Impact of hydrogen peroxide and peracids on enzymatic activity of protein tyrosine phosphatase CD45

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PUBLICATIONS AND CONFERENCE PRESENTATIONS OF THE DISSERTATION DATA AND RESULTS

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The results of the project have been partially presented by the author at the international conferences: in Nagoya, Japan (2010), Yalta, Ukraine (2009), and Gdansk, Poland (2008) as well as at the seminars in Edmonton, Canada (2012) and Palermo, Italy (2012).

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ABSTRACT

Protein tyrosine phosphatases (PTPs) dephosphorylate proteins at phosphotyrosine residues, and together with protein tyrosine kinanses are responsible for the regulation of tyrosine phosphorylation status controlling numerous cellular processes, such as cellular growth, differentiation, metabolism, cell-cell communication and immune response.

CD45 is a transmembrane phosphatase mostly expressed on the surface of nucleated hematopoietic cells. CD45 dephosphorylates, among others, the Src-family kinases, and thus is a key positive regulator of T or B cell receptor-mediated signaling leading to lymphocyte activation. Moreover, CD45 plays an important role in allergic response being required in mast cells for the degranulation process, and subsequent histamine release. In pancreatic acinar cells CD45 has been found to operate as a negative regulator of cytokine receptor signaling pathway via suppression of JAK kinases. It has been reported that CD45 might be implicated in carcinogenesis as the overexpression of CD45 encoding gene correlates with increased proliferation rate of myeloma cells. CD45 is expressed in multiple isoforms as a result of alternative splicing of variable exons. Abnormalities in the expression of CD45 splice variants cause severe combined immunodeficiency syndrome or autoimmune disorders, and possibly multiple sclerosis. Modulation of the enzymatic activity of different CD45 isoforms can prevent the rejection of transplanted organs, as well as β-amyloid peptide-induced microglial activation observed in Alzheimer's disease.

The hallmark defining the classical PTP enzymes is the strictly conserved active site sequence $C(X)_5R$ within the catalytic domain which constitutes the phosphate-binding pocket of the enzyme. The cysteine residue inside the signature motif participates directly in the removal of phosphate group from certain phosphoproteins. The cysteine residue located at the bottom of the active site cleft exists in the thiolate anion form, and is highly susceptible to oxidation. Oxidation of cysteine residue leads to the formation of a reversible form of sulfenic acid residue, while highly oxidizing environment can induce further oxidation yielding physiologically irreversible sulfinic and sulfonic acid residues, all of which consequently cause inactivation of the enzyme.

Peracids are a class of highly oxidizing chemicals, and thus may induce inactivation of PTPs via oxidation of the catalytic cysteine residue. The peroxycarboxyl group (O=C-O-OH) in peracid is an oxidized derivative of the regular carboxyl group (O=C-OH). Peracids can undergo decomposition, forming carboxylic acids, and releasing active oxygen. Peracids can be produced as a result of the reaction between carboxylic acids and hydrogen peroxide.

Living organisms naturally produce hydrogen peroxide (mitochondrial respiratory chain, oxidases, and specifically in white blood cells) that may be potentially responsible for the conversion of a carboxylic acid (O=C-OH) into a peroxy acid (O=C-O-OH). A possibility of peracid formation increases with the overproduction of carboxylic acids, like in some liver disorders or psoriaris.

The experiments, I have performed, demonstrate that nanomolar concentrations of peracids can inactivate recombinant phosphatase CD45 as well as CD45 natively present in Jurkat cell line. Interestingly, the obtained results point to a unique correlation between the length of a peracid's hydrocarbon chain and its inhibitory effect on CD45. Medium-chain peracids (C8, C10) proved to have a higher impact on CD45 deactivation than short- or long-chain peracid analogs. The experiments demonstrate that carboxylic acids have no inhibitory effect on CD45 activity, which allows to assume, that the peroxycarboxyl group rather than nonspecific impact of hydrocarbon chain is directly implicated in the mechanism of enzymatic inhibition.

According to the computational analysis, peroxy acids of C8, C9 and C10 hydrocarbon chains have been predicted to fit best in the CD45 active site, with C10 having the binding affinity of –5.8 kcal/mol. The maximum binding affinitiy was calculated for peroxy acid C9. These data are in accordance with the experimental results showing that peroxy acids C8 and C10 have the strongest inhibitory effect on PTPs as observed for both the recombinant protein and cell culture experiments. Thus, the computational docking analysis seems to support the hypothesis that the observed differences in the inhibitory effect on CD45 activity depend mainly on the peracid chain length, and consequently different binding affinities for the PTP active site. Interestingly, the calculated binding affinities of the peroxy acids as compared with the respective carboxylic acids are slightly higher (~1 kcal/mol). The overall binding affinity trend as dependent on the number of carbon atoms in an acid chain is comparable for both the peroxy acids and the carboxylic acids. However, according to the experimental data carboxylic acids do not display any inhibitory effect on CD45. In conclusion, the obtained data suggest that peroxycarboxyl group of peroxy acids is directly implicated in the mechanism of CD45 inhibition with the strongest inhibitory effect observed for medium-chain peracids of optimal binding affinity. Moreover, the experimental data suggest that inactivation of PTP CD45 by peracids is caused by the oxidation of the cysteine residue in the catalytic center, rather than by steric hindrance due to strong ligand binding in the catalytic center. The studies on inhibitory properties of peracids against PTPs may be a good starting point for the synthesis of analogous inhibitors of higher selectivity and potential clinical application.

LIST OF ABBREVIATIONS

PTP - protein tyrosine phosphatase

PTK - protein tyrosine kinase

RPTP - receptor protein tyrosine phosphatase

ROS - reactive oxygen species

JAK - Janus kinase

STAT - signal tranducer and activator of transcription

*p*NPP - *para*-nitrophenylphosphate

DTT - dithiotreitol

OPD - *ortho*-phenylendiamine

PDB - Protein Data Bank

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1. INTRODUCTION

1.1. PROTEIN TYROSINE PHOSPHATASES

Protein tyrosine phosphatases (PTPs) are enzymes that remove phosphate groups from phosphorylated tyrosine residues (dephosphorylation) present on various proteins (Figure 1). Protein tyrosine phospahatases form a large family of enzymes discovered in diverse organisms including bacteria, yeast, insects and vertebrates [Fauman, Saper, 1996]. The human genome contains over a hundred genes for different protein tyrosine phosphatases (similarly to what can be observed in the genes of the protein tyrosine kinase family) encoding approximately five hundred types of phosphatases. A large number of protein tyrosine phosphatases is due to the fact that their genes are able to generate alternatively spliced products, which can further undergo posttranslational modifications [Alonso et al., 2004]. Protein tyrosine phosphatases modulate the cellular level of tyrosine phosphorylation under normal and pathological conditions, having both positive and negative effects on cellular signal transduction [Tonks, Neel, 1996]. A disregulation of the activity of PTPs has drawn attention of pharmacists and scientists due to its association with the development of numerous human diseases [Ferreira et al., 2006]. A critical role in pathogenesis of various disorders, and physiological significance of protein tyrosine phosphatases were one of the main reasons for human genome sequencing studies as well as mice gene knockout projects [Li, Dixon, 2000].

