Protein mimicry and the design of bioactive cell penetrating peptides: The genesis of STOPSPERM bioportides

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Abstract

The mature spermatozoon, a highly differentiated cell equipped for the sole purpose of fertilisation, lacks the protein machinery required for conventional endocytotic mechanisms. Perhaps contrary to expectation, cell penetrating peptides (CPPs) rapidly translocate across the unique sperm plasma membrane to accrete within distinct intracellular compartments. Confocal microscopy, employing red-fluorescent CPPs and bioportides, is a convenient platform to study this membrane translocation process. In the virtual absence of genetic expression, rapid physiological responses of human sperm are dependent upon protein-protein interactions that may be regulated by post-translational modifications including phosphorylation. This chapter provides an outline of the design of bioactive CPPs, or bioportides, which include protein-mimetic sequences from the interaction domains of sperm proteins. Protocols are included which enable the biological assessment of the impact of bioportides upon the viability and motility of spermatozoa.

Keywords Bioportide, Spermatozoon, Motility, Viability, Confocal microscopy

1. Introduction

Imagine a multi-dimensional *space of all possible molecules* within which the highly variable constituents are organised into groupings based upon their biochemical characteristics [1]. This hypothetical space resembles many of those pseudo-coloured images generated by the Hubble space telescope, a mostly empty void occasionally punctuated by matter clumped together in irregular patterns. One important sector of hypothetical molecular space contains carbon-biased organic molecules including genomes, carbohydrates, lipids and molecules assembled from AAs, the peptides and proteins. Collectively, these diverse assemblies include the biogenic building blocks of life. Located close by there is a relatively insignificant nucleus of amino acids (AAs), a grouping which includes the 20 proteinogenic L-forms now favoured by life on Earth. This sub-division of the *space of all possible organic molecules* is populated by many other AAs of comparative minor relevance to biogenic processes. This sector is comparatively ancient; aromatic AAs, including tryptophan, were likely produced by abiotic inorganic synthesis at deep sea hydrothermal vents on this planet [2]. The buried lakes of Mars and Saturn's icy moon Enceledus are alternative sites for organic synthesis in an aqueous environment. Volatile glycine has been detected in the coma of comet 67P/Churyumov-Gerasimenko [3], providing compelling evidence for an alternative and extra-terrestrial origin of some organic precursors which contributed to the early development of life. Purely chemical processes, including thermal copolymerisation [4], could have assembled AAs into peptides or perhaps structurally-related branched peptoids, in a non-random pattern to yield larger organic molecules with catalytic activity [5]. Thus, short

amyloid peptides which promote the sequence-selective, regioselective and stereoselective condensation of amino acids [5] satisfy the requirement for the self-replication of information-containing molecules fundamental to the generation of prototypic cell-like structures.

Following the poorly understood, though likely multi-stage, conclusion of abiogenesis, peptides emerged as key mediators of cellular physiology. As championed by John Stewart [6], the linear nonapeptide bradykinin (BK; RPPGFSPFR) and related homologues regulate all human physiological processes and many pathologies. BK, in common with many peptide mediators, specifically binds receptor proteins of the G protein-coupled receptor (GPCR) family (B1 and B2), located in the plasma membrane, to produce acute changes in cellular biology. The molecular determinants of BK:GPCR interactions must have been solved at a very early stage of biogenesis. It is likewise obvious that this intrinsic *recognition and binding capacity* of most human proteins is essential to their multi-functionality. Numerous intracellular signalling proteins contain one or more conserved domains, including SH2 and SH3, which determine their spatio-temporal localization within larger signalling complexes. Intriguingly, the accumulation of discrete functional domains within human proteins probably occurred at different times during their evolutionary history [7]. As this process of domain accretion continues, powered by processes such as gene duplication and the divergence of pre-existing genes and *de novo* gene origination, proteins are becoming increasingly larger in size [7,8]. One intuitive consequence of this molecular evolution is the recognition that human proteins rarely function in isolation but instead act within larger complexes or *protein machines*.

The advent of cell penetrating peptides (CPPs) has enabled more efficient access to intracellular protein complexes assembled by discrete protein-protein interactions (PPIs) and the capacity to selectively modulate the activity of intracellular processes. The *space of all possible CPPs*, a still relatively minor component of *peptide and protein space,* became visible only recently but is currently rapidly expanding. Within this sector there is not always a clear distinction between “inert” CPP vectors and their biologically active siblings. Cationic CPPs are generally particularly water soluble and can be exogenously applied to cultured cells at concentrations exceeding 30 µM to negatively impact upon cellular morphology, viability and function.

Considering the intrinsic binding capacity of peptides *per se*, it is surely no surprise that, within a given CPP population, some examples will interact with intracellular protein targets or perhaps other macromolecules including lipids. Observed differences in the intracellular accretion of CPPs (**Fig. 2**), including studies with sperm [9], may well be the consequence of this process [9,10]. Fortunately, it is possible to exploit this phenomenon. Thus, the penetratin CPP [11] (RQIKIWFQNRRMKWKK) can be utilised as a CPP vector to direct delivery of peptide cargoes to the sperm flagellum [9].

