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Delivering non-hormonal contraceptives to men: advances and obstacles

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Abstract

There have been major advances in male contraceptive research during the past two decades. However, for a contraceptive to be used by men, its safety requires more stringent scrutiny than therapeutic compounds for treatment of illnesses because the contraceptives will be used by healthy individuals for an extended period of time, perhaps decades. A wide margin is therefore required between the effective dose range and doses that cause toxicity. It might be preferable that a male contraceptive, in particular a non-hormone-based compound, is delivered specifically and/or directly to the testis and has a rapid metabolic clearance rate, reducing the length of exposure in the liver and kidney. In this article, we highlight the latest developments regarding contraceptive delivery to men and with the aim of providing useful information for investigators in future studies.

Introduction

Hormonal contraception for men has made significant advances in recent years with several products currently under development, and it is anticipated that hormonal-based male ‘pills’ will become available on the market within the next decade. Male contraception also includes testosterone undecanoate, testosterone enanthate-progestin combinations (both delivered via injection) and testosterone gels and transdermal patches (for reviews see [1,2]). For the past three decades much work has also been done in the development of non-hormonal male contraceptives. The most notable examples of these are gossypol derived from cotton seeds (for reviews see [3,4]) and extracts from *Tripterygium wilfordii*, a Chinese herb shown to be effective in inducing male infertility [5] when administered orally. Although both compounds caused germ cell exfoliation from the seminiferous epithelium of the testis, clinical studies have shown these plant-derived extracts to display low efficacy and high toxicity. In addition, gossypol-induced infertility was non-reversible in a large percentage of human subjects, even though earlier studies in rodents showed an acceptable margin between safety and efficacy (for review see [6]). Thus, the rationale of developing non-hormonal contraceptive approaches for men is based on possible advantages over hormonal methods. For instance non-hormonal approaches are not likely to interfere with androgen-dependent functions and/or organs (e.g. prostate) if they selectively target cellular

events in the testis and/or epididymis, such as germ cell adhesion and maturation, because the hypothalamic–pituitary–testicular axis is not affected. As such, non-hormonal side-effects, if any, will probably be minimal. Furthermore, non-hormonal male contraceptives might elicit a more rapid onset of infertility versus hormonal approaches, which take several weeks to become effective. However, although there are several compounds currently under investigation, as well as several possible targets for fertility intervention, no reversible nonhormonal contraceptive method for men (other than surgical vasectomy) is available or in clinical development [6].

At present, different non-hormonal approaches are being investigated. These include interfering with sperm maturation in the testis and the epididymis, interfering with sperm morphology, motility and metabolism, as well as with sperm–egg fusion, and also with Sertoli-germ cell interactions (for reviews see [6,7]). One notable example is the use of the alkylated imino sugar, miglustat, which disrupts epididymal sperm motility and morphology (miglustat is prescribed for the treatment of type I Gaucher disease in which patients show abnormal deposition of glucocerebro-side in the liver, spleen, lung and bone marrow because of a genetic deficiency in glucocerebrosidase). Although miglustat was reported to induce reversible infertility in mice [8], a small-scale study involving five subjects showed that this drug did not disrupt spermatogenesis in humans [9]. Nonetheless, the anti-fertility effects of miglustat in mice should be further examined, particularly because this line of research will probably shed new knowledge on the role of imino sugars in spermatogenesis, particularly in sperm motility and morphogenesis. These findings might also lead to the development of new derivatives of miglustat that have antispermatogenic effects in humans. In addition to this approach, sperm-specific antigens are being explored for the development of vaccines to block sperm function and/or sperm–egg interactions as the means to affect fertility (for reviews see [10,11]). For example, if a high titer anti-sperm antibody could be developed in either males or females following administration of a sperm-specific antigen as a vaccine, it could affect sperm motility, metabolism and/or sperm–egg interactions. Finally, the most recent approach to non-hormonal contraceptive development in males involves targeting Sertoli–germ cell interactions, such as germ cell adhesion, in the seminiferous epithelium (for a review see [12]). This research is based on findings illustrating that the Sertoli cell functions as a nurse cell, providing nutritional and structural support to developing germ cells [13,14] – premature detachment of germ cells from the Sertoli cell leads to germ cell death and infertility [13,15,16].

The testis is a relatively good target for non-hormonal contraceptive development because of the many cellular and biochemical facets of spermatogenesis and spermiogenesis that take place in the seminiferous epithelium resulting in the daily production of 100–200 million spermatozoa in normal men. Thus, there are numerous potential targets for fertility intervention if the molecular and biochemical events of spermatogenesis are completely elucidated. Several studies have identified a new lead compound, Adjudin™ (see Glossary), which can specifically compromise adhesion between Sertoli cells and elongating or elongated spermatids in the seminiferous epithelium of the testis without affecting cell adhesion in other organs (for review see [7]). This causes most germ cells to deplete prematurely from the epithelium, leading to infertility. Because adhesion between Sertoli cells and spermatogonia (primitive germ cells that subsequently give rise to spermatocytes

and spermatids during spermatogenesis, Figure 1) is not affected by Adjudin™, these germ cells subsequently divide, differentiate and repopulate the seminiferous epithelium once this drug is metabolically cleared [12]. Here, we summarize some of the recent advances in this area of research.