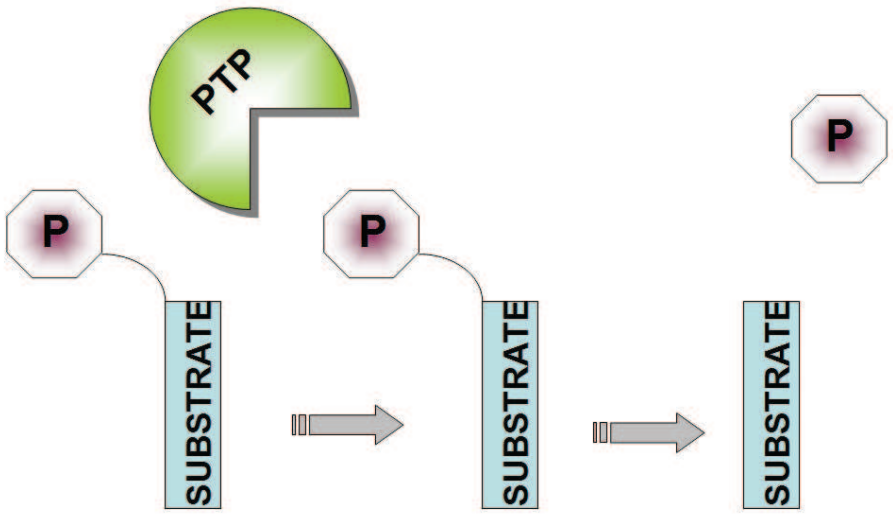


Figure 1. Dephosphorylation reaction - phosphatase removing a phosphate group.

1.1.1. The reversible phosphorylation

The protein tyrosine phosphorylation and dephosphorylation processes are evolutionarily conserved mechanisms of signal transduction in eukaryotic cells, that are fundamentally important for controlling cellular physiology, e.g. cell proliferation, differentiation, migration and oncogenic transformation [Hunter, 2009]. The phosphorylation process modifies target proteins causing conformational changes, inducing relocation within a cell or exposing new binding sites for ligands [Stoker, 2005]. The reversible phosphorylation of proteins at specific tyrosine residues is regulated by delicate balance between the antagonistic activities of protein tyrosine phosphatases and protein tyrosine kinases [den Hertog et al., 2005]. Protein tyrosine kinases (PTKs) are responsible for the phosphorylation reaction as opposed to protein phosphatases which remove the phosphate groups (Figure 2). This balanced and opposing action is of critical importance for maintaining homeostasis, and any disturbance may contribute to disease development including cancer, diabetes mellitus and immune deficiency [Neel, Tonks, 1997]. In the recent decades, PTPs have been believed to play mostly a housekeeping role in the cell, together with PTKs regulating tyrosine phosphorylation. Now, several studies have changed this perception and put PTPs in a new perspective as partner enzymes equally contributing to the regulation of reversible protein phosphorylation [Soulsby, Bennet, 2009]. Protein tyrosine phosphatases regulate signaling pathways involved in numerous processes including cell-substrate adhesion, cell-cell adhesion, and insulin signaling [Stoker, 2005].

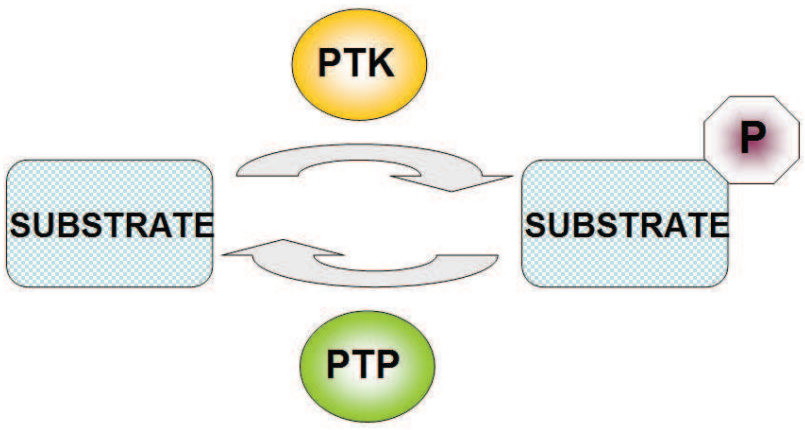


Figure 2. The reversible phosphorylation - opposing activity of PTPs and PTKs.

1.2. ROLES OF PTPS IN PATHOLOGICAL PROCESSES

1.2.1. Tumorigenesis

Diregulation of tyrosine phosphorylation may lead to cancer development characterized by abnormal replication, growth or metastatic potential [Hunter, 1995]. Multiple protein tyrosine kinases have been identified as proto-oncogenes; their enzymatic counterparts - phosphatases were believed to be tumor suppressors [Luo et al., 2008]. There is evidence that PTPs can function as tumor suppressors (Figure 3) [Scott, Wang, 2011], but some PTPs upregulating certain growth-factor receptors, display some oncogenic potential [Ostman et al., 2006].

PTP	Tumor suppressive function
LAR	mutations identified in colon cancer
DEP1	overexpression reverts the transformed phenotype of i.a. colon cancer cell
SHP1	is inactivated in leukaemias, lymphomas and multiple myeloma
GLEPP1	is inactivated in lung and colorectal cancer; re-expression is associated with tumour suppression

Figure 3. Tumor suppression activity of selected PTPs

The role of PTP enzyme family members in development and progression of tumors has been presented in many studies. PTPs has been proven to be implicated, among others, in gliomagenesis [Navis et al., 2010], breast cancer [Aceto, Bentires-Alj, 2012], colon cancer [Wang et al., 2004] or multiple myeloma (CD45 phosphatase) [Bataille et al., 2003; Dawes, et al., 2006]. The involvement of PTPs in tumorigenesis might be partly explained by the increased generation of reactive oxygen species in cancer cells as compared to normal cells, which can lead to pathologically enhanced oxidation of PTPs [Liou, Storz, 2010].

1.2.2. Protein tyrosine phosphatases and pathogenic microorganisms

Many protein tyrosine phosphatases were found to play important roles in controlling physiology and pathogenicity of various microorganisms. The PTPs are often exploited by pathogenic viruses and bacteria to cause infection, or proliferate in a host cell. The bacterial infection process involves the modification of the host signaling pathways by certain effectors, some of which are protein tyrosine phosphatases [Cozzone et al., 2004].

A few structures of viral PTPs have been successfully determined in *Vaccinia* virus, *Baculovirus* and *Variola* virus. Protein tyrosine phosphatase from *Variola* virus seems to act as a causative agent in smallpox being essential for the virus viability [Koksal et al., 2009].

Numerous protein tyrosine phosphatases have been reported in bacteria, and except for differences in the primary structure and electrostatic surface charge, protein folding pattern resembles that of mammalian phosphatases. The bacterial enzymes are mainly virulence effectors causing disregulation of cellular functions (*Yersinia pestis*, *Yersinia enterocolitica*) or cytoskeletal rearrangements in the host cell (*Salmonella enterica*). *Yersinia* species utilize type III secretion system for translocation being essential for virulence effects on the host cell, like inhibiting the innate immune response and inducing infection. Protein tyrosine phosphatase YopH plays a role of a bacterial agency, controlling signaling pathways required for phagocytosis [Bohmer et al., 2012]. The YopH phosphatase is one of the most active protein tyrosine phosphatases characterized to date, and it is speculated that YopH gene might have a eukaryotic origin [Trosky et al., 2008].

1.2.3. Implication of PTPs in human disease

Disfunction of protein tyrosine phosphatases contribute to the development of human diseases (Figure 4), including autoimmunity disorders, diabetes mellitus, cardiovascular and neurological pathology [Ferreira et al., 2006]. Currently research on pathophysiological roles of protein tyrosine phosphatases has focused on transgenic or knockout mice studies, best highlighted by the discovery of the role of protein tyrosine phosphatase 1B in type II diabetes, and obesity. It has been found that mice lacking PTP1B phosphatase are healthy, and have enhanced sensitivity to insulin. Moreover, mice with PTP1B deletion are lean and protected from diet-induced obesity [Klaman et al., 2000].

