurally, MARK4 kinase domain has a unique “DFG-in/ α C helix out” conformation in their inactive form along with

other essential features of Ser/Thr kinases.³⁸ Besides, its role in spermatogenesis, it also functions as mediator of Wnt signaling network, where it is involved in phosphorylation and activation of Dvl protein which helps in translocating β -catenin to the nucleus and regulating its target genes.³⁷ The unique architecture of the catalytic cleft imposed by the presence of “DFG-in/ α C helix out” conformation renders it difficult to use the existing kinase inhibitors for MARK4 to inhibit the L isoform. Recently 9-oxo-9H-acridin-10-yl core containing molecules, which are known to exert inhibitory action on MARK2³⁸, and their mode of interaction with MARK2 and MARK4 was studied in detail by Jenardhanan et al.³⁹ Later, they also identified new potential molecules against MARK4 and found 1-hydroxy-3-[2-(3-nitrophenyl)-2-oxoethoxy]-9H-xanthen-9-one (Ligand name: 411) and 5 other molecules to bind specifically with MARK4 and their binding induced closure of the catalytic cleft. Their binding was stabilized by interaction with catalytic lysine K88, D199 of DFG motif, A138 of hinge region (Fig. 1C & D). They establish strong contacts with residues of MARK4 which are also involved in interaction with ATP suggesting that these newly identified inhibitors can act as potent ATP competitors that can specifically recognize the unique “DFG-in/ α C helix out” conformation of MARK4 and inhibit its activity making them promising lead compounds against MARK4 and thus could alter the microtubule dynamics of Sertoli cells and spermatids during spermatogenesis.

Tissue specific serine kinases (TSSKs)

Tissue specific serine kinases of CAMK Ser/Thr protein kinase family are unique post-meiotic kinases that are crucial in spermiogenesis. Right from TSSK1 to TSSK6, members of this

Table 3 . Structural details of kinases and their inhibitors dealt in the review

S.No.	Kinase	PDB ID	Inhibitor	
			Name	Structure
1	PAK6	4KS8	Sunitinib
2	MARK4	Predicted model	1-hydroxy-3-[2-(3-nitrophenyl)-2-oxoethoxy]-9H-xanthen-9-one
3	TSSK1	Predicted model	K-252a
4	TSSK2	Predicted model	K-252a
5	TSSK6	Predicted model	K-252a
6	FAK	2JKK	2-({5-chloro-2-[(2-methoxy-4-morpholin-4-yl)phenyl]amino}pyrimidin-4-yl)amino)-n-methylbenzamide
7	Fyn	2DQ7	Staurosporine	

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Table 3 . Structural details of kinases and their inhibitors dealt in the review (Continued)

S.No.	Kinase	PDB ID	Inhibitor	
			Name	Structure
8	Hck	1QCF	1-ter-butyl-3-p-tolyl-1 h-pyrazolo[3,4-d]pyrimidin-4-ylamine

family are targets for development of male contraceptives.^{20,40-43} TSSK1 and TSSK4 are found to be expressed in the anterior head and flagellum of sperm, while TSSK2 and TSSK6 are exclusively expressed in the sperm head. Both TSSK2 and TSSK6 (SSTK) are localized near the post-acrosomal region and near the acrosome tip.²⁰ TSSK1 and TSSK2 knockout studies in mice resulted in production of morphologically abnormal spermatids. These proteins are essential for the distal migration of chromatin bodies (CB) and their impaired expression results in improper movement of CB that affects maturation of sperm mitochondria and mitochondrial sheath formation. This eventually affects the cytodifferentiation events in sperm leading to male infertility (For lesions see **Fig. 4**, Shang et al., 2010⁴⁴).^{44,45} Similarly, mice with knocked out TSSK6 produced sperm with abnormal morphology and decreased motility rates. TSSK6 is exclusively involved in phosphorylation of histones H1, H2A, H2AX, and H3 of condensing chromatin during the nuclear condensation step of spermiogenesis. Upon disruption of TSSK6, chromatin condensation is affected resulting in the formation of sperms with abnormal morphology (For lesions see **Fig. 5**, Spiridonov et al., 2005).⁴³ Expression of TSSK3 is observed in round and elongated spermatids²⁰, indicating that the disruption of TSSK1-3 and TSSK6 can be used to develop potent and specific male contraceptives.

