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ダイジェスト

JB REVIEW

Membrane traffic governs the STING inflammatory signalling

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The cGAS-STING innate immune pathway has recently emerged as a critical driver of inflammation in a variety of settings, such as virus infection, cellular stress and tissue damage. The pathway detects microbial and host-derived double-stranded DNA (dsDNA) in the cytosol, and triggers the production of the type I interferons through the activation of IRF3. The detailed mechanistic and biochemical understanding of the pathway has enabled the development of pharmacological agents for the treatment of chronic inflammation and cancer. STING is an endoplasmic reticulum (ER)-localized transmembrane protein. Upon emergence of cytosolic dsDNA, STING exits the ER and migrates sequentially to the Golgi, recycling endosomes and lysosomes. Importantly, the intracellular translocation of STING is essential for the activation and inactivation of the STING signalling. In this review, I summarize the recent insights into the regulators of the membrane traffic of STING and STING-associated autoinflammatory diseases.

Keywords: innate immunity, membrane traffic, microautophagy, palmitoylation, STING

Mechanisms that regulate the production of secondary siRNAs in plants

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Many organisms produce secondary small interfering RNAs (siRNAs) that are triggered by primary small RNAs to regulate various biological processes. Plants have evolved several types of secondary siRNA biogenesis pathways that play important roles in development, stress responses and defense against viruses and transposons. The critical step of these pathways is the production of double-stranded RNAs by RNA-dependent RNA polymerases. This step is normally tightly regulated, but when its control is released, secondary siRNA production is initiated. In this article, we will review the recent advances in secondary siRNA production triggered by microRNAs encoded in the genome and siRNAs derived from invasive nucleic acids. In particular, we will focus on the factors, events, and RNA/DNA elements that promote or inhibit the early steps of secondary siRNA biogenesis.

Keywords: microRNA, ribosome, RNA silencing, secondary siRNA, siRNA

REGULAR PAPER
BIOCHEMISTRY
Protein Structure

A three-state mechanism for trifluoroethanol denaturation of an intrinsically disordered protein (IDP)

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Relating the amino acid composition and sequence to chain folding and binding preferences of intrinsically disordered proteins (IDPs) has emerged as a huge challenge. While globular proteins have respective 3D structures that are unique to their individual functions, IDPs violate this structure–function paradigm because rather than having a well-defined structure an ensemble of rapidly interconverting disordered structures characterize an IDP. This work measures 2,2,2-trifluoroethanol (TFE)-induced equilibrium transitions of an IDP called *At*PP16-1 (*Arabidopsis thaliana* phloem protein type 16-1) by using fluorescence, circular dichroism, infrared and nuclear magnetic resonance (NMR) methods at pH 4, 298 K. Low TFE reversibly removes the tertiary structure to produce an ensemble of obligate intermediate (I) retaining the native-state (N) secondary structure. The intermediate I is preceded by a non-obligate tryptophan-specific intermediate Iw whose population is detectable for *At*PP16-1 specifically. Accumulation of such non-obligate intermediates is discriminated according to the sequence composition of the protein. In all cases, however, a tertiary structure-unfolded general obligate intermediate I is indispensable. The I ensemble has higher helical propensity conducive to the acquisition of an exceedingly large level of α-helices by a reversible denaturation transition of I to the denatured state D as the TFE level is increased. Strikingly, it is the same N$\rightleftarrows$I$\rightleftarrows$D scheme typifying the TFE transitions of globular proteins. The high-energy state I characterized by increased helical propensity is called a universal intermediate encountered in both genera of globular and disordered proteins. Neither I nor D strictly show molten globule (MG)-like properties, dismissing the belief that TFE promotes MGs.

Keywords: protein–TFE interactions, TFE denaturation of proteins, three-state protein denaturation by TFE, α-helical propensity of proteins in aqueous TFE

Biomolecular Structures

Cryo-EM and biochemical analyses of the nucleosome containing the human histone H3 variant H3.8

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Histone H3.8 is a non-allelic human histone H3 variant derived from H3.3. H3.8 reportedly forms an unstable nucleosome, but its structure and biochemical characteristics have not been revealed yet. In the present study, we reconstituted the nucleosome containing H3.8. Consistent with previous results, the H3.8 nucleosome is thermally unstable as compared to the H3.3 nucleosome. The entry/exit DNA regions of the H3.8 nucleosome are more accessible to micrococcal nuclease than those of the H3.3 nucleosome. Nucleosome transcription assays revealed that the RNA polymerase II (RNAPII) pausing around the superhelical location (SHL) −1 position, which is about 60 base pairs from the nucleosomal DNA entry site, is drastically alleviated. On the other hand, the RNAPII pausing around the SHL (−5) position, which is about 20 base pairs from the nucleosomal DNA entry site, is substantially increased. The cryo-electron microscopy structure of the H3.8 nucleosome explains the mechanisms of the enhanced accessibility of the entry/exit DNA regions, reduced thermal stability and altered RNAPII transcription profile.