If a CPP includes mimetic sequences intrinsic to proteins it is feasible that, following intracellular accumulation into an appropriate cellular compartment, a predictable influence upon biological activity is manifest. The term bioportide was introduced simply to distinguish this class of bioactive CPP from the more common vector sequences [12]. The studies described herein with bovine and human spermatozoa serve to illustrate this relatively simple principle. The highly differential male gamete lacks all conventional

energy-dependent endocytotic processes and yet CPPs and bioportides readily accumulate within distinct intracellular sites [9,13]. STIM$^{371-392}$ (KQLLVAKEGAEKIKKKRNTLFG) is a poylcationic sequence mimicking a highly helical segment of the Orai activating region of the regulatory protein stromal interaction molecule 1. This exemplar of a rhegnylogically-organised bioportide [12,14] was predicted from consideration of the role of STIM1 as a modulator of Ca^{2+}-transients. Pre-treatment of human sperm with STIM$^{371-392}$ significantly slowed the decay of $[Ca^{2+}]_i$ in cells stimulated with progesterone [14].

Finally, we will report upon more recent developments [13] which have utilised sychnologically-organised protein-mimetic bioportides to disrupt Protein Phosphatase 1 (PP1) complexes and so inhibit sperm motility. Some of these studies [13] utilised chimeric bioportides consisting of penetratin and sequences from AKAP4, a sperm-specific PP1 interactor. Molecular modelling of AKAP4 bound to PP1 facilitated the identification of an optimised PP1 binding sequence (SVITF) utilised in our current lead compound, MSS1, a protein-mimetic bioportide with increased potency of action.

The methodologies described below are sufficient to support additional studies of CPP transduction pathways and intracellular trafficking in a highly differentiated cell type that is seemingly endocytosis incompetent. Since the mature human spermatozoon is virtually incapable of genetic expression, bioportide technologies could be utilised to interrogate additional intracellular signalling mechanism that regulate unique features of sperm cell physiology including capacitation and fertilisation. Located within the *space of all possible CPPs*, *bioportide space* is now rapidly expanding [10,12-15] to include

STOPSPERM examples as prototypical male contraceptives. Navigation of this embryonic sector is accompanied by an appropriate *music of the spheres*, a mellifluous and descriptive refrain:

She plays her mouth into a smile
And offers that he stay a while
Two hearts that beat as one
And eyes that hardly ever saw the sun
Plan 9 Channel 7, The Damned (Scabies, Sensible, Vanian, Ward), 1979.

2. Materials

2.1. Isolation of spermatozoa

Bovine sperm

1. Bovine semen (BULLSEMEN.COM, Inimex Genetics Ltd). Straws containing approximately 150 µl semen.
2. Liquid nitrogen storage facility.
3. CO_2 incubator.
4. Laminar Flow Class II tissue culture hood.
5. Supplemented Earle's Balanced Salt Solution (sEBSS) phenol red-free, filter-sterilized: 1.8 mM $CaCl_2.2H_2O$, 5.37 mM KCl, 0.81mM $MgSO_4.7H_2O$, 26.2 mM $NaHCO_3$, 1.0 mM $NaH_2PO_4.2H_2O$, 116.4 mM NaCl, supplemented with 55.6 mM D-glucose, 2.73 mM Na pyruvate, 41.8 mM Na lactate supplemented with 0.3 % charcoal-delipidated/fatty acid free Fraction V Bovine Serum Albumin (BSA) (*see* **Note 1)**.
6. Sterile syringes (50 ml), sterile syringe filters (45 µm) and 50-100 ml sterile universal tubes.

7. Sterile 10 ml tubes.
8. 1.5 ml sterile Eppendorf tubes.
9. Microcentrifuge.

Highly motile human sperm

1. $C0_2$ incubator.
2. Laminar Flow Class II tissue culture hood.
3. Ham's F-10 medium (non-capacitating medium): To 750 ml of purified water add 7.4 g NaCl, 1.2 g $NaHCO_3$, 0.285 g KCl, 0.154 g Na_2HPO_4, 0.153 g $MgSO_4.7H_2O$, 0.083 g KH_2PO_4, 0.044 g $CaCl_2.2H_2O$ and 1.1 g D-glucose. Adjust the pH to 7.4 with NaOH. Make up to 1000 ml with purified water.
4. Isotonic density-gradient medium (80% and 40%).
5. Sterile 10 ml tubes.
6. 1.5 ml sterile Eppendorf tubes.
7. Microcentrifuge.

2.2. Confocal analysis of CPP import into sperm compartments

1. Laminar Flow Class II tissue culture hood.
2. 5% (w/v) poly-D-lysine hydrobromide (mW 30,000-70,000) in filter-sterilized dH_2O.
3. CO_2 incubator.
4. TAMRA-labelled purified and lyophilized peptides
5. Distilled H_2O (dH_2O).
6. Sterile syringes, syringe filters and Eppendorf tubes.