Structurally, these proteins are similar to MARK4 and to the ELKL motif kinase EMK1.⁴³ They all share the expected features of a serine/threonine kinase, but they are different in terms of their activation. TSSK1-2 and TSSK6 undergo autophosphorylation and they do not need any upstream kinases to become activated.^{43,46} Their activity is found to be inhibited by K-252a, an alkaloid isolated from *Nocardia* sp. soil fungi. It inhibits both TSSK1 and 2 with an inhibitory constant of 0.8 μ M and

16.6 μ M respectively.³³ Since there are no crystallographically solved structures of TSSKs, we have predicted the 3 dimensional structure of TSSK1, 2 and 6 using homology modeling and in order to study the mode of inhibition, molecular docking of K-252a with TSSKs was performed and the interaction profile shows that they bind within the ATP catalytic cleft, and form hydrogen bonds with K30, and K127. It also engages in hydrophobic contacts with E49, D143 which are crucial residues in engaging ATP within the cleft. Similar kinds of interactions were also observed between K-252a and TSSK2, and forms hydrogen bonds with catalytic Lysine K30, D86, and D143 of DFG motif. Binding of K-252a to TSSK6 forms hydrogen bond with E80, A82 of hinge region, and N129 which is prone to interact with magnesium ion. The binding of K-252a within ATP binding cleft and formation of stable interactions with catalytic conserved lysine, aspartic acid of DFG motif indicates that this molecule can also act as an effective ATP competitor (**Fig. 2A-F**). This interaction profile would help in understanding the mode of TSSK inhibition and the mechanism behind the pathological changes observed during spermatogenesis in the absence of TSSK.

Protein Tyrosine Kinases in Spermatogenesis

Protein Tyrosine kinases (PTK), members of non-receptor tyrosine kinases (NRTKs) are downstream regulators activated by receptor tyrosine kinases (RTKs), which altogether triggers signaling cascades that are vital to cell survival, differentiation and growth. Members of PTKs are widely expressed in various cells and the SRC family of kinases form the largest subfamily of PTKs, with 9 members (Src, Lck, Hck, Fyn, Blk, Lyn, Fgr, Yes, and Yrk),

Figure 1. Structure and mode of inhibition of PAK6 and MARK4. **(A–B)** Structure of PAK6 in complex with Sunitinib (PDB ID: 4KS8) and the interaction profile as 2 dimensional plot. **(C–D)** Three dimensional structure of MARK4 in complex with identified lead compound 1-hydroxy-3-[2-(3-nitrophenyl)-2-oxoethoxy]-9H-xanthen-9-on (411) and its 2D interaction plot. (Color code for 2D plot: Pink lines: H-bond (backbone); Pink dotted lines: H-bond (side chain); Pink: Negatively charged residues; Blue: Positively charged residues; Cyan: Polar residues; Green: hydrophobic residues; Red circle: Water; Yellow halo: ligand exposure)

and regulate mitogenesis, T and B-cell activation and cytoskeleton restructuring (**Table 1**). Members of this family have a C-terminal kinase domain [SH1], a central SH2 and SH3 domain, followed by an N terminal SH4 domain. SH2 and SH3 domains are involved in protein-protein interactions, while SH4 domain is always either myristoylated or palmitoylated followed by a domain of 50–70 domains that are divergent among the members. Each member has a C-terminal tail that has a unique auto-inhibitory phosphorylation site.⁴⁷ Expression of these kinases regulates most of the spermatogenic events and is correlated with migration of primordial germ cells to genital ridges, and its phosphorylation activities are found in meiosis during spermatogenesis. They regulate sperm morphogenesis and Sertoli cell tight junction at the blood-testis barrier (BTB).⁶ We have discussed a few relevant Src family of kinases that are crucial in spermatogenesis (**Table 2**) and their mode of inhibition (**Table 3**).

