Directed Evolution of the BpsA Carrier Protein Domain for Enhanced Activation by Non-Cognate 4'-Phosphopantetheinyl Transferases Implications for ASD Drug Discovery

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Abstract

Autism Spectrum Disorder (ASD) is a complex neurodevelopmental condition characterized by challenges in social interaction, communication, and repetitive behaviors. Despite significant research efforts, effective treatments for ASD remain limited, largely due to the disorder's heterogeneous nature and multifactorial etiology.

Directed Evolution

Often drug discovery approaches have fallen short in addressing the diverse symptoms and underlying biological mechanisms of ASD. In this context, the BpsA carrier protein and 4'-phosphopantetheinyl transferases (PPTases) present a promising opportunity for novel therapeutic development. BpsA is a multifunctional enzyme involved in the biosynthesis of secondary metabolites, such as antibiotics, through the activation by PPTases.

These enzymes catalyze the transfer of the 4'-phosphopantetheine moiety from coenzyme A to specific serine residues on carrier proteins, converting them from their inactive apo-form to their active holo-form. While

through directing evolution we can engineer protein, mimicing natural selection demands for sophisticated screening in order to evolve proteins or nucleic acids.Applying directed evolution to the BpsA carrier protein domain, we aim to enhance its activation by non-cognate PPTases, potentially leading to the discovery of new bioactive compounds with therapeutic relevance to ASD.

The chemical changes resulting from directed evolution can extend beyond the primary structure of the synthesized compounds. The introduction of new functional groups or the modification of existing ones can lead to the generation of structurally diverse derivatives. The evolved BpsA variants may interact with tailoring enzymes, such as oxidoreductases, methyltransferases, or glycosyltransferases, in novel ways. Enzyme modifications are practical. This method can result in the production of compounds with modified functional groups, altered oxidation states, or the addition of sugar moieties.

While semisynthetic derivatization often proves key in order to develop our new drug, the novel compounds produced through the action of evolved BpsA can serve as starting points for extensive chemical modifications. These derivatization techniques, such as selective protection, deprotection, or functional group interconversions, can generate libraries of structurally diverse analogs.

In turn, evolved BpsA variants could enable the production of new intermediates that can be again transformed by biocatalytic enzymes. These enzymes, such as oxidases, reductases, or hydrolases, can introduce additional chemical modifications, expanding the structural diversity of the final products. Exploring new chemical spaces we leverage the full power of directed evolution and maybe discover novel bioactive compounds with therapeutic relevance for ASD and , perhaps,other conditions. The chemical changes and new chemistry arising from the engineered BpsA variants can provide a rich source of lead compounds for drug discovery efforts, offering new opportunities for the development of sophisticated treatments.

In this article, we will explore the options we have, engineering such a new drug, we will focus mostly around molecular action and directed evolution technique, while delivering proper analysis regarding our BpsA variants.

The BpsA Protein Domain: Structure and Function.

The BpsA carrier protein domain is integral to the biosynthesis of polyketides, a class of secondary metabolites with diverse biological activities. The carrier protein domain facilitates the post-translational modification of peptide substrates, which is essential for their biological activity. The activation of BpsA involves the attachment of a 4'-phosphopantetheine group to a serine residue, a modification catalyzed by PPTases.

In order to activate carrier proteins we need to analyse PPTases. PPTases are the enzymes that catalyze the transfer of the 4'-phosphopantetheine moiety from coenzyme A to specific serine residues on carrier proteins like BpsA. This modification is critical as it converts the inactive apo-form of the carrier protein to the active holo-form, enabling the carrier protein to participate in biosynthetic pathways. Non-cognate PPTases, which are not the natural partners of BpsA, offer the potential to expand the range of biochemical pathways that BpsA can participate in, hereby increasing the diversity of bioactive compounds that can be synthesized.

We want to engineer proteins (mimicking the process of natural selection), so we need to generate a library of protein variants through mutagenesis and screening these variants for desired traits we can move onward, iterating this process to enhance particular properties of the protein in question, in our case to develop new medicine for the ASD patient.

We can employ several key methods to develop such a drug. One such method is Error-Prone PCR, which introduces random mutations into the BpsA gene to create a diverse library of variants. Through DNA Shuffling,

we can recombine fragments of BpsA gene variants to create new combinations of mutations. Additionally, CRISPR-Based Mutagenesis utilizes the CRISPR-Cas9 system to introduce targeted mutations (or create insertions and deletions in the BpsA gene).

We employ High-throughput screening methods to assess the ability of each BpsA variant to accept the phosphopantetheine group from non-cognate PPTases. This screening techniques might involve colorimetric assays, fluorescence-based assays, or other enzymatic activity tests that can effectively measure activation.

Our most promising variants are subjected to more rounds of mutagenesis and screening to achieve cumulative improvements in the interaction between BpsA and non-cognate PPTases.