7. 35 x 10 mm sterile petri dishes with 15 mm glass bases (GPE Scientific Limited, UK).
8. Supplemented EBSS phenol red-free, pre-warmed to 37^{o}C.
9. MitoTracker® Deep Red FM (Ex/Em 644/665 nm) or MitoTracker® Green FM (Ex/Em 490/516 nm) (Molecular Probes, Thermo Fisher Scientific, UK): using phenol red-free sEBSS, prepare to a final assay concentration of 500 nM for MitoTracker® Deep Red FM and 200 nM for MitoTracker® Green FM. 10 ml of the above probes are sufficient for 10 x 1 ml samples.
10. LysoTracker® Green DND-26 (Ex/Em 504/511 nm), (Molecular Probes, Thermo Fisher Scientific, UK): using phenol red-free sEBSS, prepare to a final assay concentration of 2 µM.
11. 1% (w/v) sterile trypsin.
12. Carl Zeiss LSM 880 Confocal Microscope equipped with a live cell imaging chamber.

2.3. Measurement of sperm viability

1. Plate reader.
2. CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (Promega) (includes MTS and PMS solutions).
3. 96-well plates.

2.4. Determination of sperm motility

1. Computer-assisted sperm analysis (CASA) system.
2. Disposable counting chambers (20 µm deep).

3. Methods

3.1 Peptide design

As recently reviewed [15], a variety of different strategies can be adopted to support the identification and molecular optimisation of bioportides. Cationic helices are a proven source of protein-mimetic bioportide sequences, most likely because such domains are commonly involved in PPIs at which sites the relative abundance of arginine is enriched. Moreover, the adoption of a helical secondary structure may be a critical determinant to enable bioportides to translocate biological membranes and bind intracellular proteins. We have re-introduced the term rhegnylogic to describe the molecular organisation of bioportides such as $STIM^{371-392.}$ [14]. This intrinsically cell penetrant sequence contains the important KIKKK motif from STM1 intimately involved in the regulation of store-operated Ca^{2+} channels (SOCs). Pre-treatment of human sperm with $STIM^{371-392}$ delayed the closure of SOCs to prolong the Ca^{2+}-transient in sperm stimulated by progesterone [14].

FIGURE 1 HERE

Protein phosphatase 1 (PP1) is a major regulator of sperm motility acquisition during epididymal transport [13]. The activity of PP1γ2, a testis- and sperm-specific isoform localized within head regions and entire flagellum, is negatively regulated by forming holoenzymes with Regulatory Interactors of Protein Phosphatase One (RIPPOs) [16]. Thus, the molecular interfaces between PP1γ2 and RIPPOs are attractive targets for bioportide technologies (**Fig. 1**). Indeed, the binding of RIPPOs to PP1 is mediated by short linear

motifs of which RVxF is the most common. The design of bioportides to target the PPI between PP1γ2 and the sperm specific RIPPO AKAP4 were necessarily sychnologic in nature since native sequences flanking the degenerate binding motif of AKAP4$^{41-45}$ (KVICF) were unlikely CPPs. One example, AKAP4-BM, comprised AKAP4$^{34-53}$ in chimeric combination with penetratin (underlined) as the C-terminal:

AKAP4-BM: GQQDQDRKVICFVAVSTLNV<u>RQIKIWFQNRRMKWKK</u>

Subsequently, molecular modelling simulations of AKAP4 bound to PP1γ2 identified an optimised decapeptide binding sequence, *YRSVITFVAV,* lacking problematic Asp and Cys residues observed in the native AKAP4-BM [13]. Subsequently, this sequence was utilised in the second-generation STOPSPERM bioportide MSS1:

MSS1: *YRSVITFVAV*<u>RQIKIWFQNRRMKWKK</u>

3.2. Sperm isolation

Bovine sperm

The method for the isolation of bovine sperm presented below is adapted from Nash and co-workers [17] and advocates the “swim-up” method in which highly motile sperm migrate to an upper phase of medium. This method also includes the addition of BSA which acts as a cholesterol acceptor to mediate capacitation *in vitro* [18]. For the purpose of assessing whether CPPs translocate an intact outer sperm membrane, assayed samples should clearly

not be allowed to undergo capacitation which involves alterations in sperm membrane fluidity and a cholesterol efflux. However, this method can provide temporal flexibility for assessing CPP translocation across the dynamic spermatozoa plasma membrane spanning its journey from ejaculation to inside the uterine tract.

1. Warm supplemented Earle's Balanced Salt Solution (sEBSS) to 37 ^{o}C.
2. Remove 1 straw of bovine semen from liquid nitrogen and warm at 37^{o}C for no longer than 30 minutes.
3. In a laminar flow class II tissue culture hood, very gently place 1 ml sEBSS in the bottom of a long sterile 10 ml tube.
4. Cut away the bottom of the straw and gently place under the sEBSS phase.
5. Carefully cut away the top end of the straw. The semen will be released into the bottom phase (*see* **Note 2**).
6. Incubate for no longer than 1 hour at 37^{o}C to allow sperm to "swim up" into the sEBSS phase (*see* **Note 3**).
7. After 1 hour, remove 200 µl of the upper phase of the sperm sample (containing approximately 3 × 10^{6} cells/ml) and place in a sterile 1.5 ml Eppendorf (*see* **Note 4**).
8. Centrifuge at 4000 rpm (1500 x g) for 5 minutes in a microcentrifuge.
9. Gently remove the supernatant.
10. Suspend the sperm pellet in 1ml of prepared treatments and incubate at 37^{o}C for the desired time period (*see* **Section 3.3, step 4**).