Focal adhesion kinase (FAK)

Focal adhesion kinase, also known as protein kinase 2 (FAK/PTK2), is a regulatory switch of integrin-based signaling cascades, and is found in all mammalian cells. FAK along with c-Src and c-Yes forms an integral component of Focal adhesion complex (FAC). Expression of FAK in testis is observed at BTB, apical ES and at TJ. In stages III to VI of epithelial cycle, FAK is expressed at BTB and predominant expression of p-FAK-Tyr-397 was observed at Sertoli-Sertoli cell interface.⁴⁸ Inhibition of FAK induces mis-localization of occludin and JAMA-A, integral membrane proteins of BTB and also it makes the BTB less sensitive to the treatment of environmental toxicants like cadmium.⁴⁹ Of its varied phosphorylated mimetics, p-FAK-Y397 and p-FAK-Y407 are found to have stage specific expressions and changes in their spatiotemporal expression, affects the remodeling of the actin filaments in apical and basal ES. In testis, the

Figure 2. Structure prediction and mode of inhibition of human TSSKs. (A) shows the predicted model of TSSK1 using homology modeling with template MARK2 (PDB ID: 3EIC⁸⁴) with 39.84% sequence similarity. The predicted model was generated using Modeler 9v11⁸⁵ with a DOPE score of -29648.60 Kcal/mol. The predicted model was docked with ATP competitor K25a using the binding site residues surrounding the catalytic cleft (K30, E49, D143) using Schrödinger Glide module.⁸⁶ The docked complex has a Glide score of -3.454 Kcal/mol and the interaction profile is depicted in (B) as 2D plot. (C) Shows the predicted model of TSSK2 in complex with K252a. The model was predicted with a DOPE score of -29546.37 Kcal/mol. The docked complex has a Glide score of -5.116 Kcal/mol and the interactions are depicted as 2D plot in (D). (E) shows the 3 dimensional predicted structure of TSSK6 docked with inhibitor K252a. The predicted model has a DOPE score of -26851.05 Kcal/mol and the docked complex with K252a has a Glide score of -6.692 Kcal/mol. Their interaction profile as 2D plot is depicted in F. (Refer Fig. 1 for color code)

phosphorylated FAK activates various actin regulatory proteins such as Eps8, palladin, filamin A, Arp3 and N-WASP. These actin regulatory proteins are crucial for formation of bundled and un-bundled/branched actin structures at both apical and basal ES. Concurrent transition from bundled to un-bundled/branched state is vital for transporting preleptotene spermatocytes across BTB and for release of mature spermatozoa into the

lumen. The expression of p-FAK-Y397 is restricted to stage VII-VIII, during which it interacts with Eps8 and palladin, which are actin bundling regulating proteins, and induces the actin bundling events. In late stage VIII, the expression of p-FAK-Y397 is depleted while expression of p-FAK-Y407 is observed at stage VII-VIII until late stage VIII. p-FAK-Y⁴⁰⁷ activates actin bundling proteins as well as the barbed branching actin

polymerization protein Arp3.⁵⁰⁻⁵³ Thus, the stage specific expression of Y397 and Y407 phosphorylated FAKs, along with actin bundling and barbed branching polymerization proteins regulates the configuration of actin microfilaments in apical ES and thereby they regulate the release of the spermatozoa into the lumen. Disruption of actin microfilaments by environmental toxicant cadmium and male contraceptive adjuvin results in the release of premature spermatozoa into the lumen. Micrographs of testis cross section of cadmium induced toxic effects in sperm shows that the treatment of cadmium induces the mislocalization of FAK and occludin (For lesion, Fig. 4, Siu et al., 2009).⁴⁹ This disrupts the BTB and results in germ cell loss. These features identify FAK as a key molecular switch that decides the conformation of actin microfilaments and other components of BTB and apical ES.

In general, FAK has a N-terminal band 4.1 protein, ezrin, radixin, moesin homology, FERM domain, a linker region, a central kinase domain, proline-rich low-complexity region, and a C-terminal focal adhesion targeting (FAT) domain. FERM domain interacts with the C-lobe of kinase domain and renders the FAK in its auto-inhibited state, and the disruption of this interaction activates FAK, which then phosphorylate its substrates.⁵⁴ The trigger of integrin signaling initiates autophosphorylation of FAK at residue Y397 which provides high affinity binding for SH2 domain to Src kinase, which in turn phosphorylates FAK at several sites. The activity of FAK is inhibited by the use of Type I and allosteric kinase inhibitors. Type I inhibitors compete with ATP and binds at the catalytic cleft and inhibits the kinase activity, while allosteric inhibitors binds at the sites distant to the ATP binding site and induces conformational changes that disrupts the binding of ATP.