Potential male contraceptives that target Sertoli–germ cell adhesion

Adjudin™, formerly called AF-2364, 1-(2,4-dichlorobenzyl)-1*H*-indazole-3-carbohydrazide (M_r 335.18), was subsequently renamed Adjudin™ to illustrate its ability to induce Sertoli–germ cell adherens junction disruption (for reviews see [12,13]) (Figure 2). It was initially identified from a batch of approximately two dozen newly synthesized analogs of lonidamine [18]. Although lonidamine was shown to effectively inhibit spermatogenesis, the effort to develop it into a male contraceptive was abandoned because of its hepato- and nephrotoxicity in monkeys, dogs and humans [17].

By using a sensitive *in vivo* assay, Adjudin™ was identified as a compound with promise as a male contraceptive. This bioassay is based on the premise that disruption of Sertoli–germ cell contacts will transiently induce production of testin [7] (Box 1). In short, adult rats [270–300 gm b.w. (body weight)] were treated by gavage with different analogs of lonidamine using doses of 25 and 50 mg/kg b.w. at time 0. Thereafter, rats ($n = 4$ rats per time point per dose for each compound) were sacrificed on days 1, 3, 5, 7 and 14, and the steady-state mRNA level of testin was assessed by northern blot analysis. The drug that caused a transient surge in the testin mRNA level by ~10–50-fold, followed by a rapid decline to its basal level by ~2 weeks, was subsequently shown to reversibly disrupt spermatogenesis when assessed by histological and mating studies [12,18]. Adjudin™ was selected from these new analogs following seven years of work by our group [18].

Subsequent studies showed that Adjudin™ was effective in depleting elongating and/or elongated and round spermatids, as well as spermatocytes (but not spermatogonia) [19] from the seminiferous epithelium [18,20]. Equally important, at doses of 35 and 50 mg/kg b.w. (administered by gavage), which effectively induced transient and reversible infertility in adult rats, Adjudin™ did not significantly alter the serum levels of follicle stimulating hormone (FSH), luteinizing hormone (LH) and testosterone when compared with control animals, illustrating that the hypothalamic–pituitary–testicular axis was not affected. Furthermore, liver and kidney function were not affected at these doses based on serum microchemistry analysis [18,20]. These findings illustrated that Adjudin™ was a potential male contraceptive that targeted germ cell adhesion in the testis. Also, results from acute toxicity and mutagenicity studies performed according to Food and Drug Administration (FDA) guidelines have shown Adjudin™ to be safe for further development and considerably less toxic than its analog, lonidamine (Table 1). However, in a subsequent subchronic toxicity study, a narrow margin between safety and efficacy was found in male rats when Adjudin™ was administered orally for 29 days at 50 mg/kg b.w. [21] (Table 1). In this instance, 29 days of consecutive exposure to Adjudin™ resulted in adverse effects – liver inflammation and skeletal muscle atrophy were detected in 3 out of 10 adult male rats [21]. In other words, the dose that effectively induced infertility in rats was too close to the dose that caused hepato- and myotoxicity. To circumvent this toxicity issue, it was

speculated that Adjudin™ could be delivered directly to the testis, by-passing systemic absorption. This approach would also lower the effective dose of Adjudin™ required to induce germ cell depletion from the testis considerably, in turn widening the margin between its safety and efficacy. Table 2 summarizes effects of Adjudin™ in adult rats based on studies published in the past decade.

In this context, it is interesting to note that CDB-4022 [(4aRS,5SR,9bRS)-2-ethyl-2,3,4,4a,5,9b-hexahydro-8-iodo-7-methyl-5-(4-carbomethoxyphenyl)-1H-indeno(1,2-c)-pyridine-hydrochloride] [11], an indenopyridine derivative, is another antispermatogenic compound with similar phenotypic effects in testes of mice, rats, dogs and monkeys, in that it also induced germ cell exfoliation from the semi-niferous epithelium [22–24]. These anti-spermatogenic effects were reversible in all species tested except rats, which required administration of a GnRH (gonadotropin-releasing hormone) agonist or antagonist before CDB-4022 for fertility to be regained in the majority of animals [25]. In short, these studies illustrate that targeted delivery of antispermatogenic compounds to the testis is a promising approach for developing male contraceptives if these drugs can selectively perturb Sertoli–germ cell adhesion, metabolism, maturation and/or apoptosis, without harming Sertoli cells and spermatogonia. Thus, their disruptive effects on spermatogenesis would be reversible.

Sertoli–germ cell anchoring junctions as targets for non-hormonal contraceptive development