PTPs involved	Diseases
CD45	SCID, multiple sclerosis, Alzheimer'sdisease
PTP1B	diabetes, obesity
LAR	neurological diseases, diabetes
SHP1	immune, neurological disease
SHP2	neurological, infectious diseases
GLEPP1	osteoporosis
PTPε	osteoporosis, immune diseases
PTP MEG2	autism

Figure 4. Involvment of selected PTPs in human disease.

Protein tyrosine phosphatases are crucial for immune functions and perturbation of their activity may lead to development of autoimmune disorders including multiple sclerosis (SM) [Tchilian et al., 2001]. Mutations in protein tyrosine phosphatases encoding genes may be the main cause of malformation syndromes, e.g. severe combined immunodeficiency (SCID), Noonan syndrome (NS) - autosomal dominant congenital disorder, and juvenile acute myeloid leukemia [Kung et al., 2000; Zheng et al., 2009]. The key role of PTPs in the immune response is underscored by the fact that there is a higher level of PTPs genes expressed in the immune system cell than in any other cell [Mustelin et al., 2005].

1.2.4. PTPs as therapeutical targets

Protein tyrosine phosphatases are qualified as potential pharmacological targets for drug development, based on the critical roles they play in the regulation of different cellular signaling pathways in numerous pathologies including cancer [Zhang, 2001]. Some diseases are exacerbated by protein tyrosine phosphatase activity, and that is why PTPs modulation might play a significant role in the treatment of certain diseases. Such treatment has been already applied to alleviate osteoporosis symptoms via inhibition of protein tyrosine phosphatase ε. PTP-ε controls development of osteoclasts implicated in bone resorption [Bialy, Waldmann, 2005]. Protein tyrosine phosphatase CD45 is a key pharmacological target

in allergic reactions, neurological disorders and may be a new approach for Alzheimer's disease medication [Tan et al., 2000]. Because of PTP1B implication in diabetes mellitus and obesity, PTP1B has become a major target for pharmacological modulation in the treatment of these pathologies.

Because of the role of PTPs in the viability and pathogenic virulence, they can be new candidates in treatment of infections diseases, and prevention of bioterrorism (*Yersinia pestis*) [Bohmer et al., 2012]. The use of therapeutic agents causing PTPs inhibition is limited by a few major concerns. The main problem is that a single phosphatase may regulate several signal transduction pathways, and thus inhibiting its activity will result in multiple side effects [Zhang, 2001]. In the future, the studies oriented on deepening our understanding of the molecular mechanisms of PTPs regulation and their roles in human pathology, could help design protein tyrosine phosphatase inhibitors of potential clinical importance [Alonso et al., 2004].

1.3. PROTEIN TYROSINE PHOSPHATASES INHIBITORS

Based on essential roles of protein tyrosine phosphatases in numerous signal transduction pathways and their implication in pathology, there is increasing interest in identification of novel PTPs inhibitors. In general, tyrosine phosphatase inhibitors are mainly inorganic compounds, like sodium orthovanadate, nitric oxide and phenyl arsine oxide. The main disadvantage of these compounds is that they are not selective enough, and effective at relatively high concentrations. There have been many studies on novel PTPs inhibitors, more potent and selective than the already known inhibitors, but the major problem here, is the high sequence homology of the catalytic center shared by different PTPs [Bialy, Waldmann, 2005]. In the literature there are many reports on natural compounds that inhibit different types of enzymes, including protein tyrosine phosphatases. Natural PTPs inhibitors are extracted form plants, algae or some microorganisms. For example, the natural compound dephostatin being a competitive PTPs inhibitor at a micromolar concentration, was isolated from *Streptomyces* spp [Ferreira et al., 2006]. Another inhibitor, 4-isoavenaciolide, was isolated from a fungal strain. There are numerous compounds derived from fruits presenting inhibitory properties against PTPs, such as nornuciferine from *Annona muricata* or karanjin from *Pongamia pinnata*. However, the therapeutic usefulness of natural compounds is limited, because of their low stability and selectivity, but again it might be a good starting point for development of more effective synthetic analogs [Bialy, Waldamann, 2005].

Many studies, involving NMR-based screening or molecular modeling, have been focused on development of compounds that inactivate protein tyrosine phosphatases by structurally mimicking phosphotyrosine as the natural substrate. The posphotyrosyl group was replaced by the mimetic structures like sulfotyrosyl, thiophosphotyrosyl or phosphonomethylphenylalanine [Jenkins et al., 2002]. The phosphotyrosine mimicing PTPs inhibitors display a high inhibitory activity, but their permeability through the cell membrane is much limited.

The identification of a novel binding site of PTPs located about 20 angstroms away from the catalytic center, which is less conserved among phosphatases, constitutes a new paradigm for a potential inhibitor design. Such compounds may bind to both the primary catalytic center, and the secondary binding site inducing allosteric inhibition [Barr et al., 2009].

The vast majority of described PTP inhibitors do not display drug-like properties, with low cell permeability, selectivity and pharmacological activity [He et al., 2012].

1.4. CLASSIFICATION OF PROTEIN TYROSINE PHOSPHATASES

According to the primary structure of the catalytic domain, protein tyrosine phosphatases fall into four categories (Figure 5). Class I of PTPs, based on their substrate specificity, is comprised of 38 classical tyrosine phosphatases (strictly tyrosine specific) and 61 dual-specificity phosphatases (in regard to tyrosine and serine/threonine or tyrosine and threonine) [Hendriks et al., 2008].

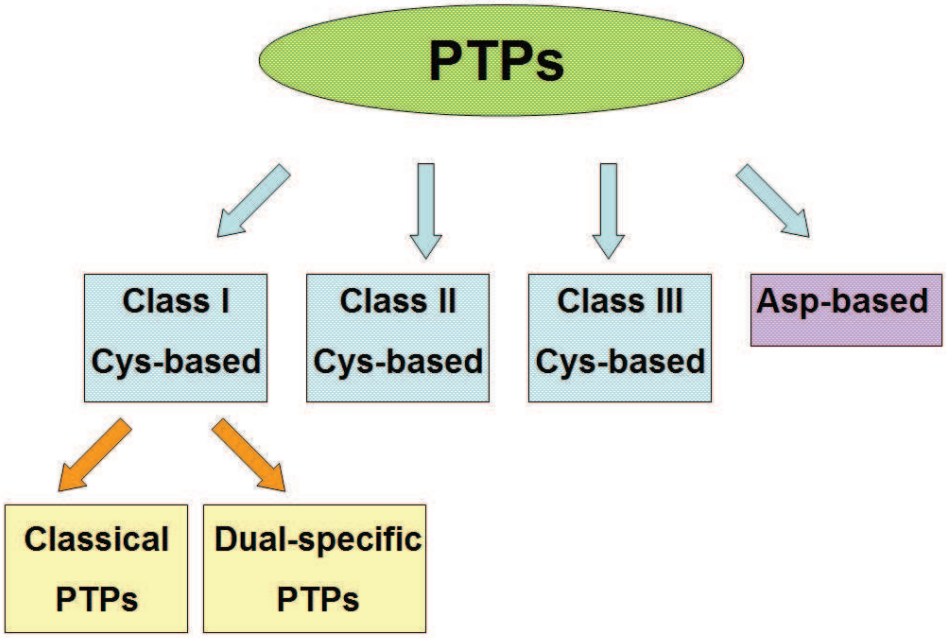


Figure 5. Classification of PTPs.