Highly motile human sperm

Considering the heterogeneous nature of the human ejaculate in terms of sperm integrity, a density gradient centrifugation method can be used to obtain only the highly motile/viable fraction.

1. Human semen should be collected by masturbation after 2-3 days of sexual abstinence.
2. Allow semen sample to liquefy for ~30 min (37°C, 5% CO_2).
3. Prepare the density-gradient medium in a test-tube by layering 1 ml of 40% (v/v) density-gradient medium over 1 ml of 80% (v/v) density-gradient medium (*see* **Notes 5 and 6**).
4. Mix the semen sample and place 1 ml of semen above the density-gradient media and centrifuge according to manufacturers' recommendations. Depending on the concentration, more than one tube per semen sample may be needed to obtain the number of sperm cells needed for the experiment.
5. Remove the supernatant from the sperm pellet and suspend the highly motile/viable fraction in 5 ml of Ham's F-10 medium and centrifuge at 500 g for 5 minutes (*see* **Note 7**).
6. Repeat the washing procedure.
7. Suspend the final pellet in Ham's F-10 medium to the final concentration desired (e.g. $1x10^6$ cells/200 μl), add the appropriate bioportides treatments (test various concentrations 1-30 μM) and incubate at 37°C, 5% CO_2 (test various time periods) (*see* **Note 8**).

3.3. Confocal studies

CPPs rapidly and efficiently enter bovine and human spermatozoa whilst being compatible with sperm physiology [9]. Moreover, a range of chemically diverse CPPs demonstrate a propensity to accumulate in differential intracellular sperm compartments (**Fig. 2**). Thus the following methodology encompasses qualitative uptake analyses of TAMRA-labelled CPPs using live-cell imaging confocal microscopy and some useful intracellular probes which can be employed for quantitative co-localization analyses (**Fig.2**). For instance, we have previously reported that the mastoparan analogue and bioportide mitoparan preferentially accumulates within the mitochondrial midpiece of bovine spermatozoa and that tat accretes within the sperm head including the acrosome [9]. The methodology below therefore includes treatment of spermatozoa with intracellular probes which label the midpiece (Mitotracker®) and the acrosome. High concentrations of LysoTracker® Green DND-26 (2 µM) can be used to label the sperm acrosome [19], which is far in excess of the concentration of LysoTracker® which is used to label lysosomes in somatic cells (50-75 nM).

1. In a laminar flow class II tissue culture hood, coat glass-bottomed confocal dishes with 5 % (w/v) poly-D-lysine hydrobromide (mW 30,000-70,000). Apply in small drops to the centre of the glass well. **(**see **Notes 9 and 10)**.
2. Leave for at least 1 hour, gently remove excess with a pipette and allow to air dry in a class II tissue culture hood with the lids removed. Before using, ensure that the poly-D-lysine coat is completely dry.
3. Dissolve purified and lyophilized TAMRA-labelled CPPs in dH_2O to a final concentration of 100 µM (*see* **Note 11**).

4. Depending on whether you wish to co-label the mitochondrial midpiece or the acrosome, or simply establish whether your TAMRA-labelled CPP is penetrant, prepare treatments. If using TAMRA-labelled CPPs alone, for each sample add 50 µl (100 µM CPP stock) in a total volume of 1 ml phenol red-free sEBSS to obtain an application concentration of 5 µM. If co-labelling with MitoTracker®, add 50 µl (100 µM CPP stock) in a total volume of 1 ml of 500 nM MitoTracker® Deep Red FM or in a total volume of 1 ml of 200 nM MitoTracker® Green FM. Similarly, if co-labelling with LysoTracker®, add 50 µl of 100 µM CPP stock in a total volume of 1 ml of 2 µM LysoTracker® Green DND-26.
5. Suspend the isolated sperm pellet (*see* **Section 3.2, step 10**) in 1 ml of pre-prepared treatments and incubate at 37ºC for the desired time period (see **Note 12**).
6. Centrifuge at 4000 rpm (1500 x g) for 5 minutes in a microcentrifuge. (*see* **Note 13**).
7. Remove the supernatant and suspend in 250 µl of sEBSS.
8. Pipette onto the poly-D-lysine-coated glass bottom of the confocal dish and view by confocal microscopy. Extra pre-warmed sEBSS without phenol red can be added to the confocal dish to prevent the sample from drying out. This procedure is best carried out once the majority of sperm have become immobilized.
9. Prior to confocal live cell imaging analyses, ensure that the live cell imaging chamber has stabilized at 37°C and contains a humidified atmosphere of 5 % CO_2.

FIGURE 2 HERE

3.4. Measurement of Sperm viability

1. After exogenous application of bioportides, transfer sperm cells to 96-well plates at 100 µl/well.
2. Measure sperm viability in each condition using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) reagent with the CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (Promega) according to manufacturer's guidelines [13,20] (*see* **Note 14**).

3.5 Determination of sperm motility

Computer-assisted sperm analysis (CASA) systems are capable of measuring sperm motility and kinematics and can evaluate sperm concentration and morphology. CASA presents two main advantages over manual methods: (1) high precision, (2) the generation of quantitative data for several kinematic parameters.