Sertoli–germ cell anchoring junctions, particularly those between Sertoli cells and spermatids [step 8 (see Glossary) and beyond in the rat] (Figure 1) have unique features compared with those found in other epithelia. For instance, the anchoring junction that confers adhesion between Sertoli cells and elongating and/or elongated spermatids in the seminiferous epithelium is known as the apical ectoplasmic specialization (apical ES), a testis-specific actin-based adherens junction (AJ) (Figure 1). Other than the apical ES, no other junction type (e.g. desmosome-like junction) is known to mediate adhesion between Sertoli and elongating and/or elongated spermatids. The apical ES also has a structural counterpart, known as the basal ectoplasmic specialization (basal ES), which is present between adjacent Sertoli cells at the blood–testis barrier (BTB) and is thought to reinforce tight junction integrity (Figure 1). Cell adhesion constituted by the ES is regulated by the following protein complexes: integrins and laminins, nectins and afadins, JAM-C and ZO-1 (junctional adhesion molecule-C and zonula occludens-1), and cadherins and catenins, which work in concert with adaptors, phosphatases and kinases, and actin-binding proteins (for reviews see [13,26]). Interestingly, the integrin–laminin multi-protein complex is unique to the apical ES because in other epithelia, integrins and laminins are restricted to focal contacts at the cell–matrix interface [27]. Recent studies have reported some of these protein complexes (e.g. integrins and laminins), as well as their downstream signaling pathways (e.g. $\text{ROCK} \rightarrow \text{LIMK} \rightarrow \text{cofilin}$ and $\text{PI3K} \rightarrow \text{p130}^{\text{Cas}} \rightarrow \text{pFAK} \rightarrow \text{p-PKB} \rightarrow \text{ERK}$), to be transiently during activated Adjudin™-mediated germ cell loss from the epithelium (for review see [28]). These findings raise the possibility that some of these signaling molecules might become targets for male contraceptive development, and this was demonstrated in studies using specific inhibitors [28].

Interestingly, a recent study using a micropipette pressure transducing system (MPTS) to quantify the strength of adhesive force between Sertoli cells and either spermatocytes, pre-step 8 spermatids or step 8 spermatids, reported that almost twice as much force was required to detach step 8 spermatids from Sertoli cells compared with spermatocytes and pre-step 8 spermatids, which use classical adherens and desmosome-like junctions for adhesion [29]. These results illustrate that the apical ES, which is found between Sertoli cells and step 8 spermatids, is a more stable cell adhesive structure compared with the desmosome-like junction [29] (Figure 1). Nevertheless, Adjudin™ was shown to preferentially pull elongating spermatids away from Sertoli cells [30]. This conclusion was also supported by another study that investigated the kinetics of germ cell loss from the seminiferous epithelium in testes after treatment of adult rats with Adjudin™. Morphometric analysis revealed that elongating and/or elongated spermatids depleted from the epithelium significantly faster than other germ cell types, namely round spermatids and spermatocytes [19]. Taken together, these results indicate that the apical ES is the initial target of Adjudin™, even though its adhesive force is stronger than that conferred by the desmosome-like junction [29,30], suggesting that the apical ES would be a prime target for interfering in fertility.

However, many questions remain unanswered, such as: which protein complex(es) at the apical ES is the putative target of Adjudin™? How do different multi-protein complexes at the apical ES coordinate or communicate with each other during the loss of elongating and/or elongated spermatids from the seminiferous epithelium? Although crosstalk between different protein complexes at the apical ES has been proposed to maintain ES integrity, as well as to coordinate its restructuring during spermatogenesis [27], the molecule(s) and the mechanism(s) that regulate such crosstalk and/or coordination remain to be identified. In this context, it is of interest to note that Adjudin™ did not affect epithelial cell adhesion in other organs (e.g. kidney, liver and brain) at doses that effectively induced massive germ cell depletion from the seminiferous epithelium, causing transient infertility [12]. Clearly, these findings illustrate the uniqueness of the apical ES at the Sertoli–spermatid interface, but additional research is needed to address these important outstanding questions.

Targeted drug delivery to the testis

Figure 3 summarizes the mechanisms and/or approaches that have been the subject of considerable research, by which a non-hormonal contraceptive drug might be delivered to the testis. These include simple diffusion of a small lipophilic compound, that is if the drug can penetrate through the BTB, plus three other more specific mechanisms or approaches, briefly discussed below (Figure 3). These include drug delivery across the BTB by understanding its timely restructuring (e.g. disassembly and reassembly) during spermatogenesis, utilizing specific drug transporters on the Sertoli cell for drug entry, and using the FSH receptor, which is restricted to the Sertoli cell plasma membrane, as a specific drug ‘carrier’ to the testis.

The blood–testis barrier (BTB): an obstacle for drug delivery

Several blood–tissue barriers exist in mammals, including the blood–brain barrier, blood–retina barrier and blood–testis barrier (BTB). The BTB, which is constituted by adjacent

Sertoli cells (Figure 1), is one of the tightest blood–tissue barriers known to exist to date. This is crucial for spermatogenesis because post-meiotic germ cell development must occur in a unique microenvironment sequestered from the systemic circulation. In addition, the BTB is unique compared with other blood–tissue barriers because it must ‘open’ (or destabilize) transiently at stages VII–VIII of the seminiferous epithelial cycle of spermatogenesis to accommodate the transit of preleptotene and leptotene spermatocytes across the barrier. This enables these germ cells to translocate from the basal to the adluminal compartment for further development [31] (Figure 1). Although the tightness of the BTB poses an obstacle to the delivery of contraceptives into the seminiferous epithelium, a narrow physiological window exists at stages VII–VIII whereby a drug can still enter the testis, that is, if the biology and regulation of the BTB are known (Figure 3). For instance, recent studies have shown that cytokines (e.g. TGF (transforming growth factor)- β 2 and - β 3 [32,33] and TNF (tumor necrosis factor)- α [34,35]) and natriuretic peptides (e.g. C-type natriuretic peptide [CNP] [36]) reversibly perturb BTB function *in vivo*, as well as Sertoli cell tight junction and basal ES function *in vitro*. These findings are significant because cytokines and CNP secreted by Sertoli and/or germ cells locally into the BTB microenvironment during spermatogenesis activate different signal transduction pathways (e.g. TGF- β 3 activates p38 MAPK or ERK [32,33,37,38]), which can downregulate the steady-state levels of integral membrane proteins at the BTB and lead to a transient disruption of the BTB. Thus, cytokines and CNP might perturb the tight junction and/or basal ES at the BTB at stages VII–VIII of the seminiferous epithelial cycle. Indeed, immunohistochemistry studies have illustrated that the production of CNP, cytokines and/or their corresponding receptors are highest at these stages [33,34,36].