Class II is represented in human genome by only one gene encoding a relatively small (18 kDa) tyrosine-specific low molecular weight phosphatase (LMWPTP). Three tyrosine-threonine phosphatases, which play a role of the cell cycle regulator, belong to Class III [Alonso et al., 2004]. Class IV comprises four tyrosine and serine-tyrosine phosphatases, that differ from the other ones with respect to catalytically active amino acid residue being aspartic acid instead of the most commonplace cysteine residue typical for PTPs of Class I, II and III [Rayapureddi et al., 2003].

1.4.1. Receptor and non-receptor PTPs

Based on cellular localization, the classical PTPs are categorized as transmembrane (receptor-like PTPs) and intracellular (cytosolic) phosphatases (non-receptor PTPs) (Figure 6). Cytosolic PTPs are localized in a variety of intracellular compartments, such as cytosol, plasma membrane or endoplasmic reticulum, while receptor-like PTPs are predominantly found in the plasma membrane [Andersen et al., 2001].

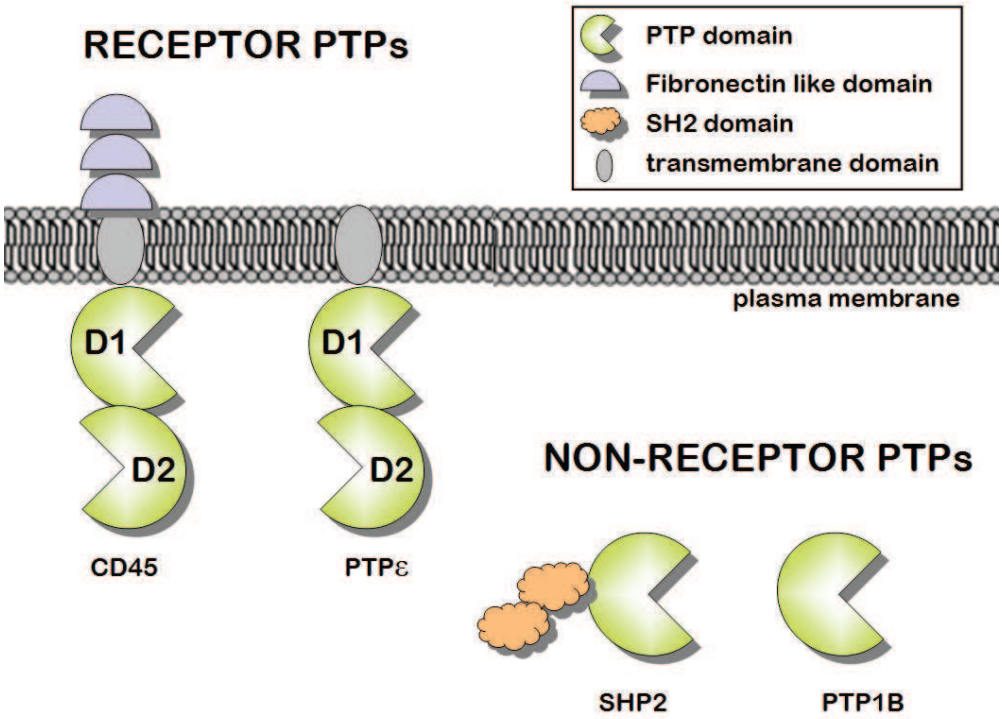


Figure 6. Schematic illustration of representative receptor and none-receptor PTPs.

Every non-receptor tyrosine phosphatase contains a single catalytic domain connected to variable sequences modulating the activity and intracellular localization of the enzyme [Takakura et al., 1999]. The structure of receptor PTPs is perfectly designed to transduce signal across the plasma membrane. The majority of receptor protein tyrosine phosphatases have an extracellular region, a single transmembrane domain and two intracellular conserved PTP domains (D1 and D2) [Tabernero et al., 2008]. The catalytic activity has been reported only in D1 domain being the closest to the cell membrane PTP domain. The other PTP domain, i.e. D2 has been proposed to play only regulatory roles. Although D2 domain manifests no catalytic activity, it is still necessary for maintaining specificity and stability of the enzyme [Stoker, 2005; Persson et al., 2004].

1.5. PROTEIN TYROSINE PHOSPHATASE CD45

Like many other receptor PTPs, CD45 consists of a transmembrane domain, extracellular flanking region and intracellular tandem domains. The catalytic activity is observed only in the PTP domain that is directly linked to the cell membrane. The extracellular region of CD45 varies among vertebrates, but the intracellular region is conserved in different species, be it

a shark, mouse or human [Nam et al., 2005].

CD45 is a sort of prototype member of the transmembrane receptor PTPs, and plays a crucial role in the immune reaction processes. Phosphatase CD45, also known as the leukocyte common antigen, is important for development and antigen-induced activation of T and B white blood cells [Holmes, 2005].

B cell as well as T cell antigen receptor signaling is dependent on Src family kinases. CD45 plays a critical role in activating the Src family kinases by removing the inhibitory phosphate group from the tyrosine residue of Lck kinase (in T cells) and Lyn/Fyn/Lck (in B cells) [Hermiston et al., 2009]. Increased expression of the CD45 phosphatase leads to a positive regulation of Src family kinases activity in both T and B cells (Figure 7). However, in T cells CD45 may also negatively regulate the T cell specific Src family kinase (Lck) via dephosphorylation of the tyrosine residue in the activation loop [Zikherman et al., 2012].

CD45 phosphatase may regulate cytokine production as well as response to cytokines. It regulates cytokine and chemokine formation by NK cells, IL-6 in neutrophils and histamine degranulation in mast cells. CD45 can dephosphorylate JAK (Janus kinase), therefore negatively or positively regulating cytokine receptor signaling through activation of STAT (signal tranducer and activator of transcription) pathway [Tchilian, Beverley, 2006; Huntington, Tarlington, 2004].

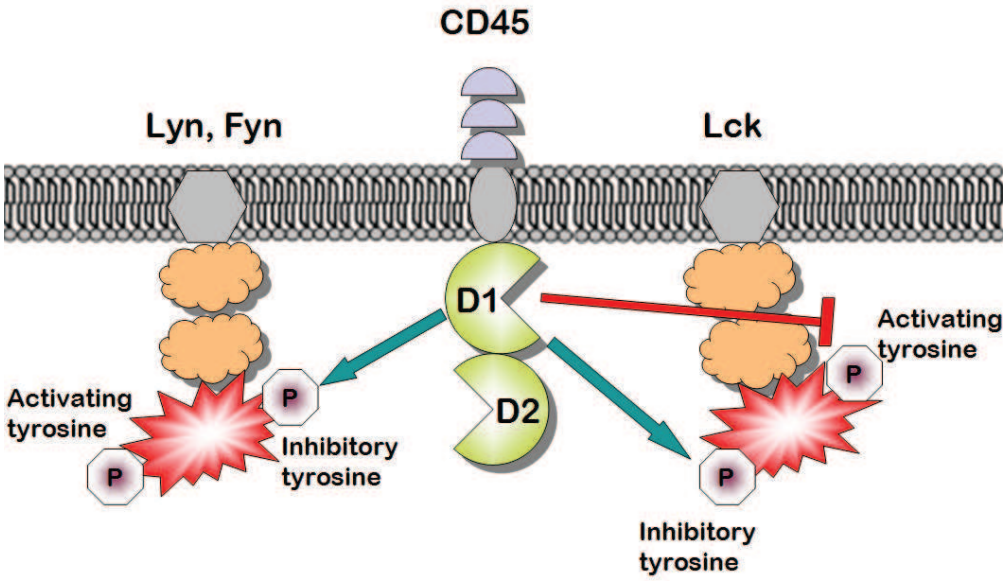


Figure 7. Regulation of Src family kinases via dephosphorylation by CD45.