1. After treatment with bioportides, evaluate sperm motility by CASA. Several manufacturers produce CASA systems and each one must be correctly set up for human samples to ensure optimal performance.
2. Load sample (2 µl) into individual disposable counting chambers, 20 µm deep, which should be pre-heated and maintained at 37°C (*see* **Note 15**).

3. Several representative fields should be examined. Evaluate at least 1000 sperm cells per measurement.
4. Record the percentage of progressive motile (fast and slow), non-progressive motile and immotile spermatozoa in each condition (*see* **Note 16**).

FIGURE 3 HERE

4. Notes

1. Preparation of sEBSS: phenol red-free EBSS can either be purchased from Sigma or made up in the lab according to the materials highlighted in Section 2.1. Whichever option is selected, add supplements, 4.5 g D-glucose, 150 mg Na pyruvate, 2.34 g Na lactate and 1.5 g BSA to 500 ml phenol red-free EBSS. First weigh out the above supplements then dissolve in 50 ml of EBSS in a sterile universal. Filter sterilise back into the bottled and sterile EBSS.
2. It is very easy to disturb the bottom layer of semen and contaminate the upper layer of sEBSS with seminal fluid. Thus it is advised that the 10 ml tube is placed in a supportive rack.
3. Leaving spermatozoa in sEBSS for any longer may initiate capacitation events. To isolate “capacitated sperm” leave for 5-6 hours. If you are assessing whether your CPP translocates an intact outer sperm membrane, ensure that the duration of spermatozoa isolation and treatment times do not exceed a total of 3 hours.

4. 200 µl should contain a sufficient number of sperm for 1 confocal dish. Remove no more than a total of 600 µl and take care not to disturb the bottom layer.
5. To limit damage from the products of non-sperm cells (e.g. leukocytes), spermatozoa should be separated from the seminal plasma within 1 hour of ejaculation.
6. There are several density gradients reagents available that should be used according to the manufacturers' recommendations. Media containing silane-coated silica particles are commonly used, for example, Suprasperm™ (Origio, MediCult, Copenhagen, Denmark), SpermGrad™ (Vitrolife, San Diego, CA), SilSelect™ (Ferti Pro NV, Beernem, Belgium) and PureSperm™ (NidaCon Laboratories AB, Gothenburg, Sweden).
7. Other media such as Biggers, Whitten and Whittingham (BWW) or Earle's balanced salt solution (EBSS) can be used. For capacitation conditions, supplement the medium with bovine or human serum albumin and incubate at 37°C, 5% CO2 for 4–5 h before adding the bioportides.
8. If the incubator contains atmospheric air, the medium should be buffered with Hepes and the tube caps kept tightly closed. If the incubator atmosphere is 5% (v/v) CO_2, the medium should be buffered with sodium bicarbonate and the caps loosened to allow gaseous exchange. These protocols are sufficient to maintain a pH compatible with sperm survival.

9. Prior to confocal analyses, sperm need to be immobilized. It is advised that the preparation of the poly-D-lysine-coated glass-bottomed confocal dishes is completed before sperm isolation (**Section 3.2**) so as to ensure sufficient drying time for the poly-D-lysine coating.
10. Once finished with the 5% poly-D-lysine solution, ensure that any remainder is kept tightly sealed in a refrigerator.
11. Filter sterilize and store your stock in sterile Eppendorf tubes at -20^{o}C. Avoid repeated freeze-thaw cycles and overexposure to light.
12. 1 hour should be more than sufficient time for cellular penetration. Analyses of translocation kinetics has demonstrated that the CPPs C105Y and Tat translocate into bovine spermatozoa within 10 minutes [9].
13. To establish that detected fluorescence is not attributable to surface-associated CPP, a trypsinization step can be included here and spermatozoa can be incubated with 1% (w/v) trypsin for 5 minutes at 37^{o}C, then collected by centrifugation. This is particularly useful when assessing the intracellular uptake of a CPP for the first time into isolated spermatozoa.
14. The reduction of tetrazolium compounds provides a reliable and rigorous assessment of spermatozoa viability [13,20]. The novel tetrazolium compound 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS) and the electron coupling reagent phenazine methosulfate (PMS) are a convenient ‘in-solution’ method for the assessment of viable spermatozoa.

15. Sperm motility is highly sensitive to temperature.
16. Other movement parameters can be evaluated using the CASA system such as curvilinear velocity (VLC), straight-line (rectilinear) velocity (VSL) and average path velocity (VAP).

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References

1. Woolfson, A. (2000) *Life Without Genes*. Harper Collins, London, U.K.
2. Ménez, B., Pisapia, C., Andreani, M., Jamme, F., Vanbellingen, Q.P., Brunelle, A., Richard, L., Dumas, P., and Réfrégiers, M. (2018) Abiotic synthesis of amino acids in the recesses of the oceanic lithosphere. *Nature*, **564**, 59-65.
3. Altwegg, K., Balsiger, H., Bar-Nun, A., Berthelier, J-J., Bieler, A., Bochsler, P., Briois, C., Calmonte, U., Combi, M.R., Cottin, H., De Keyser, J., Dhooghe, F., Fiethe, B., Fuselier, S.A., Gasc, S., Gombosi, T.I., Hansen, K.C., Haessig, M., Jäckel, A., Kopp, E., Korth, A., Le Roy, L., Mall, U., Marty, B., Mousis, O., Owen, T., Rème, H., Rubin, M., Sémon, T.,Tzou, C-Y., Waite, J.H., and Wurz, P. (2016) Prebiotic chemicals - amino acid and phosphorus - in the coma of comet 67P/Churyumov-Gerasimenko. *Sci. Adv*. **2**, e1600285.