Furthermore, changes in the homeostasis of proteases and protease inhibitors at the cell–cell interface in the seminiferous epithelium can also lead to transient BTB disruption [13]. For instance, recent studies have shown that TNF α regulates the steady-state protein levels of matrix metalloprotease-9 (MMP-9) and collagen IV in the basal compartment of the seminiferous epithelium, which, in turn, regulates tight junction dynamics at the BTB [34]. Interestingly, other *in vitro* [39,40] and *in vivo* [41,42] studies have illustrated that testosterone promotes BTB integrity. In addition, a recent study has shown that integral membrane proteins at the BTB, such as occludin, are endocytosed via the clathrin-mediated pathway, and that both TGF- β 2 and testosterone accelerate its rate of internalization [43]. TGF- β 2-mediated internalization of occludin is targeted to the late endosome where it is degraded, whereas testosterone promoted the recycling of internalized occludin back to the cell surface, perhaps to a different site via recycling [43]. Collectively, these data illustrate that during migration of preleptotene and leptotene spermatocytes across the BTB, testosterone promotes the ‘assembly’ of ‘new’ tight junction fibrils below the migrating spermatocyte, whereas cytokines induce the ‘disassembly’ of ‘old’ tight junction fibrils above the spermatocyte in transit. Perhaps the assembly of new tight junction fibrils below the germ cell in transit is facilitated via transcytosis by relocating integral membrane proteins from the ‘old’ to the ‘new’ site. This therefore maintains BTB integrity during transit of preleptotene and leptotene spermatocytes across the barrier as illustrated by a lanthanum study (Figure 1e), in which two spermatocytes in transit were ‘trapped’ between tight junction fibrils above and below the migrating cells. Although additional research is

needed to delineate the precise mechanism(s) of BTB regulation during spermatogenesis, in particular the roles of CNP, cytokines and testosterone in BTB dynamics, these findings provide a unique opportunity for exploring an entirely different approach for delivering contraceptive compounds to the seminiferous epithelium beyond the BTB by taking advantage of the ‘natural opening (or restructuring)’ of the BTB.

Drug transporters in the testis

Sertoli cells are known to express different transporters and efflux pumps that either facilitate the uptake of or limit the exposure of developing germ cells to xenobiotics and/or drugs, respectively (Figure 3). For instance, multi-drug resistance proteins (MDR; e.g. MDR1a, 1b and MDR2), efflux pumps that were originally identified in tumor cells that had gained resistance to certain anti-cancer drugs, have been shown to regulate the passage of drugs across blood–tissue barriers [44,45]. P-glyco-protein is the product of the *MDR1* gene in humans, and of the *MDR1a* and *1b* genes in mice and rats, and it is expressed in Sertoli, Leydig and peritubular myoid cells, and late spermatids [46,47]. Interestingly, it was reported that certain drugs such as digoxin, cyclosporin A and vinblastine accumulated in the testes and brains of MDR-1a^{-/-} mice beyond the corresponding blood–tissue barrier [48–50]. The significance of P-glycoprotein in drug trafficking across the BTB has also been illustrated in studies using P-glycoprotein inhibitors. For example, testicular drug entry was enhanced in mice treated with LY-335979, a P-glycoprotein inhibitor [51]. In addition to P-glycoprotein, another efflux pump, breast cancer resistance protein (BCRP), is also found in peritubular myoid cells in the testis [47]. This is interesting given that myoid cells, which reside in the tunica propria, outside of the seminiferous epithelium (Figure 1b,e), also contribute to BTB integrity, at least in rodents (for review see [52]). Collectively, these data illustrate that the testis, in particular the Sertoli cell that constitutes the BTB, is equipped with transporters and efflux pumps to regulate drug entry into and out of the seminiferous epithelium. Additional research is needed to understand the regulation of drug transporters and efflux pumps, which might be candidates for targeted delivery of male contraceptives to the seminiferous epithelium beyond the BTB, in particular if testis-specific transporters and/or efflux pumps are identified in the testis.

A novel approach for delivering contraceptives to the testis via the FSH receptor

As discussed above, the narrow margin between effective and toxic doses of Adjudin™ has prompted investigation of the possibility of specifically delivering this drug to the testis to lower its effective dose, thereby reducing its systemic toxicity. Because FSH receptors are restricted to Sertoli cells in the testis of mammals [53], FSH is an excellent candidate for the targeted delivery of Adjudin™. It has previously been shown that FSH variants with mutations and/or deletions of glycosylation sites in α and/or β subunits would lose their hormonal activity without significantly affecting their receptor-binding ability [54–60]. We therefore used a mutant FSH in which a free aldehyde group was generated on the N-terminus of the β subunit following mild oxidation of the FSH mutant when all other amino acids in both subunits were unaffected. Thereafter, the free aldehyde group was allowed to form a stable hydrazone bond with an Adjudin™ molecule [21]. The efficacy of Adjudin™ in inducing reversible infertility in rats was several orders of magnitude higher following its conjugation to the FSH mutant [21]. Although this approach of drug delivery is technically