Protein tyrosine phosphatase CD45 is abundantly expressed in leukocytes, however the similar level of expression has been reported in pancreatic acinar cells, where CD45 can operate as a negative controller of pro-inflammatory cytokine production due to suppression of JAK kinases [Dios et al., 2005]. It has been found that CD45 expression is down-regulated in pancreatic acinar cells during acute pancreatitis, which suggest its potential role in pathogenesis of the disease [Dios et al., 2006].

1.5.1. Isoforms of CD45

CD45 is expressed in multiple isoforms (from 180 kDa up to 220 kDa) as a result of alternative splicing of variable exons, which changes the amino-terminal region of ~200 amino acids (Figure 8). The extracellular CD45 domain in each isoform has different architecture. The same isoforms can be expressed in different cell types [Holmes, 2005].

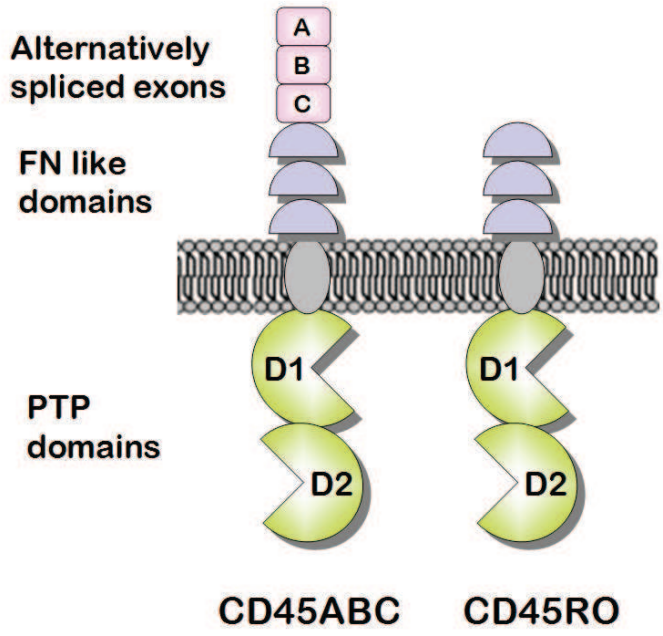


Figure 8. Structure of CD45 the largest splice variant CD45ABC, and the smallest CD45RO.

Any disturbance in the expression of CD45 splice variants may potentially cause autoimmune disorders, multiple sclerosis, Alzheimer's disease, and is implicated in the rejection of transplant organs [Dawes et al., 2006]. Modulation of the activity of various CD45 isoforms may be a novel approach to treatment of Alzheimer's disease (due to inhibition of β-amyloid peptide-induced microglial activation) [Sasaki et al., 2001], and has been proven effective in

the prevention of transplant organs rejection [Lazarovits et al., 1996] or graft-versus-host disease.

1.6. STRUCTURE OF PTPS

An impressive number of three-dimensional structures of PTPs has been determined by X-ray analysis of the obtained crystal structures. The numerous enzymology studies, and comparison of 3D structures with amino acid sequences of PTP family members, have provided valuable information regarding evolutionary conservation, intrafamily PTP diversity, substrate recognition, catalytic activity and other characteristic features. The superimposition analysis of all known structures of D1 and D2 domain of classical protein tyrosine phosphatases showed that they have a highly conserved topology, and the single domain is folded with β sheets surrounded by α helices. The sequence comparison between the human PTP domains allowed to identify the conserved residues and motifs, and particularly the highly conserved active site sequence VHCSXGXGR (Figure 9) [Andersen et al., 2001].

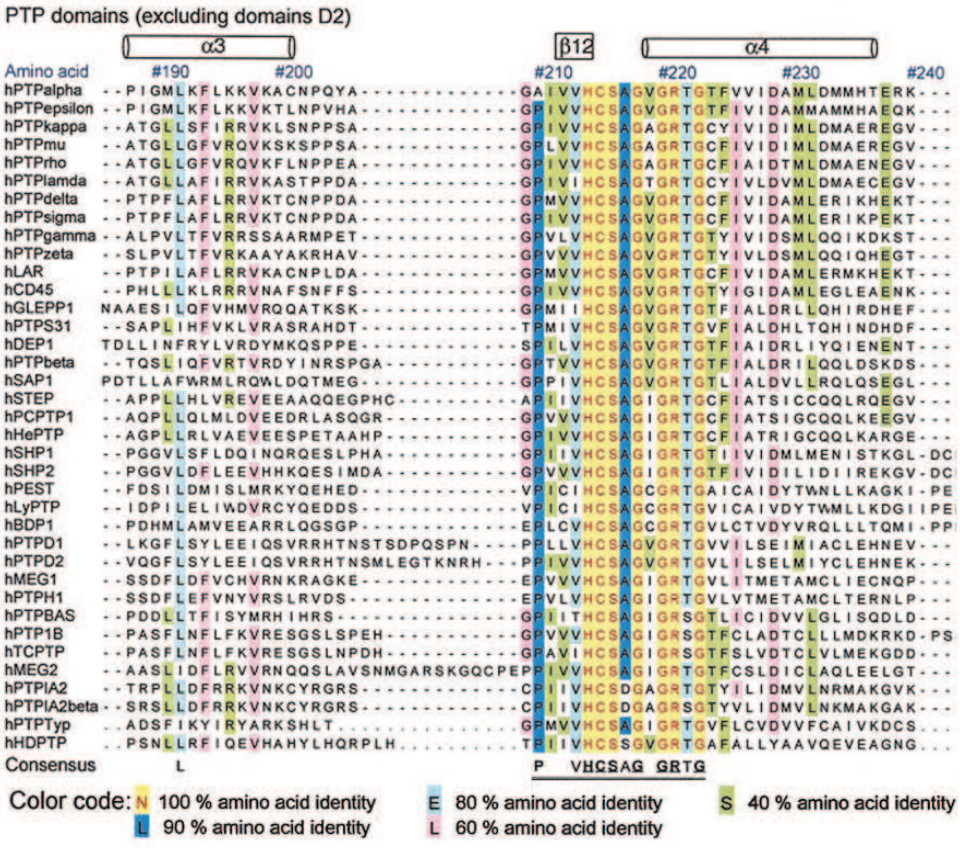


Figure 9. Amino acid sequence comparison of 37 human PTP catalytic domains within the active site (based on Andersen et al., 2001).

In contrast to the similarity of structural architecture, PTPs differ in the electrostatic surface potential, which may result in the observed variation of substrate specificity. The mapping studies of PTPs surface determined only a few conserved surface patches in the vicinity of the active site. The active site of PTPs is highly positively charged (Figure 10) [He et al., 2012].

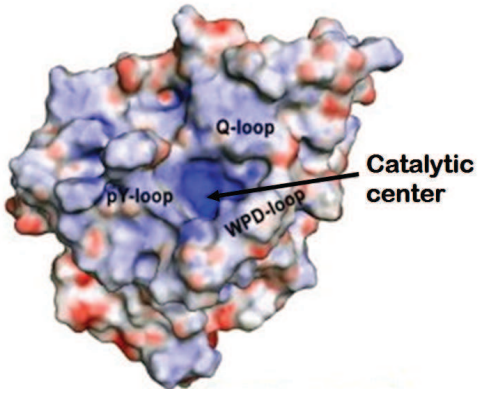


Figure 10. The electrostatic potential (blue - positive, red – negative) at the surface of PTP1B phosphatase (based on He et al., 2012).