Keywords: chromatin, epigenetics, H3.8, histone variant, nucleosome

Glycobiology and Carbohydrate Biochemistry

Curcumin analog GO-Y030 inhibits tumor metastasis and glycolysis

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Tumor metastasis is one of the worst prognostic features of cancer. Although metastasis is a major cause of cancer-related deaths, an effective treatment has not yet been established. Here, we explore the antitumor effects of GO-Y030, a curcumin analog, via various mechanisms using a mouse model. GO-Y030 treatment of B16–F10 melanoma cells inhibited TGF-β expression and glycolysis. The invasion assay results showed almost complete invasion inhibition following GO-Y030 treatment. Mouse experiments demonstrated that GO-Y030 administration inhibited lung tumor metastasis without affecting vascular endothelial cells. Consistent with this result, GO-Y030 treatment led to the downregulation of MMP2 and VEGFα, inhibiting tumor invasion and metastasis. The silencing of eIF4B, a downstream molecule of S6, attenuated MMP2 expression. Our study demonstrates the novel efficacy of GO-Y030 in inhibiting tumor metastasis by regulating metastasis-associated gene expression via inhibiting dual access, glycolytic and TGF-β pathways.

Keywords: curcumin analog, matrix metallopeptidase 2, migration assay, scratching assay, tumor metastasis

Biochemistry in Diseases and Aging

Phosphorylated SARM1 is involved in the pathological process of rotenone-induced neurodegeneration

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Sterile alpha and Toll/interleukin receptor motif-containing protein 1 (SARM1) is a NAD^+ hydrolase that plays a key role in axonal degeneration and neuronal cell death. We reported that c-Jun N-terminal kinase (JNK) activates SARM1 through phosphorylation at Ser-548. The importance of SARM1 phosphorylation in the pathological process of Parkinson's disease (PD)

has not been determined. We thus conducted the present study by using rotenone (an inducer of PD-like pathology) and neurons derived from induced pluripotent stem cells (iPSCs) from healthy donors and a patient with familial PD PARK2 (FPD2). The results showed that compared to the healthy neurons, FPD2 neurons were more vulnerable to rotenone-induced stress and had higher levels of SARM1 phosphorylation. Similar cellular events were obtained when we used PARK2-knockdown neurons derived from healthy donor iPSCs. These events in both types of PD-model neurons were suppressed in neurons treated with JNK inhibitors, Ca^{2+}-signal inhibitors, or by a SARM1-knockdown procedure. The degenerative events were enhanced in neurons overexpressing wild-type SARM1 and conversely suppressed in neurons overexpressing the SARM1-S548A mutant. We also detected elevated SARM1 phosphorylation in the midbrain of PD-model mice. The results indicate that phosphorylated SARM1 plays an important role in the pathological process of rotenone-induced neurodegeneration.

Keywords: JNK, PARK2, Parkinson's disease, Phosphorylation, SARM1

Neurochemistry

Proline-rich transmembrane protein 2 knock-in mice present dopamine-dependent motor deficits

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Mutations of proline-rich transmembrane protein 2 (PRRT2) lead to dyskinetic disorders such as paroxysmal kinesigenic dyskinesia (PKD), which is characterized by attacks of involuntary movements precipitated by suddenly initiated motion, and some convulsive disorders. Although previous studies have shown that PKD might be caused by cerebellar dysfunction, PRRT2 has not been sufficiently analyzed in some motor-related regions, including the basal ganglia, where dopaminergic neurons are most abundant in the brain. Here, we generated several types of *Prrt2* knock-in (KI) mice harboring mutations, such as c.672dupG, that mimics the human pathological mutation c.649dupC and investigated the contribution of Prrt2 to dopaminergic regulation. Regardless of differences in the frameshift sites, all truncating mutations abolished Prrt2 expression within the striatum and cerebral cortex, consistent with previous reports of similar Prrt2 mutant rodents, confirming the *loss-of-function* nature of these mutations. Importantly, administration of L-dopa, a precursor of dopamine, exacerbated rotarod performance, especially in *Prrt2*-KI mice. These findings suggest that dopaminergic dysfunction in the brain by the *PRRT2* mutation might be implicated in a part of motor symptoms of PKD and related disorders.