Various selective inhibitors are used to inhibit autophosphorylation of FAK, as well as many molecules that are targeted to hamper kinase activity by binding with kinase domain. Recently, one from Novartis [NVP-TAE226] and 2 from Pfizer [PF-573 228 and PF-573 271] were tested. The candidate from Novartis, NVP-TAE226 was discontinued due to its off-target effects, while the PF-573 228 of Pfizer did not show any inhibitory effects in in vivo and in vitro.⁵⁴⁻⁵⁷ However, PF-573 271 does inhibit kinase activity of FAK and also its homolog PYK-2. Another inhibitor, PND-1186 was reported by Walsh et al. and it selectively inhibited the autophosphorylation activity of FAK.^{55,58-60} Golubovskaya et al. have also designed an allosteric inhibitor, Y15 [1,2,4,5-benzenetetraamine tetrachloride] that selectively inhibits the autophosphorylation of FAK by targeting Y397. This compound binds to N-terminal domain of FAK that contains Y397 and decreases the autophosphorylation of FAK. In their work⁶⁰, Lietha et al., solved the structure of FAK kinase domain with bis-anilino-pyrimidine analogs [TAE226, TAF089, TAF672 and AZW592 PDB ID: 2JOI], and showed that these molecules bind into the ATP binding cleft of FAK kinase domain. All these molecules share a common, 5-chloro-2-orthomethoxyanilino-4-anilinopyrimidine core that binds with the hinge region by forming hydrogen bonds with E500 and C502. Inhibitor TAE226 acts as an ATP competitive inhibitor and it inhibits FAK with an IC_{50} of 6.2460 ± 0.519 (nm).⁶¹

Interestingly, the binding of TAE226 within catalytic cleft (Fig. 3A & B) near DFG motif [D564-G566] induces the formation of a short helical turn with DFG motif, and this change is seen only in FAK kinase.⁶¹ This particular conformational change confers high potency and specificity of TAE226 over FAK. Recently, Iwatani et al., have performed a high throughput screening and identified novel tricyclic sulfonamides with allosteric inhibitory profile against FAK. These compounds have 1,5-dihydropyrazolo[4,3-c][2,1]benzothiazine scaffold and they act as ATP non-competitive inhibitors with an inhibition range of 0.88 to 1.0 μ M.⁶² On the whole, these novel tricyclic sulfonamides, TAE226 and Y15 can be used to prevent the activation of FAK and also, they might be effectively used to develop male contraceptives.

Fyn

Fyn kinase, a member of Src family of kinases, is vital in integrin-mediated signaling events. It is highly expressed in Sertoli cells especially below the layer of actin filaments in both apical and basal ES. It plays a role in development of the testis in early stages of growth, germ cell survival and sperm head morphology. Disruption of Fyn kinase by gene mutation or by toxicants or drugs would lead to several defects in spermatogenesis. It has been observed that in the absence of Fyn in 3 wk old mice, the development of seminiferous tubule is defective and the lumina was not fully developed (For lesion, refer Fig. 4B, Maekaw et al., 2002).⁶³ Its absence also induced formation of abnormal vesicular structures at both apical and basal ES (For lesion, refer Figs. 6 and 7, Maekaw et al., 2002⁶³). Inhibition of Fyn kinase in the early stages of testis development would lead to germ cell necrosis as evidenced by loss of nuclear envelope, degenerated mitochondria, and multivesicular vacuoles (A Fyn $-/-$ necrotic germ cell is shown in Fig. 5, Maekaw et al., 2002⁶³). It is also essential for ensuring proper sperm head morphology and its fertilization ability⁶⁴ and the absence of Fyn drastically affects the physical shaping of sperm that becomes evident from the presence of club-shaped and triangular sperm heads with impaired head to neck connections in Fyn null mice (Refer Fig. 3 in Luo et al., 2012⁶⁵).