1.6.1. PTPs catalytic center and cysteine residue

The catalytic domain of PTPs consists of about 280 amino acids with several characteristic motifs. The key structural features of the catalytic PTP domain pertain to the most conserved regions and include phosphate binding loop (PTP loop), WPD loop, catalytic water motif (Q loop) and the phosphotyrosine recognition loop (Figure 11) [Barr et al., 2009]. The active center of classical PTPs is located in a crevice at the protein surface; the size of the groove is related to the high selectivity of substrates. Howewer, a considerable plasticity of active site pocket of PTPs has been reported indicating that compounds significantly larger than phosphotyrosine may interact inside the catalytic center [He et al., 2012].

The signature motif VHCSXGXGR is located at the bottom of the catalytic center pocket, and forms a phosphate binding loop (PTP loop) [Bialy, Waldmann, 2005]. The PTP loop contains the cysteine and arginine residues, both of them being essential for enzymic activity. The highly conserved cysteine residue in classical PTPs is directly implicated in the removal of phosphate from phosphoproteins. Mutation of the active site cysteine residue results in a catalytically inactive enzyme [Barrett et al., 2005].

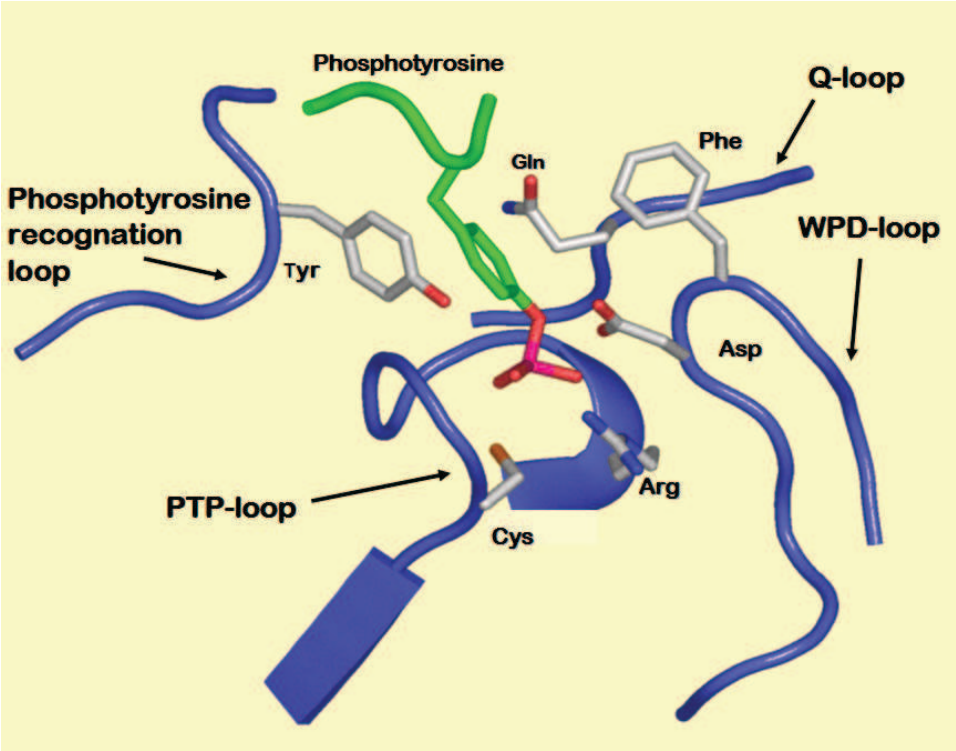


Figure 11. The architecture of the PTPs catalytic center based on PTP1B phosphatase.

A catalytically important structural element of the catalytic center is the phosphotyrosine recognition loop with the conserved amino acids residues KNRY (Lys, Tyr). This element determines the depth of the active site crevice and induces interactions between tyrosine residue and the substrate phosphotyrosine [Andersen et al., 2001].

Another key feature of the catalytic PTP domain is WPD loop that can change its conformation from "open" to "closed" position. The binding of a substrate to the PTP loop induces conformational changes moving the surrounding structure by several angstroms to close the catalytic center, and thus locking the phosphotyrosine on the inside of the active site pocket. The WPD loop movement may be induced also with small ligans, such as sulfate or phosphate. The WPD loop mobility form the open to closed position, is involved in substrate recognition, and determines catalytic efficiency. The studies of the WPD loop transition dynamics are important for drug designing attempts, and create a possibility to produce a selective inhibitor which might recognize the selected loop conformation [Barr et al., 2009].

An essential structural component of PTP active site is the catalytic water motif (Q loop) with the highly conserved glutamine residue. The glutamine residue is responsible for positioning a water molecule that is required for hydrolysis of the phosphoenzyme intermediate which being formed during the catalysis process [Tabernero et al., 2008].

1.6.2. Catalytic mechanism

The mechanistic studies of numerous PTPs, X-ray crystallography and directed site mutagenesis, suggest that all PTPs share the common catalytic mechanism [Zhang, 2001].

The catalysis proceeds in a two-step manner (Figure 12). In the first phase, the thiolate ion of the catalytic cysteine residue performs nucleophilic attack on the substrate phosphate, and simultaneous protonation of the tyrosyl-leaving group of the substrate by the side chain of the conserved aspartic acid residue acting as a "general acid". The proton donation is a critical step of the catalysis process (Figure 12 A). The phosphoryl group of the substrate is thus transferred toward the nucleophilic cysteine, resulting in the formation of phosphoenzyme intermediate. The catalytic cysteine residue of the phosphoenzyme intermediate is localized right underneath the phosphoryl group. The arginine residue in the signature motif facilitates the substrate binding process, and helps stabilize the intermediate via ionic interactions [Tonks, 2003].

In the second step, the phosphoenzyme intermediate is hydrolyzed with a water molecule, leading to the dissociation of an inorganic phosphate and regeneration of the enzyme (Figure 12 B). In the second phase of the catalysis process, the aspartic acid residue of the WPD loop, extracts proton from the water molecule acting as a "general base" [Li, Dixon, 2000].

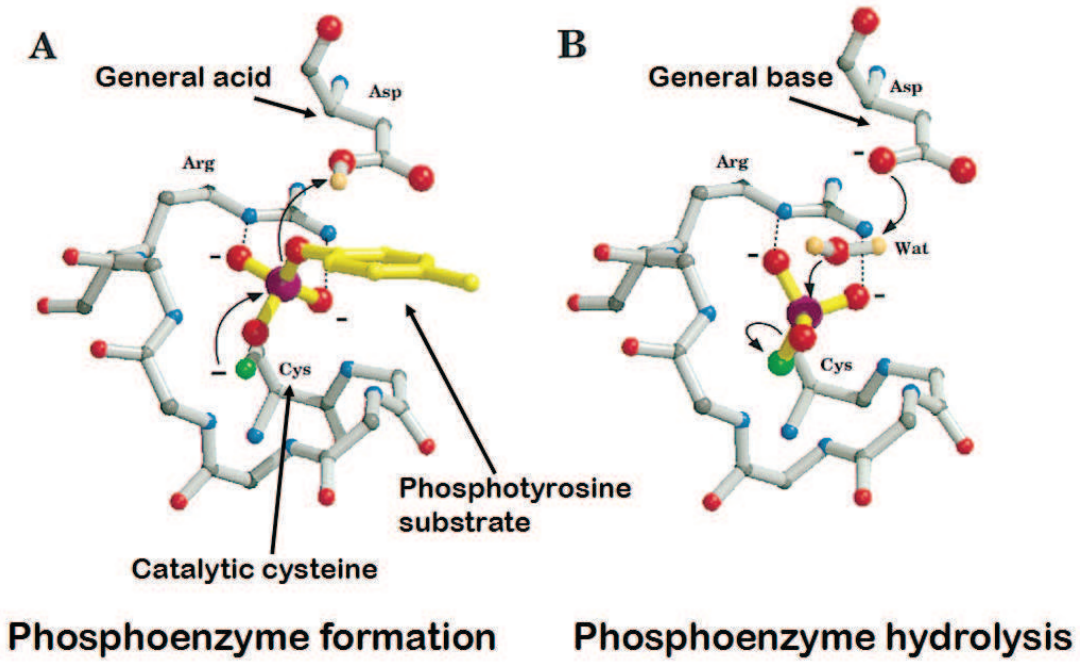


Figure 12. The catalytic mechanism of PTPs (based on Li, Dixon, 2012).