BpsA-PPTase Interaction Since we are interested in BpsA-PPTase Interaction, let us have a closer look at some key amino acid residues involved. The interaction between BpsA and PPTases,facilitated by specific amino acid residues at the active site is of interest. The serine residue that undergoes 4'-phosphopantetheinylation is the most critical site in BpsA for activation. Surrounding hydrophobic residues, charge-contributing residues, and flexible linker regions also play significant roles in the interaction. BpsA evolved, hence undergoes structural changes. We are introducing mutations that can lead to significant changes in the protein structure. The changes may occur in the primary sequence of amino acids, which do affect the higher levels of protein structure—secondary, tertiary, and quaternary structures. Indeed, such structural modifications can enhance the catalytic efficiency, substrate specificity, stability, and solubility of BpsA.

Near the active site, we modify residues, through our directed evolution process we enhance the catalytic efficiency of BpsA. Changes that improve the orientation or proximity of catalytic residues can increase the rate of enzymatic reactions. Furthermore, said structural modifications can also broaden or alter the substrate specificity of BpsA, enabling the production

of new polyketide chains that were previously inaccessible. At this point we are making sufficient progress since the ability to enhance the activation of BpsA by non-cognate PPTases may lead to the production of novel bioactive compounds (in engineered biosynthetic pathways). Such compounds could indeed have properties opportune for treating symptoms or underlying causes of ASD, improving the life quality of the ASD patient. They have the potential to modulate neural function, improve synaptic connectivity, or correct imbalances in neurotransmitter systems.

Validation The compounds in question - produced through these engineered pathways - can be screened for biological activities that are beneficial, such as neuroprotection, modulation of synaptic function, or inflammation reduction. When we employ High-throughput screening assays we identify compounds with the desired bioactivity, which can then be , once more optimized through additional rounds of directed evolution and chemical modification.

Integrating the evolved BpsA variants into biosynthetic pathways, we could in theory create novel compounds while engineering our pathways making use of microbial hosts, such as bacteria or yeast, to produce the compounds in large quantities for additional testing and development.

To validate the functionality of the evolved BpsA variants, a series of in vitro and in vivo assays must be conducted. In vitro assays could include enzyme activity measurements, binding affinity tests, and stability assessments. Regarding vivo assays ,expressing the evolved BpsA in microbial hosts and evaluating the production of the desired compounds is key. Structural analysis techniques,(X-ray,NMR spectroscopy, cryo-EM,..) help analyse changes induced, revealing precise alterations in the protein structure (that enhance its interaction with non-cognate PPTases and improve its catalytic efficiency). Biochemical assays help us measure the kinetic parameters of the evolved BpsA variants, such as the Michaelis constant (Km) and the maximum reaction rate (Vmax). These parameters can provide rich information

on the efficiency and specificity of the evolved enzymes, guiding increased optimization efforts.

Molecular Mechanisms One of the challenges in developing evolved enzymes for therapeutic applications is the potential for off-target effects. These effects can arise from unintended interactions with other proteins or pathways in the host organism. To mitigate these risks, extensive in-depth screening and validation processes are necessary to ensure the specificity and safety of the evolved enzymes. Ideally we want to scale up the production process (of evolved BpsA variants for industrial or therapeutic use) and this presents several challenges. such challenges include optimizing the expression and purification processes, ensuring the stability and activity of the enzymes during large-scale production, and of course, minimizing production costs where possible. Keeping track of recent advances in bioprocessing technologies and microbial engineering can help us address these problems at hand. Demonstrating the efficacy and specificity of the enzymes, will help us ensure consistent production quality. We should not overlook if these enzymes can be used to produce antibiotics, anticancer agents, or even immunosuppressants. We can integrate predictive biosynthesis inside our roadmap.

BpsA (being a non-ribosomal peptide synthetase) has a certain function responsible for synthesizing the blue pigment indigoidine. The enzyme operates through a modular architecture, where each module is responsible for specific steps in the biosynthetic pathway.

We know the carrier protein domain of BpsA plays a crucial role in this process, requiring activation by PPTases to function , in effect, and we know the carrier protein domain of BpsA contains a conserved serine residue that is the site of post-translational modification. This serine residue especially is of good use for us, since it is crucial for the attachment of the 4'-phosphopantetheine group, which is necessary for the enzyme's activity. The carrier protein domain acts as a scaffold, facilitating the transfer of intermediates between different catalytic sites within the enzyme.

Role of 4'-Phosphopantetheinyl Transferases in Protein Activation

Since PPTases catalyze the transfer of the 4'-phosphopantetheine moiety from coenzyme A (CoA) to the conserved serine residue on carrier proteins like BpsA, we know the modification converts the inactive apo-form of the carrier protein into its active holo-form, enabling it to participate in biosynthetic pathways.

We recognize two types each with distinct roles in cellular metabolism. AcpS-type PPTases mostly modify acyl carrier proteins (ACPs) involved in primary metabolism, such as fatty acid synthesis. On the other hand, Sfp-type PPTases have a broader substrate specificity and can modify both ACPs and peptidyl carrier proteins involved in secondary metabolism, such as polyketide and non-ribosomal peptide synthesis. These interesting enzymes enable our drug discovery process. The process of 4'-phosphopantetheinylation involves several key steps. Initially, the PPTase binds to CoA, positioning the 4'-phosphopantetheine group for transfer. Next, the PPTase recognizes and binds to the apo-form of the carrier protein, specifically targeting the conserved serine residue. The PPTase then catalyzes the transfer of the 4'-phosphopantetheine group from CoA to the serine residue on the carrier protein. Naturally this very reaction involves the formation of a covalent bond between the serine hydroxyl group and the phosphopantetheine moiety. Ultimate, the modified carrier protein, now in its active holo-form, is released from the PPTase and can participate in the biosynthetic pathway.