Keywords: dopamine, l-dopa, paroxysmal kinesigenic dyskinesia, Prrt2, rotarod

CELL

Development and Aging

A crucial stem cell plasticity regulation pathway: identification of key elements using the NCCIT human embryonic carcinoma cell line

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Upon removal of stemness factors, a small subpopulation of embryonic stem cells (ESCs) spontaneously extrudes the t-SNARE protein syntaxin-4, which upregulates the cell adhesion molecule P-cadherin and induces the onset of epithelial-mesenchymal transition (EMT)-like behaviors with loss of stemness in each cell. In this study, we identified a series of molecular elements responsible for this phenomenon using several small-molecule inhibitors and the human embryonic carcinoma cell line, NCCIT. We found that the syntaxin-4–triggered morphological changes and a decrease in stemness signatures were independently induced by the activation of Rho-associated kinase (ROCK) and the abrogation of PI3K/Akt signaling. We also found that the extracellular expression of syntaxin-4 inactivated focal adhesion kinase (FAK) in association with the augmented expression of P-cadherin, and comparable controls of either of these downstream elements of syntaxin-4 accelerated both ROCK-induced F-actin stress fiber formation and P13K/Akt–suppressed loss of stemness signatures. Cells expressing P-cadherin inactivated FAK but FAK inhibition did not affect P-cadherin expression, demonstrating a causal relationship between P-cadherin and FAK in the event of syntaxin-4 induction. These results reveal a novel signaling axis in stem cells and shed new light on the crucial elements for stem cell plasticity and the maintenance of stemness.

Keywords: FAK, P-cadherin, PI3K/Akt, Rho/ROCK, syntaxin-4

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ダイジェスト

JB REVIEW

Insights into the regulation of mitochondrial functions by protein kinase A-mediated phosphorylation

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Cyclic AMP (cAMP)—protein kinase A (PKA) signaling is a highly conserved pathway in eukaryotes and plays a central role in cell signaling cascades in response to environmental changes. Elevated cAMP levels promote the activation of PKA, which phosphorylates various downstream proteins. Many cytosolic and nuclear proteins, such as metabolic enzymes and transcriptional factors, have been identified as substrates for PKA, suggesting that PKA-mediated regulation occurs predominantly in the cytosol. Mitochondrial proteins are also phosphorylated by PKA, and PKA-mediated phosphorylation of mitochondrial proteins is considered to control a variety of mitochondrial functions, including oxidative phosphorylation, protein import, morphology and quality control. In this review, we outline PKA mitochondrial substrates and summarize the regulation of mitochondrial functions through PKA-mediated phosphorylation.

Keywords: cAMP, mitochondria, phosphorylation, PKA

The Nuclear Cap-Binding Complex, a multitasking binding partner of RNA polymerase II transcripts

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In eukaryotic cells, RNAs transcribed by RNA polymerase-II receive the modification at the 5′ end. This structure is called the cap structure. The cap structure has a fundamental role for translation initiation by recruiting eukaryotic translation initiation factor 4F (eIF4F). The other important mediator of the cap structure is a nuclear cap-binding protein complex (CBC). CBC consists of two proteins, which are renamed as NCBP1 and NCBP2 (previously called as CBP80/NCBP and CBP20/NIP1, respectively). This review article discusses the multiple roles CBC mediates and co-ordinates in several gene expression steps in eukaryotes.

Keywords: 7-methyl guanosine (m7G) cap, cap-binding protein complex (CBC), eIF4E, gene expression, RNA polymerase II

Access and utilization of host-derived iron by Leishmania parasites

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Iron is involved in many biochemical processes including oxygen transport, ATP production, DNA synthesis and antioxidant defense. The importance of iron also applies to *Leishmania* parasites, an intracellular protozoan pathogen causing leishmaniasis. *Leishmania* are heme-auxotrophs, devoid of iron storage proteins and the heme synthesis pathway. Acquisition of iron and heme from the surrounding niche is thus critical for the intracellular survival of *Leishmania* inside the host macrophages. Moreover, *Leishmania* parasites are also exposed to oxidative stress within phagolysosomes of macrophages in mammalian hosts, and they need iron superoxide dismutase for overcoming this stress. Therefore, untangling the strategy adopted by these parasites for iron acquisition and utilization can be good targets for the development of antileishmanial drugs. Here, in this review, we will address how *Leishmania* parasites acquire and utilize iron and heme during infection to macrophages.