Owing to its crucial functionality, Fyn kinase is an important target for development of male contraceptives and understanding the causes for male infertility. The structure of Fyn kinase has been solved with Staurosporine.⁶⁶, which is a well-known non-specific protein kinase inhibitor that acts as an ATP competitor. In Fyn, Staurosporine binds in the catalytic cleft located between the N- and C-lobes of kinase domain. It forms 2 hydrogen bonds with E342 and M344 of hinge region and it forms CH $\cdots$ O interaction, and 8 CH $\cdots$ π interactions with the residues S348 and G277 respectively. It also engages the following residues: L276, V284, and A296 of N-lobe, and G347 and L390 of C-lobe through hydrophobic interactions (Fig. 3C & D). Owing to the off-target nature of Staurosporine, many groups have been developing Fyn specific inhibitors and recently Poli et al., identified 3 specific inhibitors of Fyn through an in silico based approach and evaluated their ability to inhibit the kinase activity of Fyn. From the resulting potential candidates, the authors identified 9

Figure 3. Structure and mode of inhibition of Src kinases. (A–B) shows the 3 dimensional structure of FAK in complex with benzamide derivative (2-((5-chloro-2-((2-methoxy-4-morpholin-4-yl)phenyl)amino)pyrimidin-4-yl)amino)-n-methylbenzamide) (PDB ID: 2JKK) and its 2D plot. (C–D) shows the structure of Fyn in complex with Staurosporine (PDB ID: 2DQ7) and its 2D plot. (E–F) shows the structure of Hck in complex with 1-ter-butyl-3-p-tolyl-1h-pyrazolo[3,4-d]pyrimidin-4-ylamine (PDB ID: 1QCF) and its 2D plot. (Refer Fig. 1 for color code)

Figure 4. A comprehensive view of the protein kinases involved in spermatogenesis and chemical modulators disrupting their functions.

complex, PPI is bound within the catalytic cleft and forms hydrogen bonds with T338, E339 and M331 of hinge region (Fig. 3E & F). PPI renders the Hck in its auto-inhibited form and acts as a selective inhibitor of Hck. Anastassiadis et al., have studied the efficiency of various kinase inhibitors to inhibit the action of Hck and other kinases and they found that Dasatinib could potentially inhibit the Hck with 9.46 pKd. Similarly they also found that Bosutinib, Foretinib and Lestaurtinib can also inhibit the action of Hck.³³ All these are known kinase inhibitors used in treatment of cancer and this interaction profile could give an insight into the underlying mechanism

with an inhibitory constant ranging from 5 to 71 μ M. Among them, the top compounds [VS6, VS4 and VS3] (named as in the original paper) possessed inhibitory constants of 4.8, 11.5 and 15.0 μ M IC₅₀ values respectively. These inhibitors form hydrogen bonds with residues E343 and M345 of hinge region [E343-G348]. Due to their specificity, these molecules can be considered for further evaluation of their potential to inhibit the action of Fyn in testis.⁶⁷

Haematopoietic cell kinase (Hck)

Haematopoietic cell kinase (Hck) is expressed in spermatocytes, spermatids and also in the principal piece of the flagellum. Bordeleau et al., in their studies demonstrated, for the first time, the presence of both full-length and truncated forms of Hck kinase in bull testis.⁶⁴ The truncated form is expressed in the round and elongating spermatids and regulates the acrosome formation, suggesting that this form of truncated Hck may be involved in regulating the sperm head shape but the morphological changes produced by inhibiting this kinase have not been characterized yet. This truncated form of Hck has SH2, SH3 domains, and the first lobe is involved in binding of ATP, but lacks a C-lobe involved in phosphorylation and substrate binding and a C-terminal domain. It also has an N terminus unique 8 amino acid motif and a C-terminal 40 amino acid long tail regions. Nevertheless, the expression of full length Hck is also observed in the bovine testis suggesting that both the forms of Hck are involved in spermatogenesis.⁶⁸ The crystal structure of HCK in complex with PP1 (4-amino-5-(4-methylphenyl)-7-(*t*-butyl)pyrazolo [3,4-*d*]pyrimidine) is available and PPI acts as an ATP competitor which binds within the catalytic cleft.⁶⁹ In this

affecting sperm development in case of Hck inhibition.

Future Perspectives

With the advent of increased abnormalities in reproductive function in men across the world, the need for understanding their root cause at the structural level would help in reversing these effects. Since protein kinases play a major role in almost all signaling cascades that govern the normal functioning of testis, they form the molecular targets for most of the anti-cancer drugs that cause testicular toxicity, as well as male contraceptives. Exposure to environmental toxicants can also cause adverse effects on the male reproductive system and many of these toxicants target kinases (Fig. 4). The structural analysis of these inhibitory mechanisms will help us to circumvent the effects of toxicants as well as aid in developing better drugs and contraceptives with minimal nonspecific toxicities.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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