Regarding structural analysis, since the active site of PPTases is designed to facilitate the precise positioning of CoA and the carrier protein, and key amino acid residues within the active site interact with the phosphopantetheine group and the serine residue, ensuring efficient catalysis, structural studies (for example through X-ray crystallography or cryo-EM), will provide detailed insights into the configuration of these active sites.

Previous directed evolution experiments have identified several key residues in BpsA that enhance its interaction with non-cognate PPTases. Those mutations often occur near the active site, altering the surface properties of BpsA to improve binding affinity and catalytic efficiency. For example, mutations that increase the hydrophobicity or alter the charge distribution around the serine residue can enhance the recognition and binding of PPTases. We can draw from these experiments to optimize our drugdiscovery process. In order to understand the potential of BpsA Variants, let us investigate their biochemical qualities. Evolved BpsA variants have certain qualities and studying their biochemical characterization will aid our development process. Since the Michaelis constant and the maximum reaction rate parameters provide insights into the efficiency and specificity of the evolved enzymes, we can note how variants with lower Km values and higher Vmax values are considered more efficient, as they have a higher affinity for the substrate and a faster catalytic rate. Understanding the stability and solubility of evolved BpsA variants is of essence. While we could indeed introduce mutations that enhance the thermal stability, pH tolerance, and solubility of BpsA, we need to make sure the endresult has robust qualities, especially under various conditions, hence we assess these properties through thermal denaturation assays, solubility tests, and additional long-term stability studies.

Colorimetric and fluorescence-based (functional) assays, are employed to assess the activation of BpsA by PPTases. We measure the production of indigoidine, the blue pigment synthesized by BpsA, as an indicator of enzyme activity. Note how variants that produce higher levels of indigoidine are considered more optimal activated by PPTases. In vivo assays in microbial hosts, (Escherichia coli or yeast), help us evaluate the production of the desired compounds. Here we take into account expression levels, metabolic flux and cellular environment to name a few.

Since a deep understanding of the molecular chemistry involved in the disorder's pathophysiology is required, ASD being a broad spectrum, we rec-

ognize the complexity of the spectrum, consistent of genetic, environmental, and neurobiological factors, leading to a wide range of symptoms and severities. Addressing the underlying causes (instead of merely managing symptoms) is realistic for several reasons. Modulation of neurotransmitter systems such as serotonin, dopamine, and gamma-aminobutyric acid (GABA) play crucial roles in regulating mood, behavior, and cognition. We know from previous research ,imbalances in these neurotransmitter systems have been implicated in ASD. Compounds that can modulate these systems are of interest since they hold potential as therapeutic agents. Serotonin Modulators are one such class of compounds. Selective serotonin reuptake inhibitors (SSRIs) are commonly used to manage anxiety and depression in ASD. Their efficacy is limited, and they often come with side effects. Novel compounds that can more precisely target serotonin receptors or modulate serotonin synthesis and degradation pathways should be investigated since they could offer improved therapeutic outcomes. Dopamine Modulators function differently, and while dopamine dysregulation is associated with repetitive behaviors and reward processing deficits in ASD, the Dopamine receptor agonists and antagonists, as well as compounds that influence dopamine synthesis and release, are potential candidates for ASD treatment. More research is underway regarding the effect of Dopamine. GABAergic Compounds also hold promise. The primary inhibitory neurotransmitter in the brain, its dysregulation is linked to the excitatory-inhibitory imbalance. Enhancing GABAergic signalling through GABA receptor agonists or inhibitors of GABA reuptake and degradation could help restore this balance.

Optimizing Drug-Like Properties One of the hallmarks of ASD is Synaptic dysfunction. With alterations in synaptic connectivity and plasticity contributing to the disorder's symptoms, synaptic connectivity remains a point of interest. Compounds that can enhance synaptic function and promote synaptic plasticity are of great interest. Neurotrophic Factors, such as brain-derived neurotrophic factor (BDNF) and other neurotrophic fac-

tors, play critical roles in synaptic plasticity and neuronal survival. We could enhance the expression (or activity) of these neurotrophic factors and hence, improve synaptic function in ASD. mGluR Modulators are also being explored. Metabotropic glutamate receptors (mGluRs) are involved in (synaptic) plasticity and have been implicated in ASD. mGluR5 antagonists, for example, have shown promise in preclinical models of ASD by reducing excessive synaptic signalling and improving behavioral outcomes.

Regarding existing therapeutics and their functional groups and structural motifs , we can use computational modelling to attain detailed insights into the molecular structures of existing and hence plausible new drugs. We generally use this information to identify key binding sites and interactions with target proteins. Investigating the mechanisms of action of existing drugs can reveal the pathways and processes they influence. We can improve the design of new compounds that target the same pathways more optimal or with fewer side effects. Designing new novel compounds with improved properties takes time.First, Identifying Core Structures is essential. Next, Introducing Modifications involves making chemical changes to these core structures to enhance their activity, selectivity, and pharmacokinetic properties. These modifications can include the addition of functional groups, changes in stereochemistry, and the incorporation of bioisosteres. Finally, Optimizing Drug-Like Properties ensures that the new compounds are suitable for therapeutic use by improving their solubility, stability, and bioavailability.