Keywords: *leishmania*, macrophage, iron, heme

REGULAR PAPER
BIOCHEMISTRY
Biochemistry General

Characterization of recombinant photoconverting green fluorescent Akanes

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Akanes are fluorescent proteins that have several fluorescence maxima. In this report, Akane1 and Akane3 from *Scleronephthya gracillima* were selected, successfully overexpressed in *Escherichia coli* and purified by affinity chromatography. Fluorescence spectra of the recombinant Akanes matured in darkness, or am-

bient light were found to have several fluorescence peaks. SDS-PAGE analysis revealed that Akanes matured in ambient light have two fragments. MS/MS analysis of Akanes digested with trypsin showed that the cleavage site is the same as observed for the photoconvertible fluorescent protein Kaede. The differences between the calculated masses from the amino acid sequence of Akane1 and the measured masses of Akane1 fragments obtained under ambient light coincided with those of Kaede. In contrast, a mass difference between the measured N-terminal Akane3 fragment and the calculated mass indicated that Akane3 is modified in the N-terminal region. These results indicate that numerous peaks in the fluorescent spectra of Akanes partly arise from isoproteins of Akanes and photoconversion. Photoconversion of Akane1 caused a fluorescence change from green to red, which was also observed for Akane3; however, the fluorescent intensity decreased dramatically when compared with that of Akane3.

Keywords: Akane, green fluorescent protein, multicolour fluorescent protein, photoconversion, Scleronephthya gracillima

Escherichia coli tRNA (Gm18) methyltransferase (TrmH) requires the correct localization of its methylation site (G18) in the D-loop for efficient methylation

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TrmH is a eubacterial tRNA methyltransferase responsible for formation of 2′-*O*-methylguaosine at position 18 (Gm18) in tRNA. In *Escherichia coli* cells, only 14 tRNA species possess the Gm18 modification. To investigate the substrate tRNA selection mechanism of *E. coli* TrmH, we performed biochemical and structural studies. *Escherichia coli* TrmH requires a high concentration of substrate tRNA for efficient methylation. Experiments using native tRNA SerCGA purified from a *trmH* gene disruptant strain showed that modified nucleosides do not affect the methylation. A gel mobility-shift assay reveals that TrmH captures tRNAs without distinguishing between relatively good and very poor substrates. Methylation assays using wild-type and mutant tRNA transcripts revealed that the location of G18 in the D-loop is very important for efficient methylation by *E. coli* TrmH. In the case of tRNA Ser, tRNA Tyrand tRNA Leu, the D-loop structure formed by interaction with the long variable region is important. For tRNAGln, the short distance between G18 and A14 is important. Thus, our biochemical study explains all Gm18 modification patterns in *E. coli* tRNAs. The crystal structure of *E. coli* TrmH has also been solved, and the tRNA binding mode of *E. coli* TrmH is discussed based on the structure.

Keywords: tRNA, RNA modification, tRNA *methyltransferase*, SPOUT, *Escherichia coli*

Distribution and role of D-glutamate, a novel D-amino acid identified in animals, in the reproductive tissues of male kuruma prawn Marsupenaeus japonicus

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Some aquatic invertebrates contain free D-alanine. We previously showed copious amounts of free D-glutamate, a novel D-amino acid, in the tissue of the male reproductive organs of *Marsupenaeus japonicus*. Herein, we clarified the distribution and potential role of D-glutamate and D-alanine in male reproductive tissues, namely the testis, vas deferens and seminal receptacle at different growth stages of *M. japonicus*. The percentage of D-glutamate to total glutamate was over 50% in these tissues. In particular, the content of D-glutamate was the most abundant in the vas deferens, the ratio of D-glutamate to total glutamate was approximately 80%. In contrast, D-alanine content was the lowest in the vas deferens among these tissues. D-Glutamate content was the highest when the prawn weighed 12 g, indicating that D-glutamate is actively synthesized in the younger stage. Our findings suggest that D-glutamate plays an important role in the reproductive functions of *M. japonicus*.