4. Fox, S.W., and Harada, K. (1960) The thermal copolymerization of amino acids common to protein. *J. Am. Chem. Soc*. **82**, 3745-3751.
5. Rout, S.K., Friedmann, M.P., Riek, R., and Greenwald, J. (2018) A prebiotic template-directed peptide synthesis based on amyloids. *Nat. Commun*. **9**, DOI: 10.1038/s41467-017-02742-3.
6. Stewart, J.M. (2004) Bradykinin antagonists: discovery and development. *Peptides*, **25**, 527-532.
7. Toll-Riera, M., and Albà, M.M. (2013) Emergence of novel domains in proteins. *BMC Evol. Biol*. **13**, DOI: 10.1186/1471-2148-13-47.
8. Andersson, D.I., Jerlström-Hultqvist, J., and Näsvall, J. (2015) Evolution of new functions de novo and from preexisting genes. *Cold Spring Harb. Perspect. Biol*. **7**, a017996.
9. Jones, S., Lukanowska, M., Suhorutsenko, J., Oxenham, S., Barratt, C., Publicover, S., Copolovici D.M., Langel, Ü., and Howl, J. (2013) Intracellular translocation and differential accumulation of cell penetrating peptides in bovine spermatozoa: Evaluation of efficient delivery vectors that do not compromise human sperm motility. *Hum. Reprod*. **28**, 1874-1889.
10. Jones, S., Uusna, J., Langel, Ü., and Howl, J. (2016) Intracellular Target-Specific Accretion of Cell Penetrating Peptides and Bioportides: Ultrastructural and Biological Correlates. *Bioconjugate Chem*. **27**, 121-129.
11. Derossi, D., Joliot, A.H., Chassaing, G., and Prochiantz, A. (1994) The third helix of the antennapedia homeodornain translocates through biological membranes. *J. Biol. Chem*. **269**, 10444-10450.

12. Howl, J., Matou-Nasri, S., West, D.C., Farquhar, M., Slaninová, J., Östenson, C-G., Zorko, M., Östlund, P., Kumar, S., Langel, Ü., McKeating, J., and Jones, S. (2012) Bioportide: An emergent concept of bioactive cell penetrating peptide. *Cell. Mol. Life Sci*. **69**, 2951-2966.
13. Silva, J.V., Freitas, M.J., Santiago, J., Jones, S., Guimarães, S., Vijayaraghavan, S., Publicover, S., Colombo, G., Howl, J., and Fardilha, M. (2020) Modulation of Phosphoprotein Phosphatase 1 complexes by the use of bioportides as a novel approach to target sperm motility. *Fertil. Steril*, in press.
14. Morris, J., Jones, S., Lukanowska, M., Howl, J., and Publicover, S. (2015) Cell penetrating peptides, targeting the regulation of store-operated channels, slow decay of the progesterone-induced $[Ca^{2+}]_i$ signal in human sperm. *Mol. Hum. Reprod.* **21**, 563-570.
15. Howl, J. and Jones, S. (2020) A new biology of cell penetrating peptides. *Pept. Sci.* DOI: 10.1002/pep2.24154.
16. Silva, J.V., Yoon, S., De Bock, P-J., Goltsev, A. V., Gevaert K., Mendes. J.F.F. and Fardilha, M. (2017). Construction and analysis of a human testis/sperm-enriched interaction network: Unravelling the PPP1CC2 interactome. *Biochim. Biophys. Acta Gen. Subj.***1861**, 375–85.
17. Nash, K., Lefievre, L., Peralta-Arias, R., Morris, J., Morales-Garcia, A., Connolly, T., Kirkman-Brown, J.C., and Publicover, S.J. (1996) Techniques for imaging Ca^{2+} signalling in human sperm. *J. Vis. Exp*. **40**, DOI:10.3791/1996.

18. Go, K.J., and Wolf, D.P. (1985) Albumin-mediated changes in sperm sterol content during capacitation. *Biol. Reprod*. **57**, 539-546.
19. Thomas, C.A., Garner, D.L., DeJarnette, J.M., and Marshall, C.E. (1997) Fluorometric assessments of acrosomal integrity and viability in cryopreserved bovine spermatozoa. *Biol. Reprod.* **56**, 991–998.
20. Iqbal, M., Aleem, M., Ijaz, A., Rehman, H., and Yousaf, M.S. (2010) Assessment of buffalo semen with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reduction assay. *J. Anim. Sci*. **88**, 922–925.