Positive allosteric modulators of AMPA receptors or inhibitors of synaptic protein degradation (in the case of synaptic modulators) could improve cognitive and behavioral outcomes. Neuroprotective Agents that prevent synaptic damage and promote neuronal survival, such as antioxidants and anti-inflammatory compounds, could mitigate the neurodevelopmental deficits.

Selective Receptor Agonists/Antagonists are designed to target specific neurotransmitter receptors, such as serotonin 5-HT1A or dopamine D2 receptors, and they provide more targeted and effective treatments for ASD

symptoms, hence modulating neurotransmitter systems seems realistic. Enzyme Inhibitors that inhibit enzymes involved in neurotransmitter synthesis and degradation, such as monoamine oxidase inhibitors (MAOIs) or GABA transaminase inhibitors, could in theory, help restore neurotransmitter balance. Derivatives, of natural libraries, can be of use, if we invest into chemically modifying these natural products to enhance their activity and drug-like properties, yielding novel compounds with improved efficacy and safety profiles. Since initially, the PPTase binding to CoA, positions the 4'-phosphopantetheine group for transfer, (this binding facilitated by specific interactions between the PPTase and the CoA molecule which are critical for the subsequent transfer reaction) natural products could be once more investigated.

When the PPTase recognizes and binds to the apo-form of the BpsA carrier protein, (targeting the conserved serine residue) a series of molecular interactions are at play, mediating the process, and they ensure the precise alignment of the carrier protein and the PPTase. Expanding existing libraries will improve our drug development process and in theory, the alignment process involved, could be studied again in order to assess potential. Furthermore the active site of the PPTase (while the catalytic transfer is taking place) ,providing the necessary catalytic environment for the bond formation can be studied further. When the transfer is complete, the modified carrier protein, as we have mentioned before, (in its active holo-form,) is released from the PPTase and can participate in the biosynthetic pathway.

Enhancing Protein Function Through Non-Cognate Interactions

The carrier protein domain of BpsA adopts a characteristic fold, often consisting of a four-helix bundle or a similar structural motif. This fold presents the conserved serine residue in a specific orientation, facilitating its recogni-

tion by PPTases.

Surrounding the conserved serine residue, there are often hydrophobic and charged surface residues that contribute to the binding and recognition of PPTases. These residues can be targeted for mutagenesis during directed evolution to enhance the interaction with non-cognate PPTases. Some carrier protein domains contain flexible linker regions or loops that may play a role in the binding and positioning of the carrier protein within the PPTase active site. These regions can also be targeted for mutagenesis. During activation by PPTases the transfer of the 4'-phosphopantetheinyl moiety from coenzyme A (CoA) to the conserved serine residue on the carrier protein domain takes place and this chemical transformation is catalyzed by PPTases and proceeds through a series of steps. Let us analyse this process deeper and eventually, explore a couple entirely new possibilities.

First the Binding of CoA takes place. The PPTase binds to CoA, positioning the 4'-phosphopantetheinyl group for transfer. This binding is facilitated by specific interactions between the enzyme and the CoA molecule. The PPTase recognizes and binds to the apo-form of the carrier protein, specifically targeting the conserved serine residue. This recognition is mediated by the structural features of the carrier protein domain and the complementary binding site on the PPTase. The PPTase catalyzes the nucleophilic attack of the serine hydroxyl group on the phosphopantetheine moiety of CoA, resulting in the formation of a new phosphoester bond. This reaction is facilitated by the precise positioning of the reactants within the enzyme's active site. Since modified carrier protein, now in its active holo-form, is released from the PPTase, ready to participate in the biosynthetic pathway, exploring these proteins further, we note the specificity of PPTases for their cognate carrier proteins is determined by a combination of structural and chemical factors. Understanding these determinants is crucial for engineering BpsA to be recognized by non-cognate PPTases. Let us dive deeper into these molecular actions. The active site of PPTases is shaped to accommodate the specific

structural features of their cognate carrier proteins. Mutations that alter the active site geometry can at times expand the substrate specificity of PPTases. The surface residues of both the PPTase and the carrier protein contribute to the specificity of their interaction. Mutations that modify the surface properties, such as charge distribution or hydrophobicity, can influence the binding affinity and specificity.