feasible, preparation of recombinant FSH protein, as well as its conjugation to Adjudin™, are costly. If this method of family planning is to be used by men, particularly those residing in developing countries where the largest population growth is expected to occur, production costs will have to be curbed significantly. Moreover, the Adjudin™–FSH conjugate would probably evolve as an injectable because the FSH polypeptide would be proteolytically cleaved in the gastrointestinal tract if administered orally. Thus, alternative administration routes should be explored. It should also be noted that this delivery approach would be applicable to other contraceptives besides Adjudin™, that is, if they exert their effects locally in the testis to perturb Sertoli–germ cell adhesion, germ cell metabolism and/or apoptosis.

Alternative approaches to non-hormonal male contraceptive administration

Perhaps the issue of cost can be partially resolved by preparing recombinant FSH mutants in *E. coli*. However, the Adjudin™–FSH conjugate would still require parenteral rather than oral administration because it would be subjected to proteolytic degradation in the gastrointestinal tract [21]. Because parenteral administration has poor patient acceptance [61], other delivery systems should be explored.

Nasal administration

Nasal administration of drugs has been shown to be ideal for the delivery of small hydrophilic and low-dose-acting drugs (for review see [62]). Nasal delivery offers greater drug efficiency often leading to less frequent dosing, increased speed of action and improved patient compliance. Although this approach is advantageous in many respects, it faces several obstacles. First, the nasal mucosa is a barrier to the diffusion of macromolecules [63]. Second, nasal secretions contain proteases that would proteolytically degrade a protein- or peptide-based drug [64]. Third, the relatively small surface area of the nasal mucosa in adult humans and rats, ~160–180 and 10–14 cm², respectively, limits the amount of drug that can be absorbed from a single administration [65]. Fourth, most proteins in the nasal mucosa have a low residence time (~15–30 min), which is the result of rapid mucociliary clearance [66]. Nevertheless, these obstacles might be overcome if permeation enhancers, which act primarily by decreasing tight junction barrier resistance, protease inhibitors and mucoadhesive agents are included in the formulation [62,64]. For instance, chitosan [poly(D-glucosamine)], a cationic polysaccharide derived from the crab shell, is one of the most effective enhancers used to increase the residence time of a drug in the nasal cavity – essentially it improves absorption [67–69]. As such, its potential use for delivering the Adjudin™-conjugate nasally should be considered.

Transdermal delivery

Transdermal delivery of Adjudin™ or the Adjudin™–FSH mutant conjugate should also be explored based on recent advances in this field of drug delivery [70–72]. Local delivery of Adjudin™ or its FSH-conjugate through one of the thinnest layers of skin known to exist in the male (i.e. the skin covering the scrotum) would take advantage of the unique microvasculature of this organ [73,74], which allows rapid heat exchange between the extensive networks of the veins and arteries to maintain the lower temperature of the testis.

This is crucial for spermatogenesis and fertility. Although the passage of Adjudin™ (which has a low molecular weight) through tight junctions present in the skin would probably not be impeded, entry of the Adjudin™–FSH conjugate (in which the molecular weight is increased by 40 kDa) would require use of a protein transduction domain (PTD). Inclusion of a PTD in the Adjudin™-conjugate formulation might facilitate the entry of this complex through the skin, but would most likely require that FSH exist initially as an unfolded protein. Adjudin™ could also be dissolved in a co-solvent system of ethanol and water (70:30, vol/vol) containing dodecylamine (a skin permeation enhancer, 1% vol/vol or wt/vol) and hydroxypropyl methylcellulose (HPMC, a gelling agent, 1% wt/wt) [71] to obtain a gel formulation that can then be applied directly to the scrotum and absorbed through the skin to gain entry to the testis. Thus, its effects would be exerted locally without initial first-pass at the kidney and/or liver, and this would probably lower the efficacy dose and toxicity of the compound.

Other delivery options

Alternatively, needle-free injection has recently gained significant interest [75]. Needle-free injection can be used to deliver protein- or peptide-based drugs with volumes of up to 1 ml within a few hundredths of a second. For example, a piezoelectric actuator can be used to deliver a stream of drug-containing fluid at a velocity of 50–160 m/s [75]. More importantly, needle-free injection offers increased penetration of drugs compared with traditional syringes. This results in more efficient use of the drug and the use of a smaller amount of drug to reach the same desired effect. Alternatively, an implant or pellet containing the conjugate could be tested if its absorption (i.e. bioavailability) can be enhanced – this is perhaps the most desirable administration route.

Concluding remarks

Non-hormonal contraceptives currently under development, such as Adjudin™ (Table 2), and potentially CDB-4022, face an important challenge regarding the margin between safety and efficacy. It is obvious that a wide margin between the safety and efficacy of a contraceptive drug significantly increases its chances of becoming a marketable product. However, the presence of an exceedingly ‘tight’ BTB is one of the major obstacles in widening the margin between these two parameters. Thus, research should focus on understanding the mechanism(s) that regulates the transient restructuring of the BTB, for example at stages VII–VIII when the BTB must ‘open’ (or destabilize) [31] to facilitate the transit of preleptotene and leptotene spermatocytes across the barrier (Figure 3). Once the mechanism(s) is elucidated, this information could then be applied to delivering a contraceptive to the testis beyond the BTB, thereby lowering its effective dose significantly. This, in turn, would widen the margin between safety and efficacy. As depicted in Figure 3, a drug might also enter the seminiferous epithelium to exert its action by simple diffusion via the basolateral Sertoli cell plasma membrane. Equally important, the entry of a drug into Sertoli cells might also be regulated by drug transporters. Alternatively, a drug can be conjugated to a ‘carrier’, such as an FSH or a transferrin mutant protein (Figure 3). It is anticipated that a concerted effort by investigators in the field along these lines of research

will contribute to overcoming the obstacles of delivering nonhormonal contraceptives to the testis in the years to come.

