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Research Advanced in Small Molecular Inhibitors of Human BRDT which for male contraceptives development

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ABSTRACT. *Testis specific Bromodomain proteins (BRDT) belong to the BET protein (Bromodomain and extra-terminal),which only expressed in testis . Recent studies have found that broad-spectrum BET protein inhibitor JQ1 can affect the binding between the BD1 domain of BRDT and acetylated histone H4, thus inhibiting sperm formation. Therefore, it has become a star molecule in male reproductive mechanism research and contraceptive drug development. At present, several research on BRDT inhibitors has started by using JQ1-based virtual screening and in vitro binding assay to screen out a variety of small molecular compounds that can effectively reduce the activity of BRDT. In this paper, the progress in this field in recent years is reviewed, and the screening direction of this kind of small molecule compounds in the later stage is analyzed and discussed.*

Keywords: *Testicular specific bromine domain protein; Male antifertility; Spermatogenesis; Virtual screening*

0. Introduction

Male antifertility drugs have been studied for more than ten years, and some hormone and non-hormone antifertility drugs have been found. These drugs generally achieve the purpose of contraception by inhibiting sperm production, reducing the number of sperm and blocking sperm swimming. However, there is no oral antifertility drug that can be used in clinic besides testosterone analogues and gamendazole, so it has far-reaching social significance to develop male antifertility drugs[1].However, with the development of biology, a series of new drug targets for male contraceptives have been discovered. Among them, BRDT is the most attractive star drug target[2,3].

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1. BRDT and its expression

Testicle-specific bromodomain-containing protein (BRDT) is a member of the bromodomain and extra-terminal (BET) protein family,which included BRD2, BRD3 and BRD4. BRDT is a nucleoproteins of 947 amino acids (180 kDa), and consists of two bromine domains: BD1 (26-121)and BD2 (276-364) which like other BET protein family members. But unlike other BET protein family members, its BD2 domain has no physiological function in existing research[4].

Another difference from other members of the BET protein family is that BRDT is specifically expressed in testis. Researchers found that BRDT expression was limited to normal testicular tissues through the detection of MR, and BRDT was not detected in 10 lung cancer cell lines without histological definition and 8 cancer cell lines with different organs, which indicated that BRDT expression was very strict tissue specificity.It has been reported that BRDT may have a trace expression in brain tissue and oocyte, but it has not been confirmed[5].

2. The function of BRDT in male fertility

The genome-wide association analysis of congenital male infertility confirmed that the single nucleotide polymorphism of BRDT was significantly associated with oligozoospermia and azoospermia in European men. And the absence of BD1, the first bromine domain of BRDT, results in wrong elongation of round sperm cells and serious defects in sperm morphology. The lack of BRDT in spermatocytes and sperm cells will lead to changes in transcription and alternative splicing processes. Therefore, BRDT is necessary for spermatogenesis. Further in-depth studies have found that BRDT is involved in sperm formation and maturation at many levels. For example, BRDT can regulate gene expression in spermatocyte, promote the formation of chromatin nucleolus in round spermatocyte after meiosis and participate in maintaining the structure and morphology of nucleolus. In addition, BRDT also plays an important role in sperm cell elongation and is involved in nuclear chromatin concentration at the late stage of spermatogenesis. The BRDT mutant phenotype test found that the BRDT mutation caused excessive acrosin, excessive cytoplasm, abnormal head or tail, and decreased motility. Therefore, full-length BRDT, especially the BD1 of BRDT, is closely related to maintaining

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male fertility[6,7].

Some studies have found that BRDT participates in the process of chromatin recombination in spermatogenesis of testis. After meiosis, BRDT recombines highly acetylated histones via paired acetyl-lysine recognition modules mediated by its first bromine domain , which binds to histone H4-4 acetylated amino acid terminal (H4Kac4) at a moderate titer (20 um). By studying the structure of BRDT protein in rodents, it was found that BD1 binds to diacetylated histone 4 peptide (H4K5ac8ac) through conserved aspartic acid residues and selectively deletes BRDT1 coding region, which is enough to sterilize suballele homozygous male mice.Although BD1 plays a key role, BD2 is also involved in sperm formation. Point mutation in BD2 is also associated with human infertility, but its mechanism is still unclear[6-8].

3. BET inhibitor JQ1 is a potential lead compound for male contraceptives

In view of the important role of BRDT in spermatogenesis, BRDT will soon become a star target in the development of male contraceptives. This is also due to several characteristics: 1) the protein is expressed almost exclusively in testis; 2) BD1 is a conserved bromine-containing domain, but it differs most from other BET members and is very promising to obtain specific ligands; 3) Most importantly, BDRT is not expressed in mitotic spermatogonia, which means that drugs targeting it will not affect spermatogonia. In theory, it can be inferred that the growth of stem cell population is reversible for spermatogenesis[2,3].

Matzuk et al. discovered that a small molecule compound (+) -JQ1 targeting human bromine domain proteins could strongly inhibit the BD1 activity of BRDT in molecular level experiments. After injecting 50 mg/kg JQ1 daily for three consecutive months, the testicular volume, seminiferous tubule area, sperm quantity and motility, testicular body of mice could be effectively reduced. The volume and area of seminiferous tubule did not affect the levels of luteinizing hormone, testosterone and mating behavior of mice. At the same time, after 4 months of discontinuation of administration, many indexes of mice could be restored to the level of control group, and had no teratogenic effect on the offspring of fertility[9]. So researchers even generally believe that small molecule inhibitors targeting BRDT will soon go into clinical practice.