The signature motif forms the base of the active-site cleft and recognizes the phosphate of the target substrate. The catalytic cysteine residue playing a role of a nucleophile is localized underneath the phosphoryl group in the vicinity of arginine residue being involved in phosphate binding. The positively charged arginine attracts the negatively charged phosphate phosphotyrosine residue. The phosphate group is then oriented and stabilized by numerous hydrogen bonds with -NHx groups of the surrounding amino acid residues. The two amino acids form a cradle-like conformation, which is responsible for the nucleophilic attack on a substrate phosphotyrosine [van Montfort et al., 2003]. The catalytically essential aspartic acid residue is localized on a flexible WPD loop within of 30-40 amino-acid distance from the catalytic cysteine residue, and is protonated. A substrate binding to the active center induces conformational changes of the WPD loop, bringing it over to the active pocket, and stabilizing substrate binding process. This translocation moves the aspartic acid residue closer to the substrate, where it plays the role of a general acid. The aspartic acid residue, in the second step of catalysis, enhances hydrolysis of the phosphoenzyme intermediate [Bialy, Waldmann, 2005].

The recent structural analysis unexpectedly identified a novel binding site, only 20 angstroms away from the catalytic center of PTPs. The presence of the secondary binding pocket has been found, e.g. in PTP1B, SHP1, SHP2, DEP1 or GLEPP1 phosphatase [Villa et al., 2005].

1.7. REGULATION OF PTPS FUNCTION

Protein tyrosine phosphatases are regulated by gene expression and numerous post-translational modifications including proteolysis, phosphorylation and oxidation. In addition to that, another mechanism of receptor PTPs control of was discovered that is based on ligand binding-induced dimerization [Tonks, 2006]. The activity of CD45 phosphatase can be also modulated through changes in the selected isoform expression, indirectly affecting glycosylation and dimerization of the extracellular domain [Xu, Weiss, 2002].

Proteolytic cleavege is a common post-translational modification that controls RPTPs activity (Figure 13). It can result in extracellular domain shedding (Figure 13A), leading to RPTPs signaling termination, or facilitating internalization of the intracellular domain. Proteolysis may lead to disconnection of the catalytic domain interacting with a new ligand (Figure 13B) [Stoker, 2005]. Non-receptor protein tyrosine phosphatases, such as PTP1B, can be a subject to intracellular cleavage that occurs in calpain-dependent manner and results in the release of

PTP catalytic domain into the cytoplasm. Intracellular calpain-induced cleavage may induce activation of non-transmembrane phosphatases [Gil-Henn et al., 2001].

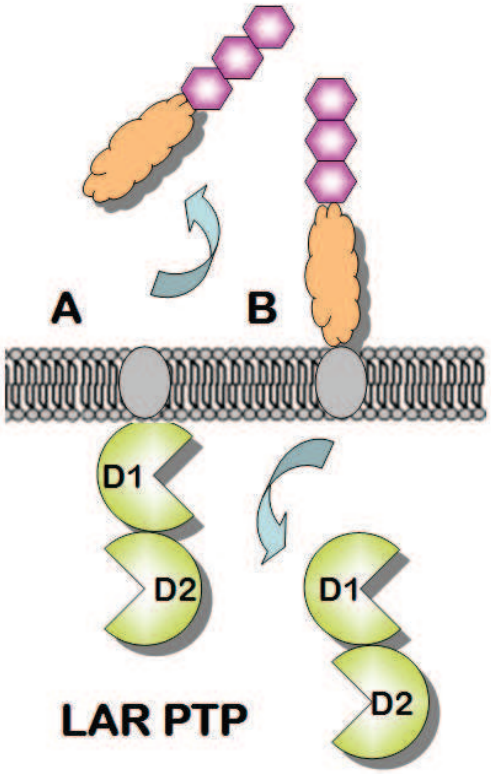


Figure 13. Regulation of RPTP by proteolytic cleavage.

Phosphorylation is another key regulatory mechanism controlling PTPs activity. It has been reported that increase of PTP1B activity may be the result of tyrosyl phosphorylation [Dadke et al., 2001]. Tyrosyl phosphorylation of SHP-2 and RPTPα provides binding sites for ligand docking, and facilitates the formation of signaling complexes [Soulsby, Bennett, 2009].

1.7.1. Dimerization

Receptor protein tyrosine phosphatases, e.g. CD45, were found to constitutively form dimers in the cell membrane. The activity of these enzymes is dependent on the relative orientation of the two monomers in the dimer and may be controlled by ligand-regulated dimerization (Figure 14) [van der Wijk et al., 2004].

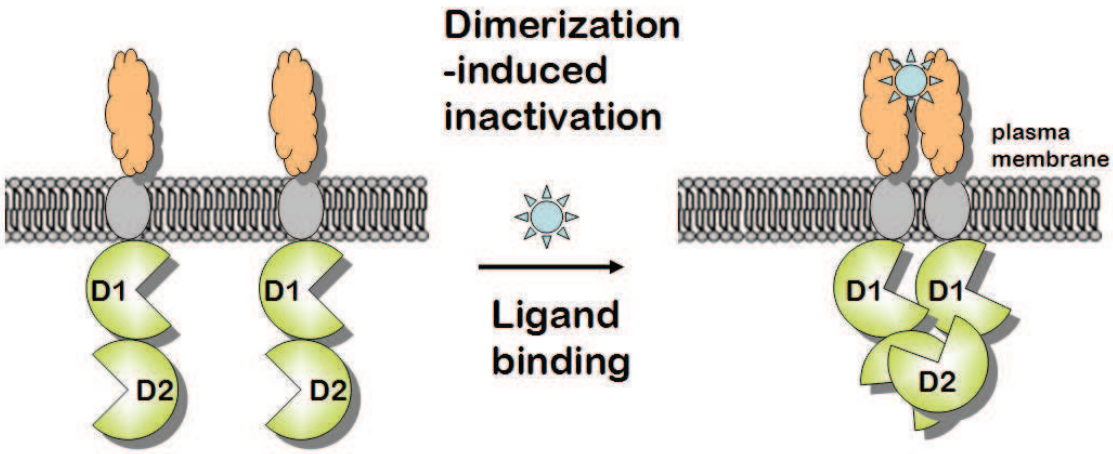


Figure 14. Regulation of RPTPs activity by dimerization.

Ligand binding has been reported to negatively regulate the RPTPs activity by inducing dimerization via blocking access to the catalytic center. In the dimeric state, there is reciprocal inhibition of the catalytic D1 PTP domains. The wedge motif in one D1 domain occludes the active site of the opposing D1 domain in the dimer [Tonks, 2006].

The dimmer formation may include homotypic interactions between catalytic D1 domains, D2 domains or heterotypic interactions between D1 and D2 domains. The dimerization is not limited to PTP domains; it may also concern extracellular domains [Bohmer et al., 2012]. However dimerization may not be a universal regulatory mechanism for every member of RPTP family of enzymes, and further studies are needed to explain whether this process may occur independently, or whether it requires the presence of a ligand [Stoker, 2005].