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Glossary

Adjudin™	formerly known as AF-2364, 1-(2,4-dichlorobenzyl)-1H-indazole-3-carbohydrazide, is a derivative of indazole-carboxylic acid and has potent antispermatogenic effects. It was shown to induce <u>adherens junction disruption</u> at the Sertoli–germ cell interface, most notably with elongating and elongated spermatids.
Anchoring junction	a cell–cell or cell–matrix junction that confers adhesion between cells leading to the formation of an epithelium or endothelium. The cell–cell or cell–matrix anchoring junction that uses actin for attachment is known as the adherens junction or focal contact, respectively. The cell–cell or cell–matrix anchoring junction that uses intermediate filaments for attachment is called the desmosome or hemidesmosome, respectively.
Blood–testis barrier (BTB)	this blood–tissue barrier found in the testis is created almost exclusively by adjacent Sertoli cells in the seminiferous epithelium instead of endothelial cells of the microvessels in other blood–tissue barriers (e.g. the blood–brain barrier, the blood–retina barrier). The BTB is considered to be one of the tightest blood–tissue barriers known to exist in mammals. In contrast to other blood–tissue barriers, which are mostly made up of tight junctions, the BTB is constituted by co-existing tight junctions, basal ectoplasmic specialization (ES), gap junctions and desmosome-like junctions.
C-type natriuretic peptide (CNP)	one of three natriuretic peptides, the others being atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP). They are related to peptide hormones and are known to regulate the vasodilatory properties of blood vessels. CNP was shown to be a product of Sertoli and germ cells. It exerts its effects on binding its receptor, natriuretic peptide receptor-B [NPR-B, also known as guanylyl cyclase receptor B (GC-B)], which leads to increases in the intracellular cGMP level. ANP and BNP only bind to and activate natriuretic peptide receptor-A (NPR-A), but not NPR-B. ANP is known to regulate Leydig cell testosterone and cGMP production.

Drug transporter	an integral membrane protein that functions in the delivery of drugs (usually small molecules) into a cell, including Sertoli and germ cells in the testis. Drug transporters are therefore important targets for drug development.
Ectoplasmic specialization (ES)	a testis-specific adherens junction type that uses actin-filaments as the attachment site. It is found at the Sertoli–Sertoli interface, known as the basal ES, and restricted to the BTB. The basal ES coexists with tight junctions, which together with the desmosome-like junctions and gap junctions constitute the BTB. The ES is also found at the Sertolispermatid (step 8 spermatid and beyond in the rat) interface, known as the apical ES. Once the apical ES forms around the head of an elongating or elongated spermatid, no other type of anchoring junction (e.g. the desmo-some-like junction) can be found at this site.
ERK	extracellular signal-regulated kinase.
Follicle stimulating hormone (FSH)	a hormone secreted by the pituitary gland that regulates testicular function via its effects on Sertoli cell function because FSH receptors are restricted exclusively to Sertoli cells in mammals.
LIM	the three genes – <i>Lin-11</i> , <i>Isl-1</i> and <i>Mec-3</i> – needed for developmental decisions and that encode the LIM domain in proteins.
LIMK	LIM kinase, also called <i>Lin-11 Isl-1 Mec-3</i> kinase.
Lonidamine	one of the earlier indazole derivatives that was used as an anti-cancer drug. It has potent anti-spermatogenic effects causing germ cell loss from the seminiferous epithelium. However, unlike most anti-cancer drugs, lonidamine affects the energy metabolism of mitochondria by inhibiting hexokinase, particularly in cells with condensed mitochondria, such as spermatids and tumor cells following exposure to irradiation. Lonidamine also induces changes in plasma and mitochondrial membranes, leading to an array of cellular responses including inhibition of respiration, depletion of ATP, blockage of DNA repair and induction of acidification in cancer cells.
p130^{Cas}	protein encoded by <i>Crkas</i> gene. Also called Crk-associated protein. p38 MAPK: p38 mitogen-activated protein kinase. p-FAK: phosphorylated focal adhesion kinase.
PI3K	phosphatidylinositol 3-kinase or phosphoinositide 3-kinase.
p-PKB	phosphorylated protein kinase B, also known as Akt.