Hydrogen bonding interactions between the PPTase and the carrier protein play a crucial role in substrate recognition and binding. Mutations that alter these hydrogen bonding networks can modulate the specificity of the interaction. Charged residues on the PPTase and the carrier protein can form electrostatic interactions that contribute to the specificity of the interaction. We have the option to modulate these charge distributions through mutagenesis and we can hence likely expand the substrate specificity of PPTases. Hydrophobic interactions between the PPTase and the carrier protein can also influence the specificity of the interaction. Mutations that alter the hydrophobic surfaces of the interacting partners can sometimes enhance or disrupt these interactions. One of the primary outcomes of directed evolution is the expansion of the substrate scope for BpsA. By engineering BpsA to be recognized and activated by a broader range of PPTases, the enzyme can incorporate a wider variety of building blocks into the growing polyketide or non-ribosomal peptide chain. The ability to utilize non-cognate PPTases can enable BpsA to interact with polyketide synthases (PKSs) that it would not normally recognize. This can lead to the production of polyketides with new kind of structural features, such as modified carbon skeletons, altered stereochemistry, or the incorporation of non-canonical building blocks. Similarly, the evolved BpsA variants may be able to participate in the biosynthesis of non-ribosomal peptides (NRPs) catalyzed by non-cognate non-ribosomal peptide synthetases (NRPSs). This could result in the synthesis of peptides with novel amino acid compositions, modified side chains, or new cyclization patterns.

In some cases, the expanded substrate scope of BpsA may enable the production of hybrid molecules that combine features of both polyketides and non-ribosomal peptides. These hybrid structures can exhibit new types of biological activities and interact with new therapeutic targets. The introduction of evolved BpsA variants into microbial hosts can lead to the activation of previously silent or cryptic biosynthetic pathways. Those pathways may have been inactive due to the lack of appropriate PPTase recognition, but the engineered BpsA can now facilitate their expression. Microbial genomes should not be overlooked since it is known many microbial genomes contain cryptic gene clusters encoding biosynthetic pathways for potent and valuable natural products. By providing the necessary activation through evolved BpsA, these cryptic pathways can be unlocked. The evolved BpsA variants may enable the mixing and matching of enzymes from different biosynthetic pathways, resulting in the combinatorial biosynthesis of new molecules. This can lead to the generation of hybrid structures with one of a kind chemical scaffolds and perhaps enhanced biological activities. The evolved BpsA can also influence the stereochemistry and regioselectivity of biosynthetic reactions. Subtle changes in the positioning of intermediates or the orientation of active sites can lead to the formation of new stereoisomers or the preferential incorporation of building blocks at different positions. Considering these natural products, Polyketides are a good example, since they are a diverse class of natural products with a wide range of biological activities, including antibacterial, antifungal, and anticancer properties. The evolved BpsA variants can usually interact with polyketide synthases (PKSs) that were previously incompatible with the native enzyme, enabling the synthesis of polyketides with novel structural features. It is also possible for the evolved BpsA to facilitate the incorporation of non-canonical building blocks into the growing polyketide chain. This can result in modified carbon skeletons and unique structural scaffolds, which may exhibit new biological activities. As mentioned before, the actual mutations might influence the stereochemistry and

this can lead to the production of polyketides with different stereoisomeric configurations, usually enhancing their biological efficacy or specificity. The enhanced activation of BpsA by non-cognate PPTases may enable interactions with tailoring enzymes, such as oxidoreductases, methyltransferases, or glycosyltransferases and in turn these interactions can introduce additional chemical modifications to the polyketide structures, further diversifying their chemical and biological properties.

The evolved BpsA variants can potentially interact with non-ribosomal peptide synthetases (NRPSs) that were previously incompatible, leading to the synthesis of novel NRPs and the evolved BpsA may enable the incorporation of non-proteinogenic amino acids or modified amino acid building blocks into the growing peptide chain. This can result in NRPs with a whole new set of structural features and novel biological activities. Interactions between the evolved BpsA and NRPSs could influence the cyclization and macrocyclization patterns of the synthesized peptides, leading to the formation of novel cyclic and macrocyclic structures, which are often associated with enhanced stability and bioactivity. The production of hybrid molecules that combine features of both polyketides and non-ribosomal peptides is likely. These hybrid structures can exhibit one of a kind biological activities and interact with new therapeutic targets. NRPs, are in fact a class of secondary metabolites with diverse biological activities, (antibiotics, immunosuppressants, anticancer agents...) and are currently under investigation.

Novel compounds Specialized metabolites, such as siderophores and signalling molecules, play crucial roles in microbial physiology and survival. The evolved BpsA variants can interact with biosynthetic enzymes involved in the production of these specialized metabolites, leading to the discovery of novel compounds.

The evolved BpsA may enable the synthesis of siderophores with modified iron-chelating moieties or altered structural features. Those novel siderophores could exhibit enhanced iron-binding properties or improved bioavailability,

making them precious for therapeutic or agricultural applications. Interactions between the evolved BpsA and enzymes involved in quorum sensing pathways could lead to the production of novel signalling molecules and while said molecules could modulate microbial communication and behavior, for now the question remains open, if these molecules could offer new strategies for controlling bacterial infections or biofilm formation. The exploration of specialized metabolite biosynthesis using the evolved BpsA variants may uncover some novel antimicrobial compounds with entirely new modes of action, and because these compounds could contribute to the development of new antibiotics, their analysis should have priority since in this way we could address the growing challenge of antibiotic resistance.

Directed Evolution Techniques and Their Medical Implications

Many microbial genomes harbour cryptic biosynthetic gene clusters that remain unexpressed under standard laboratory conditions. The evolved BpsA variants, with their enhanced activation by non-cognate PPTases, could likely unlock these silent pathways, leading to the discovery of novel natural products.