Keywords: D-alanine, D-amino acid, D-glutamate, male reproductive organs, *Marsupenaeus* japonicus

Protein Structure

Mannose oligosaccharide recognition of CGL1, a mannose-specific lectin containing DM9 motifs from Crassostrea gigas, revealed by X-ray crystallographic analysis

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CGL1 is a mannose-specific lectin isolated from the Pacific oyster *Crassostrea gigas*, and it belongs to the DM9 domain protein family. Each subunit of the CGL1 dimer consists of a tandem repeat of DM9 motifs, which were originally found in the *Drosophila melanogaster* genome. The CGL1 protomer contains two carbohydrate-binding sites: a high-affinity site A and a low-affinity site B. An assay using dendrimers containing oligomannose from yeast (*Saccharomyces cerevisiae*) revealed that CGL1 exhibited significantly higher affinity for mannotetraose (Man4)

compared to mannobiose (Man2) and mannotriose (Man3). To investigate its oligomannose-recognition mechanism, X-ray crystallographic analyses of CGL1/oligomannose complexes were performed. In the CGL1/Man2 and CGL1/Man3 complexes, Manα1–2Man and Manα1–2Manα1–2Man, respectively, were primarily bound to site A, interacting with the non-reducing mannose residue. On the other hand, in the CGL1/Man4 crystal, Man4 (Manα1–2Manα1–2Manα1–6Man) was bound at both site A and site B at the non-reducing and reducing ends, thus linking adjacent CGL1 molecules with crystallographic symmetry. These findings suggest that CGL1 can recognize both the non-reducing and reducing mannose residues of mannose oligosaccharides at its two distinct carbohydrate-binding sites. This enables efficient complex formation, making CGL1 a pattern-recognition molecule capable of recognizing diverse structures of mannose-containing carbohydrate chains.

Keywords: *Crassostrea gigas*, DM9 domain protein, innate immunity, mannose-specific lectin, X-ray crystallographic analysis

Protein Interaction and Recognition

The SH3 binding site in front of the WH1 domain contributes to the membrane binding of the BAR domain protein endophilin A2

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The Bin–Amphiphysin–Rvs (BAR) domain of endophilin binds to the cell membrane and shapes it into a tubular shape for endocytosis. Endophilin has a Src-homology 3 (SH3) domain at their C-terminal. The SH3 domain interacts with the proline-rich motif (PRM) that is found in proteins such as neural Wiskott–Aldrich syndrome protein (N-WASP). Here, we re-examined the binding sites of the SH3 domain of endophilin in N-WASP by machine learning-based prediction and identified the previously unrecognized binding site. In addition to the well-recognized PRM at the central proline-rich region, we found a PRM in front of the N-terminal WASP homology 1 (WH1) domain of N-WASP (Nt-PRM) as a binding site of the endophilin SH3 domain. Furthermore, the diameter of the membrane tubules in the presence of NtPRM mutant was narrower and wider than that in the presence of N-WASP and in its absence, respectively. Importantly, the Nt-PRM of N-WASP was involved in the membrane localization of endophilin A2 in cells. Therefore, the NtPRM contributes to the binding of endophilin to N-WASP in membrane remodeling.

Keywords: endophilin A2, N-WASP, proline-rich motifs (PRM), SH3 domain

Lipid Biochemistry

PLAAT1 expression triggers fragmentation of mitochondria in an enzyme activity-dependent manner

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The phospholipase A and acyltransferase (PLAAT) family is a protein family consisting of five members (PLAAT1–5), which acts as phospholipid-metabolizing enzymes with phospholipase A_1/A_2 and *N*-acyltransferase activities. Since we previously reported that the overexpression of PLAAT3 in mammalian cells causes the specific disappearance of peroxisomes, in the present study we examined a possible effect of PLAAT1 on organelles. We prepared HEK293 cells expressing mouse PLAAT1 in a doxycycline-dependent manner and found that the overexpression of PLAAT1 resulted in the transformation of mitochondria from the original long rod shape to a round shape, as well as their fragmentation. In contrast, the overexpression of a catalytically inactive point mutant of PLAAT1 did not generate any morphological change in mitochondria, suggesting the involvement of catalytic activity. PLAAT1 expression also caused the reduction of peroxisomes, while the levels of the marker proteins for ER, Golgi apparatus and lysosomes were almost unchanged. In PLAAT1-expressing cells, the level of dynamin-related protein 1 responsible for mitochondrial fission was increased, whereas those of optic atrophy 1 and mitofusin 2, both of which are responsible for mitochondrial fusion, were reduced. These results suggest a novel role of PLAAT1 in the regulation of mitochondrial biogenesis.