Protein transduction domain	a short stretch of amino acids usually having several positively charged lysine (Lys, K) and arginine (Arg, R) residues, which facilitates the entry of a large protein, such as β -galactosidase, into eukaryotic cells.
ROCK	<u>R</u> ho-associated protein <u>k</u> inase. Consists of two isoforms of ROCK-1 and ROCK-2.
Seminiferous epithelial cycle	indicates the unique association of Sertoli cells with germ cells at different stages of their development during spermatogenesis, which is divided into 14 stages: I through XIV in adult rats. If a specific segment of a tubule is viewed at stage VIII (during which spermiation takes place), the tubule slowly proceeds to the next stage and eventually back to stage VIII in ~14 days in adult rats, fully completing one round of the epithelial cycle.
Sertoli cells	also known as nurse or mother cells in the seminiferous epithelium, they provide both nourishment and structural support to developing germ cells. For instance, it is known that each Sertoli cell supports ~30–50 germ cells at different stages of development during the seminiferous epithelial cycle of spermatogenesis. In the rat, Sertoli cells cease to divide by post-natal day 18 and their number remains relatively constant throughout adulthood at ~40–50 million per testis.
Spermatogenesis	the development from diploid spermatogonia (2n) to haploid spermatozoa (1n) that takes place in the seminiferous epithelium under the influence of testosterone and FSH. It is composed of (i) mitosis (spermatogonial proliferation phase); (ii) meiosis (spermatocytes differentiate into spermatids); and (iii) spermiogenesis [spermatids differentiate from round to elongated spermatids (spermatozoa)]. In the rat, spermiogenesis can be divided into steps 1–19, namely step 1 to step 19 spermatids, wherein the nucleus of the spermatid becomes condensed, concomitant with the formation of the acrosome, and apical ES appears in step 8 spermatids.
Spermiation	the detachment of fully developed spermatids (spermatozoa) from the adluminal compartment into the tubule lumen occurring at late stage VIII of the seminiferous epithelial cycle of spermatogenesis.

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Box 1. Testin

Testin is a testis-specific protein [7] composed of two isoforms: testin I (35 kDa) and testin II (37 kDa) (GenBank™ Accession Numbers: U16858; NM_173132, NP_775155 for rat testin, and NM_178098, NP_835199 for mouse testin). It is a Sertoli cell secretory protein for which production is stimulated by testosterone in males and which is produced by granulosa cells in females. Following its secretion, testin binds onto the Sertoli cell surface. Although the exact function of testin has not yet been established, it seems to function as a signaling protein in some unknown aspect of cell adhesion at the Sertoli cell–elongating and/or elongated spermatid interface (apical ES), as well as at the blood–testis barrier (basal ES) [7]. Although the rat testin primary amino acid sequence contains a histidine- and an asparagine-active site characteristic of cysteine proteases, and is ~60% homologous with human cathepsin L (GenBank™ Accession Numbers: NP_666023; NM_145918), testin is not a cysteine protease. Also, Sertoli cell testin is different from a tumor suppressor gene product that shares the same name (GenBank™ Accession Numbers: NM_015641, NP_056456). Tumor suppressor testin shares no significant homology in its amino acid sequence (only ~1–3%) with Sertoli cell testin. Moreover, Sertoli cell testin does not contain LIM domain or zinc finger motifs that are characteristic of the tumor suppressor, illustrating that these two proteins are functionally diverse.

Figure 1.

Regulation of spermatogenesis in adult rats. **(a)** Schematic illustration of the functional relationship within the hypothalamic-pituitary-testicular axis, which regulates spermatogenesis. Gonadotropin-releasing hormone (GnRH) released from the hypothalamus regulates pituitary secretion of LH and FSH, which exert their effects on Leydig and Sertoli cells, respectively. Leydig cells, in turn, secrete testosterone for the maintenance of spermatogenesis. **(b)** A semi-thin section of a seminiferous tubule is shown, which consists of Sertoli and germ cells (e.g. Sg, spermatogonium; PS, pachytene spermatocyte; RS, round

spermatid; ES, elongating spermatid) in the seminiferous epithelium attached to the tunica propria. This cross section shows a seminiferous tubule at stage V of the seminiferous epithelial cycle of spermatogenesis with elongating spermatids (ES) anchored onto the Sertoli cell (SC) via the apical ES. The encircled area at the head of the elongating spermatid (a step 17 spermatid) is shown as a magnification in panel (c). Adjacent Sertoli cells (SC) residing on top of the basement membrane in the tunica propria create the blood–testis barrier (BTB), which physically divides the seminiferous epithelium into basal and adluminal compartments. A magnification of the BTB is shown in panel (d). (c) Magnification of a Sertoli cell and an elongating spermatid. The apical ES is restricted to the head of an elongating spermatid and the Sertoli cell. It is typified by the presence of actin filament bundles (green arrowheads) sandwiched between the endoplasmic reticulum (ER) and the plasma membrane of the Sertoli cell. The opposing white arrowheads indicate the plasma membranes of an adjacent Sertoli cell and an elongating spermatid. This apical ES ultrastructure is restricted only to the Sertoli cell side at the Sertoli cell–elongating spermatid interface. Abbreviation: Ac, acrosome. (d) Shown is a micrograph demonstrating the detailed composition of the BTB. Co-existing basal ES and tight junctions (white arrowheads) are clearly visible. Similar to the apical ES, the basal ES is typified by the presence of actin filament bundles (green arrowheads) sandwiched between the ER and the plasma membrane of the SC as can be seen in opposing blue arrowheads, which illustrate two plasma membranes of adjacent Sertoli cells. However, unlike the apical ES, the basal ES ultrastructure is present on both sides of adjacent Sertoli cells. The BTB segregates post-meiotic germ cell development from the systemic circulation, and only spermatogonia are found outside of the BTB. (e) Migration of preleptotene and leptotene spermatocytes through the BTB at stages VII–VIII of the seminiferous epithelial cycle. This micrograph illustrates two preleptotene spermatocytes joined by intercellular bridges (i.e. clones) (see blue arrowheads) traversing the BTB as examined using lanthanum. Germ cells in transit are ‘trapped’ between tight junction fibrils (see black arrowheads), and visualized by precipitation of lanthanum (black arrowheads). When ‘new’ TJ fibrils are being formed at the base of the preleptotene and leptotene spermatocytes in transit (see red arrowheads), tight junction fibrils above the migrating cells (white arrowheads) are to be disassembled to facilitate movement. This mechanism can be exploited as a potential drug entry route into the epithelium as illustrated in Figure 3b. Abbreviations: Nu, nucleus of a Sertoli cell; *, basement membrane in the tunica propria. Scale bars in (b), (c), (d) and (e) represent 10, 0.1, 0.08 and 1 mm, respectively.