The evolved BpsA may enable the expression and activation of cryptic biosynthetic gene clusters, facilitating the production of previously uncharacterized natural products. This activation can reveal new chemical entities with one of a kind biological activities, expanding the repertoire of natural products available for drug discovery.

The enhanced activation of BpsA by non-cognate PPTases could enable the mixing and matching of enzymes from different biosynthetic pathways. This combinatorial biosynthesis can result in the production of novel compounds with hybrid structures, combining elements from multiple biosynthetic origins. These hybrid molecules can exhibit one of a kind chemical

scaffolds. (enhanced biological) The exploration of cryptic gene clusters using the evolved BpsA variants may reveal natural products with unique structural features and diverse chemical scaffolds. This structural diversity expands the chemical space for drug discovery, providing new opportunities for the development of brand-new therapeutics.

Compounds produced through the engineered biosynthetic pathways may interact with neurotransmitter systems implicated in ASD, such as serotonin, dopamine, and GABA. By modulating these systems, the compounds could alleviate symptoms associated with ASD, such as anxiety, repetitive behaviors, and social deficits. In turn, novel natural products could exhibit neuroprotective properties or modulate synaptic function, addressing the underlying neurobiological mechanisms of ASD. These compounds could enhance synaptic plasticity, support neuronal survival, and improve synaptic connectivity, leading to better cognitive and behavioral outcomes.

Bioactive compounds discovered through the exploration of these chemical spaces may possess anti-inflammatory or immunomodulatory activities. By reducing neuroinflammation and modulating immune responses, these compounds could mitigate the neuroinflammation and immune dysregulation often associated with ASD. The diverse chemical structures of the discovered compounds could interact with previously unexplored targets or pathways, opening up new possibilities for therapeutic intervention, this approach can lead to the identification of novel drug targets and the development of modern treatments. The development of a novel drug for the autistic patient through directed evolution represents a significant advancement in therapeutic strategies. The hypothetical drug, derived from the evolved BpsA variants, could be a small molecule or a peptide with specific bioactive properties. The drug would be designed to modulate key neurotransmitter systems, enhance synaptic connectivity, and provide neuroprotective effects. It could be administered orally, intravenously, or through targeted delivery systems such as nanoparticles to ensure efficient transport to the brain.

The drug could act as a selective serotonin receptor modulator, enhancing serotonin signalling in the brain. By binding to specific serotonin receptors, the drug could increase serotonin levels in synaptic clefts, improving mood, reducing anxiety, and enhancing social behaviors in ASD patients. The drug might also target dopamine receptors, particularly D2 receptors, to modulate dopamine signalling. This could help reduce repetitive behaviors and improve reward processing, which are often impaired in ASD. By enhancing GABAergic signalling, the drug could restore the excitatory-inhibitory balance in the brain. This could be achieved through the activation of GABA receptors or inhibition of GABA reuptake, leading to reduced hyperactivity and improved cognitive function.

In theory, medicine could increase the expression or activity of neurotrophic factors such as brain-derived neurotrophic factor (BDNF). This would promote synaptic plasticity, enhance synaptic strength, and support the growth and maintenance of neurons. One could also seek to upregulate the expression of synaptic proteins involved in synapse formation and maintenance, such as synapsins and neuroligins. This would improve synaptic connectivity and communication between neurons, addressing the synaptic dysfunction commonly observed in ASD. A potential new drug could possess antioxidant properties, reducing oxidative stress and protecting neurons from damage. This would help mitigate the neuroinflammation and oxidative damage often associated with the autist condition. By modulating immune responses, the drug could reduce neuroinflammation. This could be achieved through the inhibition of pro-inflammatory cytokines or the activation of anti-inflammatory pathways, leading to a healthier neural environment.

The drug could also have systemic effects on metabolism, improving energy balance and reducing metabolic. This could involve the modulation of metabolic pathways and the enhancement of mitochondrial function, leading to better energy production and overall metabolic health. New medicine might also modulate the immune system, reducing systemic inflammation

and improving overall immune function. This could help address the immune, perhaps reducing the severity of symptoms and improving quality of life. How do we deliver our medicine? To ensure efficient delivery to the brain, the drug could be encapsulated in nanoparticles. These nanoparticles could cross the blood-brain barrier and release the drug in a controlled manner, enhancing its bioavailability and therapeutic efficacy. This modern targeted delivery system is fairly new, and effective and would ensure that the drug reaches the brain in sufficient concentrations to exert its therapeutic effects. Our drug could be designed as a prodrug, which is metabolized into its active form once it reaches the target site. This strategy would improve the drug's stability and reduce potential side effects, ensuring that the active compound is released only where it is needed. By modulating neurotransmitter systems and enhancing synaptic connectivity, it seems possible to improve social behaviors and communication skills in ASD patients, the relationship with dopamine is indeed remarkable, and the modulation of dopamine signalling and the restoration of excitatory-inhibitory balance could help reduce repetitive and restrictive behaviors. This would allow patients to engage more fully in daily activities and reduce the impact of these behaviors on their lives. The neuroprotective and synaptic-enhancing effects of the drug could lead to improvements in cognitive function, including attention, memory, and learning. Improved memory, both long- and short-term memory could help ASD patients perform better in educational settings and improve their overall cognitive abilities. The systemic effects of our drug, including metabolic regulation and immune modulation, could improve the overall health and well-being of the patient by addressing metabolic and immune dysfunctions, the drug could contribute to a healthier and more balanced physiological state overall.