Figure 1

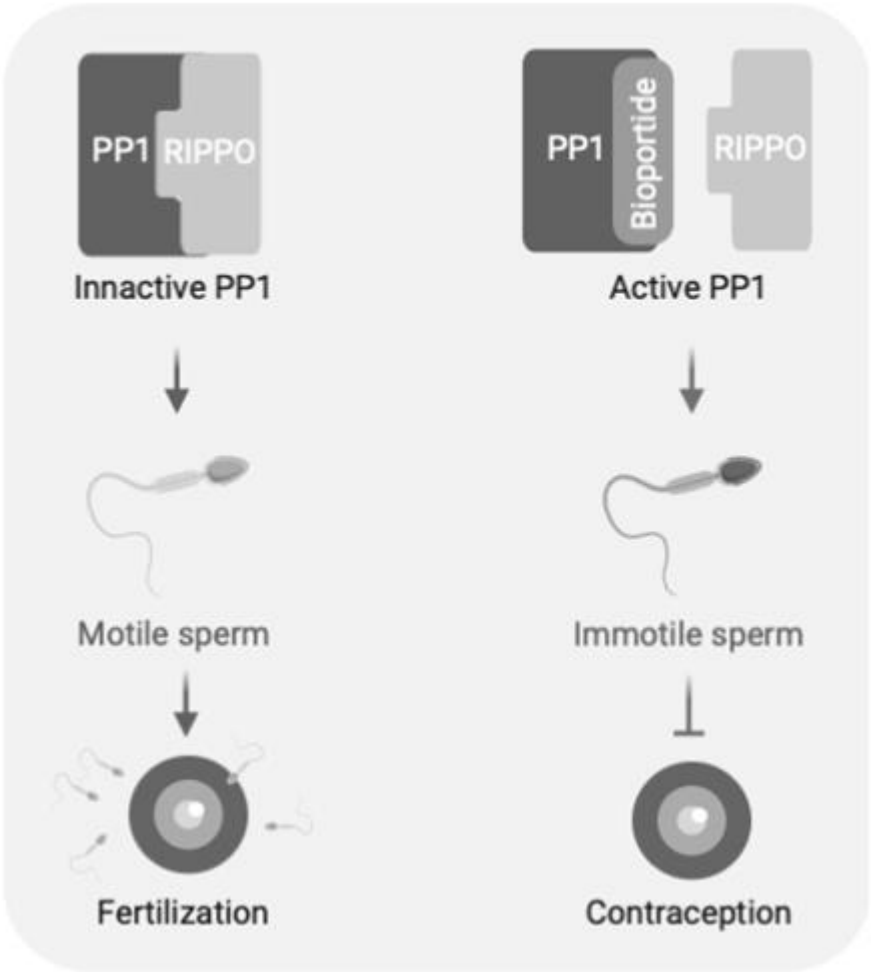

PP1
RIPPO
Innactive PP1
PP1
Bioportide
RIPPO
Active PP1
Motile sperm
Immotile sperm
Fertilization
Contraception