Figure 2. Structural formula of Adjudin™, formerly called AF-2364, 1-(2,4-dichlorobenzyl)-1*H*-indazole-3-carbohydrazide.

Figure 3.

A schematic illustration of possible routes of entry of a contraceptive, such as Adjudin™, to the testis. The male contraceptive Adjudin™ is either administered orally and absorbed in the gastrointestinal tract, or administered parenterally via an Adjudin™-FSH conjugate. In both instances, the drug enters the systemic circulation through blood vessels (bv). Adjudin™ diffuses from the bv and enters the seminiferous epithelium. Four mechanisms could be exploited to mediate entry of Adjudin™ into the testis: **(a)** The drug enters the seminiferous epithelium by simple diffusion through the intercellular space between Sertoli cells (SC) at the blood–testis barrier (BTB), which is composed of tight junctions (TJ), basal ES and desmosome-gap junctions. **(b)** The drug enters the seminiferous epithelium during the opening (or disassembly) of the BTB that occurs at stages VII–VIII of the epithelial cycle of spermatogenesis, which is thought to facilitate the transit of preleptotene and leptotene spermatocytes across the barrier. **(c)** The drug enters the seminiferous epithelium via drug transporters present on the Sertoli cell plasma membrane. This would facilitate the entry of drugs to the testis beyond the BTB. **(d)** The drug is targeted to the testis by using an

FSH mutant protein conjugate. Because FSH receptors are restricted to Sertoli cells, the drug is specifically delivered to these cells. The upper panel illustrates the possible molecular mechanism. In step (i), the conjugated drug binds to the FSH receptor. In step (ii), it is internalized, and in step (iii), it is released intracellularly. In the final step (iv), the drug exerts its effects at the apical ES. The lower panel shows the cross section of a normal control rat testis morphology (a) and the germ cell loss from the tubules at four weeks after treatment with the Adjudin^{TM}-FSH conjugate (b). Abbreviations: GC, germ cell; SC, Sertoli cell; TJ, tight junction.

Table 1A summary of toxicity study results for Adjudin™^a

Test	Regimen and dosing	Administration route	Species	Results
Acute toxicity study	100 mg/kg b.w. (1 dose)	<i>In vivo</i> , i.p.	Mouse, males (n = 3)	Negative
Acute toxicity study	2000 mg/kg b.w. (1 dose)	<i>In vivo</i> , oral	Rat, males (n = 5) and females (n = 5)	Negative
Bacterial mutation assay	0.586–18.8 µg/plate	<i>In vitro</i>	<i>Salmonella typhimurium</i> and <i>Escherichia coli</i>	Negative in both species
Mammalian erythrocyte micronucleus test	500, 1000 and 2000 mg/kg b.w. (1 dose)	<i>In vivo</i> , oral	Mouse, males (n = 5) and females (n = 5)	Negative in all three dosings
Mammalian chromosome aberration test	6.25–100 µg/ml	<i>In vitro</i>	CHO cells	Negative in inducing numerical chromosome aberrations; positive in inducing structural chromosome aberrations
29 day subchronic toxicity study	50 mg/kg b.w. <i>q</i> 1-day for 29 day	<i>In vivo</i> , oral	Rat, males (n = 10) and females (n = 10)	Negative in females; positive in 3 of the 10 males

Abbreviations: b.w., body weight; CHO cells, Chinese hamster ovarian cells; i.p., intraperitoneal administration; *q*, frequency.

^aPerformed by licensed toxicologists according to FDA guidelines. Reproduced, with permission, from [21].

Table 2 Effects of Adjudin™ in male rats^a

Changes following Adjudin™ treatment	Refs
Disrupts the apical ES, followed by desmosome-like and gap junctions, leading to germ cell loss	[19]
Causes reversible infertility in adult male rats	[12,21]
Reduces the steady-state protein levels of integral membrane proteins at the apical ES	[13]
Activates different kinases and GTPases before or at the time of germ cell loss	[13,28]
Elicits no adverse effects on the sperm reserve in the epididymis	[12]
Fails to affect the adhesion of spermatogonia; thus, infertility is transient and reversible	[12]
Fails to alter serum FSH, LH and testosterone levels	[12]
Fails to affect the BTB during germ cell loss from the epithelium	[13]
Elicits no adverse effects on cell adhesion in other organs (e.g. liver, kidney, brain)	[12]

^a Many of the original research articles were not included because of space limitations. However, these original research articles can be found in the review articles cited here