Keywords: mitochondrion, organelle, peroxisome, hospholipase A2, PLAAT

Degradation of glycosylinositol phosphoceramide during plant tissue homogenization

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A convenient method for the determination of plant sphingolipids (glycosylinositol phosphoceramide, GIPC; glucosylceramide, GluCer; phytoceramide 1-phosphate, PC1P and phytoceramide, PCer) was developed. This method includes the extraction of lipids using 1-butanol, alkali hydrolysis with methylamine and separation by TLC. The amounts of sphingolipids in the sample were determined based on the relative intensities of standard sphingolipids visualized by primulin/UV on TLC. Using this method, we found that almost all GIPCs were degraded in response to tissue homogenization in cruciferous plants (cabbage, broccoli and *Arabidopsis thaliana*). The decrease in GIPCs was compensated for by increases in PC1P and PCer, indicating that GIPC was degraded by hydrolysis at the D and C positions of GIPC, respectively. In carrot roots and leaves, most of GIPC degradation was compensated for by an increase in PCer. In rice roots, the decrease in GIPCs was not fully explained by the increases in PC1P and PCer, indicating that enzymes other than phospholipase C and D activities operated. As the visualization of lipids on TLC is useful for detecting the appearance or disappearance of lipids, this method will be available for the characterization of metabolism of sphingolipids in plants.

Keywords: glycosylinositol phosphoceramide, phospholipase C, phospholipase D, phytoceramide, phytoceramide 1-phosphate

Enzyme Inhibitors

Inhibition of human glutathione transferase by catechin and gossypol: comparative structural analysis by kinetic properties, molecular docking and their effi cacy on the viability of human MCF-7 cells

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Glutathione transferase Pi (GSTP1) expression is increased in many cancer types and is associated with multidrug resistance and apoptosis inhibition. Inhibitors of GSTP1-1 have the potential to overcome drug resistance and improve chemotherapy efficacy as adjuvant agents. This study investigated the effects of catechin and gossypol on human glutathione transferase Pi (GSTP1-1) activity and their cytotoxic effects on breast cancer cells (MCF-7) individually and in combination with tamoxifen (TAM). Gossypol effectively inhibited the enzyme with an IC_{50} value of 40 μM, compared to 200 μM for catechin. Gossypol showed stronger inhibition of GSTP1-1 activity ($Ki = 63.3 \pm 17.5$ μM) compared to catechin ($Ki = 220 \pm 44$ μM). Molecular docking analysis revealed their binding conformations to GSTP1-1, with gossypol binding at the subunit interface in an un-competitive manner and catechin showing mixed non-competitive inhibition. Gossypol had severe cytotoxic effects on both MCF-7 cells and normal BJ1 cells, while catechin had a weak cytotoxic effect on MCF-7 cells only. Combination therapy with TAM resulted in cytotoxicity of 27.3% and 35.2% when combined with catechin and gossypol, respectively. Gossypol showed higher toxicity to MCF-7 cells, but its strong effects on normal cells raised concerns about selectivity and potential side effects.

Keywords: Breast cancer cell line, Glutathione transferase Pi, Inhibition mechanism, Polyphenols, Structure–function relationship

BIOTECHNOLOGY

Biotechnology General

Construction of a T7 phage random peptide library by combining seamless cloning with in vitro translation

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T7 phage libraries displaying random peptides are powerful tools for screening peptide sequences that bind to various target molecules. The T7 phage system has the advantage of less biased peptide distribution compared to the M13 phage system. However, the construction of T7 phage DNA is challenging due to its long 36 kb linear DNA. Furthermore, the diversity of the libraries depends strongly on the efficiency of commercially available packaging extracts. To address these issues, we examined the combination of seamless cloning with cell-free translation systems. Seamless cloning technologies have been widely used to construct short circular plasmid DNA, and several recent studies showed that cell-free translation can achieve more diverse phage packaging. In this study, we combined these techniques to construct four libraries (CX7C, CX9C, CX11C and CX13C) with different random regions lengths. The libraries thus obtained all showed diversity $> 10^9$ plaque forming units (pfu). Evaluating our libraries with an anti-FLAG monoclonal antibody yielded the correct epitope sequence. The results indicate that our libraries are useful for screening peptide epitopes against antibodies. These findings suggest that our system can efficiently construct T7 phage libraries with greater diversity than previous systems.

Keywords: diversity, *in vitro*, translation, peptide screening, phage library, seamless cloning