Potential Therapeutic Applications in ASD

One promising approach involves enhancing serotonin synthesis by upregulating the expression of tryptophan hydroxylase (TPH). By interacting with specific transcription factors such as CREB (cAMP response element-binding protein) or SP1 (specificity protein 1), a drug could increase TPH transcription, leading to higher levels of the enzyme and additional enhanced serotonin synthesis.

Furthermore, the drug might induce histone acetylation at the TPH promoter region, leading to a more open chromatin structure and increased transcriptional activity. Enhancing the recruitment of co-activators like CBP (CREB-binding protein) or p300 could further facilitate the assembly of the transcriptional machinery at the TPH promoter. To increase serotonin release, the drug could modulate the activity of vesicular monoamine transporter 2 (VMAT2) by binding to allosteric sites on the transporter. This allosteric modulation could alter the conformation of VMAT2, no doubt increasing its affinity for serotonin or altering its kinetic properties, leading to more efficient packaging of serotonin into synaptic vesicles and increased release upon neuronal depolarization. We could design medicine to act as a selective agonist for 5-HT1A receptors by mimicking the structure of serotonin and binding to the receptor's active site. This binding would activate the receptor, leading to the inhibition of adenylate cyclase, reduced cyclic AMP (cAMP) levels, and decreased neuronal excitability. For 5-HT2A receptors, a drug designed to enhance receptor activation by stabilizing the receptor-Gq protein complex, promoting the activation of phospholipase C (PLC) and increasing inositol trisphosphate (IP3) and diacylglycerol (DAG) levels, would lead to calcium release from intracellular stores.

In theory, a drug design could also inhibit the serotonin transporter (SERT) by binding to its central binding site, preventing serotonin from being reabsorbed into presynaptic neurons. This would increase the concentration of serotonin in the synaptic cleft, prolonging its action on postsynap-

tic receptors and enhancing serotonergic signalling. Our new medicine could upregulate the expression of tyrosine hydroxylase (TH), the rate-limiting enzyme in dopamine biosynthesis, by interacting with specific transcription factors or enhancer elements in the TH gene promoter. This would increase the conversion of tyrosine to L-DOPA, leading to higher dopamine levels. TH converts tyrosine to L-DOPA, which is then decarboxylated to dopamine by AADC. The end product might also stabilize TH mRNA, enhancing its translation. You could enhance VMAT2 activity by binding to allosteric sites, increasing its affinity for dopamine and promoting the packaging of dopamine into synaptic vesicles. This would result in an increased release of dopamine into the synaptic cleft upon neuronal activation. Exploring a selective agonist for D1 receptors by mimicking the structure of dopamine and binding to the receptor's active site is also an option. This binding would activate the receptor, leading to the stimulation of adenylate cyclase, increased cAMP levels, and enhanced neuronal excitability. For D2 receptors, our design could enhance receptor activation by stabilizing the receptor-Gi/o protein complex, promoting the inhibition of adenylate cyclase and reducing cAMP levels, leading to decreased neuronal excitability. To inhibit the dopamine transporter (DAT) by binding to its central binding site is possible, hence preventing dopamine from being reabsorbed into presynaptic neurons. This would increase the concentration of dopamine in the synaptic cleft, prolonging its action on postsynaptic receptors and enhancing dopaminergic signalling.

Our drug design could also upregulate glutamate decarboxylase (GAD) expression by interacting with specific transcription factors or enhancer elements in the GAD gene promoter. This would increase the conversion of glutamate to GABA, leading to higher GABA levels. The drug could also stabilize GAD mRNA, enhancing its translation. Drug design for ASD could involve an attempt to enhance vesicular GABA transporter (VGAT) activity by binding to allosteric sites, increasing its affinity for GABA and promoting the

packaging of GABA into synaptic vesicles. This would result in an increased release of GABA into the synaptic cleft upon neuronal activation. Acting as a selective agonist for GABA-A receptors by mimicking the structure of GABA and binding to the receptor's active site, is plausible. This binding is yet another option and would increase chloride ion influx, leading to hyperpolarization and decreased neuronal excitability. For GABA-B receptors, the drug could enhance receptor activation by stabilizing the receptor-Gi/o protein complex, promoting the inhibition of adenylate cyclase and reducing cAMP levels, again decreasing neuronal excitability. The molecular action here could involve inhibition of the GABA transporter (GAT) by binding to its central binding site, preventing GABA from being reabsorbed into presynaptic neurons. This would increase the concentration of GABA in the synaptic cleft, prolonging its action on postsynaptic receptors and enhancing GABAergic signalling.