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4. Research advance in small molecular inhibitor of BRDT

The three-dimensional crystal structure of human BRDT has been reported in 2013, which provides a good opportunity for the virtual screening of human BRDT. Based on the crystal structure(PDB:4FLP), the pharmacophore model was established with hBRDT and its inhibitor JQ1. The established pharmacophore model was used as a 3D search query to identify effective hBRDT inhibitors from an in-house chemical database. Molecular docking analysis was next performed to filter the above 270 hit compounds from compound database by pharmacophore based screening, and then screening for 125 positive compounds according to ranking order and visual examination. By using the *in vitro* protein-based experiment, 4 novel chemical compounds were identified as hBRDT inhibitors. The half maximum inhibitory concentration (IC_{50}) of the most active compounds T480 was 9.02 μM, and the IC_{50} of the other compounds like T225,T272,T323 is around 12.77 to 32.12 μM. Although the affinity of above compound is not higher than JQ1(IC50 is 0.13 μM), but the inhibitory rate(the highest is 44.39% in T225) of above compounds is significantly higher than JQ1(70.34%). So the detailed analysis of compound T480 and T225 is the further development of new BRDT inhibitors[10].

Another group also carried out similar work. They performed the high-throughput virtual screen in the ZINC database which has six million compounds.By using the similar hBRDT structure in a low-precision mode(PDB:4FLP), they refined the list to 24,000 hits according to the molecules scoring in the top 0.4%. Next they refined the number of hits to 200 by increasing precision and applying computational filters for removal of potential pan-assay interference compounds, and then choose 22 commercially available and synthetic accessibility compounds based on applying multiple parameters and visual inspection from above 200 compounds for further testing. By using the standard fluorescence anisotropy assay, 9 compounds was testified for has unambiguous binding to hBRDT, and the best one in 9 hits was Compound 3 which contained a pyrimidine moiety. Next the Compound 3 was selected for further optimization due to its high binding potential for BRDT (IC_{50} is 5.2 μM, Ki is 0.69 μM), which is significantly lower than the known BET inhibitor I-BET762(IC_{50} is 1.4 μM) under same conditions. The further testing experiment is carried out for Compound 3 by differential scanning fluorimetry assay, and find Compound 3 caused an obvious

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increase of the hBRDT melting temperature upon its binding, which is consistent with the above screening results. Next they also choose Compound 3 to further optimization and characterization to find highly selective for the BET family of bromodomains, but unexpected result is the new Compound 3s show more selective binding to BRD4 but not to BRDT. However, the structure activity relationship data and ligand deconstruction highlight the importance of the substitution of the uracil moiety for potency and selectivity to different bromodomains in BET protein family[11].

Other research groups have also tried to develop other small molecule inhibitors for BRDT based on different thought. It is known that the BET protein family had two bromodomains, and the BD1 and BD2 bromodomains are separated by a 280 residue linker region. So researcher designed a series of bivalent ligands which has two JQ1 or I-BET151 and linked by a variably spaced polyethylene glycol (PEG) linker to either the C6 or C2 positions. By using the standard AlphaScreen Assay, they find both the homodimeric molecule JQ1 or I-BET151 or heterodimeric molecule between JQ1 or I-BET151 could effectively inhibit the binding affinity of BRDT or BRD4, and the best molecule is the homo-combination of active (S)-enantiomers of JQ1 ((6S+2S)-PEG1). Although the BD2 of BRDT is not proved to be functional, but the above result indicated that it also has potential to be drug target[12].

5. Prospect in male contraceptives targeted to BRDT

As discussed in above, BRDT plays a key role in spermatogenesis, and it is only specifically expressed in testicular cells and does not exist in mitotic spermatogonia, so BRDT becomes a potential target for the development of new male antifertility drugs, and its specific inhibitors are ideal male contraceptive in theoretically.Although several articles from 2012 have demonstrated the great potential of BRDT inhibitors as male contraceptives[9], but no real progress has been made in this area in recent years.The most important reason is the conservative sequence between BRDT and other members of BET protein family , which leads to the lack of species-specific inhibitors to BRDT. Like the several articles just mentioned above, all of the small molecule inhibitor which effective to BRDT is either also effective to other BET protein, especially effective to BRD4, or no

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experiment results to confirm its species specificity. Because other BET proteins are widely expressed *in vivo* and participate in many important physiological processes such as immunity and tumorigenesis, so the above non-specific inhibitors will cause a wide range of clinical side effects, so there is no possibility for practical application.

Therefore, the goal of developing male contraceptives targeting BRDT is to screen specific BRDT inhibitors.According to the existing research results, the screening conditions next need to consider not only the affinity between the BD1 domain of BRDT and small molecule compounds, but also the affinity of these small molecule compounds to the BD1 domain of other BET members according to the sequence differences between BRDT and other BET members.Another consideration is to select regions with low sequence homology as targets for drug screening, and the most important one should be the BD2 domain of BRDT because of its relatively lower sequence homology. Since there are several antibodies that can distinguish different BET members, so the antibody binding region of BRDT may also be the future direction of drug screening.

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