Figure 2

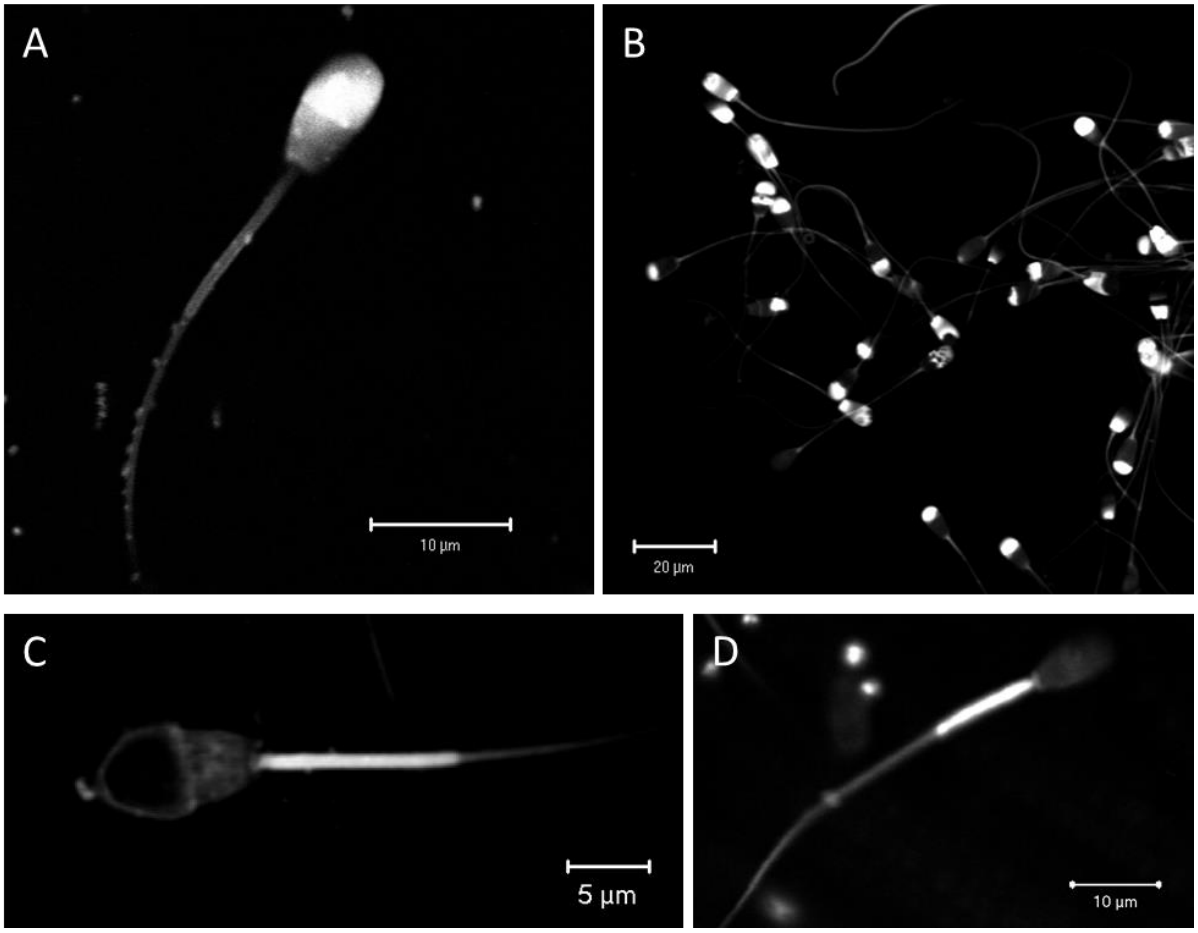
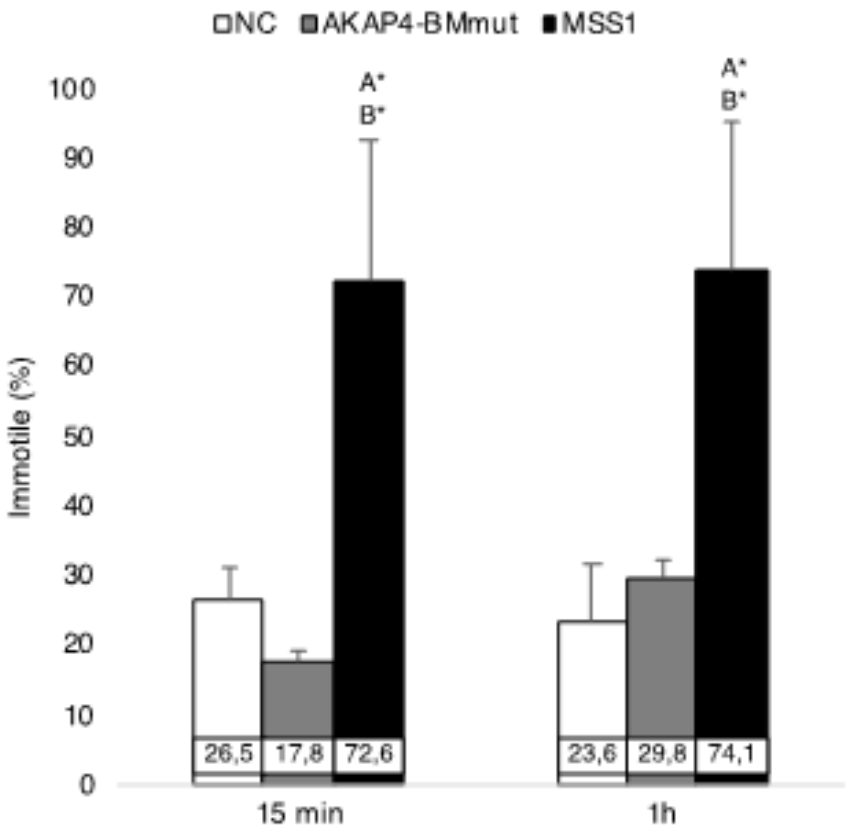


Figure 3


NC
AKAP4-BMmut
MSS1
Immotile (%)
100
90
80
70
60
50
40
30
20
10
0
A*
B*
A*
B*
26,5
17,8
72,6
23,6
29,8
74,1
15 min
1h

Legends to Figures

Fig. 1. Schematic representation of the disruption of complexes between protein phosphatase 1 (PP1) and RIPPOs by STOPSPERM bioportides. The catalytic activity of PP1 is regulated by forming holoenzymes with a variety of RIPPOs including AKAP4. PP1 inhibition, essential for sperm motility acquisition at a late stage of epididymal transport, is prevented by bioportides which act with a dominant-negative mode of action. Adapted from Silva *et al*. (2020) [13].

Fig. 2. Live cell imaging confocal microscopy can be used to establish the intracellular fate of a range of CPPs within spermatozoa. Despite fully differentiated spermatozoa lacking a variety of organelles, some intracellular probes can nevertheless be employed to label intracellular structures such as the mitochondrial midpiece and the acrosome. The fluorescent acidotropic probe LysoTracker® Green DND-26 (2 µM) labels the acrosome (A) and MitoTracker® Deep Red FM (500 nM) can be used to label the mitochondrial midpiece (C). Such molecular probes have been used to establish that TAMRA-tat (5 µM) accretes within the spermatozoa head, including the acrosome (B) and TAMRA-mitoparan (5 µM) accretes within the mitochondrial midpiece (D) [9,10].

Fig. 3. Treatment of human spermatozoa with the MSS1 StopSperm bioportide (10 μM) significantly increases the non-motile fraction. Graph bars represent the mean values of 3 independent experiments performed in triplicate. Error bars ± 95% CI. As indicated, data in samples treated with MSS1 are significantly different from the negative control (A*, NC) and samples treated with a control bioportide lacking the canonical PP1 binding motif (B*, AKAP4-BMmut) at both time periods. Adapted from Silva *et al*. (2020) [13].