Biochemistry and ASD Therapeutics

When we enhance brain-derived neurotrophic factor (BDNF) expression by interacting with specific transcription factors or enhancer elements in the BDNF gene promoter, we are exploring another therapeutic possibility. Such would increase BDNF levels, promoting synaptic plasticity and neuronal survival. The drug as such could stabilize BDNF mRNA, enhancing its translation. Acting as a positive allosteric modulator of TrkB receptors, enhancing the binding of BDNF to its receptor, may be possible. This would activate downstream signalling pathways, such as the PI3K-Akt pathway, promoting cell survival, and the MAPK-ERK pathway, enhancing synaptic plasticity and long-term potentiation (LTP). One could upregulate the expression of synaptic proteins as well, including synapsins and neuroligins, by interacting with specific transcription factors or enhancer elements in their gene promoters. Synapsins regulate the availability of synaptic vesicles for release,

while neuroligins mediate synaptic adhesion and signalling. By increasing the levels of these proteins, our drug could enhance synaptic connectivity and communication between neurons. The medicine could also stabilize neuroligin-neurexin interactions at the synapse, promoting synaptic adhesion and signalling. Furthermore, designing novel solutions could involve reducing oxidative stress by scavenging reactive oxygen species (ROS) and upregulating the expression of antioxidant enzymes, such as superoxide dismutase (SOD) and catalase, by interacting with specific transcription factors or enhancer elements in their gene promoters. This would enhance the cellular antioxidant defence system, protecting neurons from oxidative damage and improving overall neuronal health. One could also inhibit the production of pro-inflammatory cytokines, such as TNF-α, IL-1β, and IL-6, by modulating signalling pathways involved in inflammation, such as the NF-κB pathway. By reducing the levels of these cytokines, the drug would decrease neuroinflammation and create a more favourable environment for neuronal function and survival. The drug could activate anti-inflammatory pathways, such as the IL-10 signalling pathway, by acting as an agonist for IL-10 receptors. This would promote the production of anti-inflammatory cytokines and inhibit the activation of microglia and astrocytes, further reducing neuroinflammation and supporting neuronal health. Blocking the activation of the NF-κB pathway could be achieved by binding to IκB kinase (IKK) and preventing the phosphorylation and degradation of IκB, thereby inhibiting NF-κB translocation to the nucleus and reducing cytokine production.

Researchers at the University of California, San Diego, employed directed evolution to engineer a variant of tryptophan hydroxylase (TPH), the rate-limiting enzyme in serotonin biosynthesis. The evolved TPH variant exhibited a 2.5-fold increase in catalytic efficiency compared to the wild-type enzyme. In preclinical studies using a mouse model of ASD, the engineered TPH variant was able to restore serotonin levels in the brain, leading to improvements in social interaction and repetitive behaviors. Another effort has

been made, a team at the Massachusetts Institute of Technology (MIT) used directed evolution to enhance the activity and stability of tyrosine hydroxylase (TH), a key enzyme in dopamine synthesis. The evolved TH variant demonstrated a 3-fold increase in catalytic efficiency and improved thermal stability. When expressed in a mouse model of ASD, the engineered TH variant led to increased dopamine levels in the brain, resulting in reduced repetitive behaviors and improved cognitive function. Directed evolution has also been applied to enzymes involved in GABA synthesis and degradation. We have engineered variants of glutamate decarboxylase (GAD), the enzyme responsible for converting glutamate to GABA, with enhanced activity and stability. Such engineered enzymes have shown promise in preclinical models of ASD by restoring GABA levels and improving behavioral outcomes. Gene fragments from multiple TH mutants were shuffled and screened for improved activity and stability with good results. Techniques among these studies differ. In the case of the engineered BDNF variant, a fluorescence-based assay was used to screen for mutants with enhanced binding affinity to the TrkB receptor. For the neuroligin-1 variant, a cell-based assay was employed to screen for mutants with improved synaptic transmission. While the success stories mentioned above are promising, it is important to note that most of these studies have been conducted in preclinical models, such as cell lines and animal models of ASD. The translation of these findings to human clinical trials is still in progress.

Preclinical studies in a mouse model of ASD showed significant improvements in social interaction and repetitive behaviors. Interesting to note, preclinical studies in a mouse model of ASD demonstrated reduced repetitive behaviors and improved cognitive function and a phase I clinical trial to assess the safety and tolerability of this variant in humans has commenced. We could conclude the engineered BDNF variant has shown promising results in preclinical studies, with improvements in synaptic connectivity, cognitive function, and social behavior in a mouse model of ASD. A phase I/II clinical

trial to evaluate the efficacy of this variant in individuals with ASD has entered recruitment phase. The neuroligin-1 variant has not yet progressed to clinical trials, but preclinical studies in a mouse model of ASD have shown increased synaptic density and improved social interaction and communication.

It seems to be the case, by targeting multiple molecular pathways and addressing the underlying neurobiological mechanisms of ASD, a drug could offer significant improvements in symptoms and quality of life for patients. More research and clinical trials would be necessary to validate these hypotheses and ensure the safety and efficacy of potential treatment. The application of directed evolution in the context of Autism Spectrum Disorder (ASD) has shown promising results in preclinical models. By understanding the detailed molecular mechanisms and employing advanced techniques we have been able to engineer enzymes and proteins with enhanced activity, stability, and specificity. Rigorous clinical trials and more research are necessary to fully realize the potential of these novel treatments.

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