1.7.2. Oxidative stress related regulation of PTPs

Reactive oxygen species (ROS) control the function of cellular proteins due to regulation of post-translational modifications of those proteins. According to their location in the cell, ROS are generated extracellularly or in various intracellular compartments.

Many enzymes, including those of the mitochondrial electron transport chain, oxidases, oxygenases, peroxidases, produce reactive oxygen species. There are many studies on oxidative stress and its impact on pathogenesis. Oxidative stress is associated with many

pathologies such as cancer, neurodegeneration and Alzheimer's disease [Griendling, Fitzgerald, 2003].

Oxidative stress, defined as excessive reactive oxygen species formation, may induce inactivation of protein tyrosine phosphatases. Inactivation via oxidation was suggested as a mechanism of protein tyrosine phosphatases regulation [Persson et al., 2004]. A unique biochemical and structural characteristic of the PTPs catalytic cysteine engendered a hypothesis that these enzymes might be direct targets of ROS chemistry. Many PTPs have been shown to be oxidized transiently in response to various cellular stimuli. Reactive oxygen species, such as hydrogen peroxide, function as second messengers in response to extracellular stimuli and can regulate tyrosine phosphorylation-mediated signaling pathways (Figure 15) [Finkel, 2003].

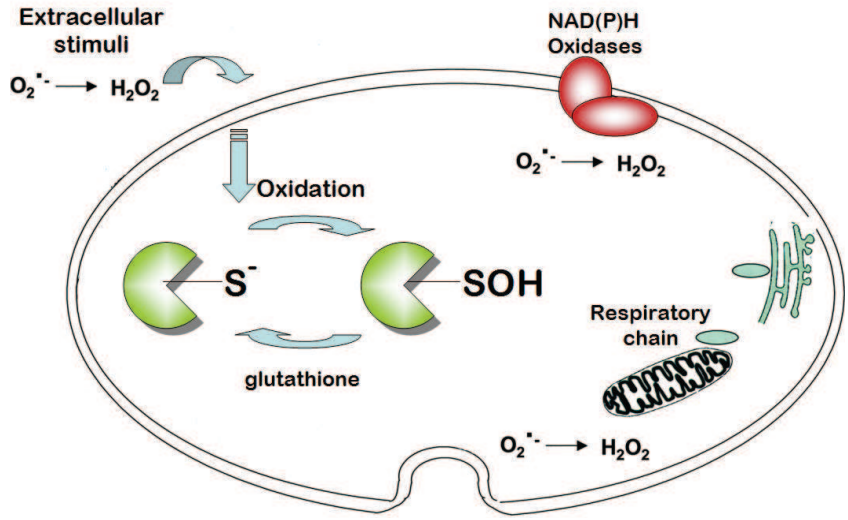


Figure 15. Hydrogen peroxide may oxidize the catalytic cysteine residues in PTPs.

Hydrogen peroxide may relatively easily cross the cell membrane in response to insulin or epidermal growth factor, and control the cellular activity of protein tyrosine phosphatases therein [Rhee et al., 2000]. Hydrogen peroxide may oxidize catalytic cysteine residue to sulfenic acid, which can be reversibly reduced to cysteine by various cellular reducing agents [Goldstein et al., 2005].

1.7.3. Reversible and irreversible oxidation of the catalytic cysteine

The essential cysteine residue in the catalytic center of PTPs exists in the form of a thiolate anion at neutral pH, and thanks to the specific amino acid microenvironment, has a relatively

extremely low pK_a. These properties allow the cysteine residue to function as a nucleophile in catalytic process, but at the same time make it highly vulnerable to oxidation [Chiarugi, Cirri, 2003]. Depending on the degree of oxidation, the catalytic cysteine residue can be converted to either sulfenic (SOH), sufhinic (SO_2H) or sulfonic (SO_3H) acid residue (Figure 16) [Tonks, 2006].

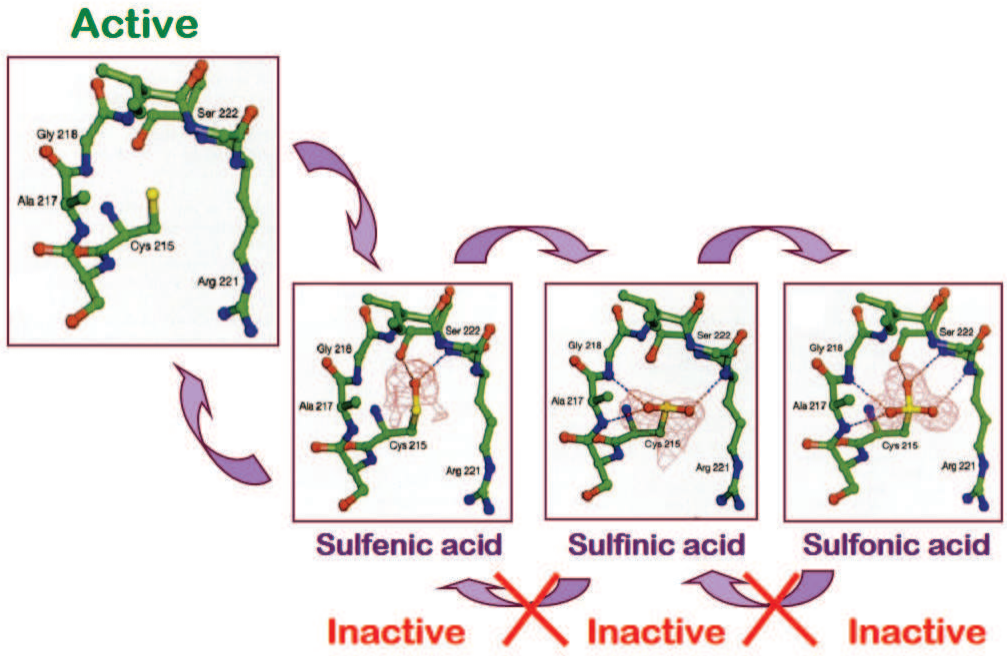


Figure 16. Reversible and irreversible oxidation of PTPs catalytic cysteine (based on von Montfort et al., 2003).

Oxidation of the cysteine residue to sulfenic acid is reversible, unlike further oxidation to sulfinic and sulfonic acid residues, which are considered irreversible. The sulfenic acid form can undergo reduction do cysteine residue via formation of a sulfenylamide intermediate [den Hertog et al., 2005]. Sulfenylamide generation induces major conformational changes in the catalytic center of the enzyme. It protects cysteine residue from irreversible oxidation to sulfinic or sulfonic acid, and facilitates enzyme reactivation. Sulfenylamide can be converted in cell by thioredoxins and glutathione into the active form of thiolate anion [Salmeen et al., 2003].

Nowadays, it has been proposed, that in some cases, conversion to sulfinic acid may be reversible. Sulfiredoxin from *Saccharomyces cerevisiae* has been reported to reduce sulfinic acid residue of peroxyredoxin Tsa1 in the presence of magnesium and ATP [Biteau et al.,

2003]. Both peroxyredoxin and protein tyrosine phosphatases contain a reactive cysteine residue in the catalytic center [Woo et al., 2003].

A reversible oxidation is considered as a novel mechanism of PTPs regulation, also observed in other proteins with the active-site cysteine, e.g. caspases, kinases, phosphatases or proteases [Krejsa, Schieven, 1998]. Reversible chemical modifications of the active site cysteine residue play a role in various physiological processes, like apoptosis, angiogenesis, cell proliferation, and receptor-mediated signaling. The reactive thiols are oxidized to either sulfenic acid residues or disulfides. Thiols are essential components of the redox chemistry in biological systems, and the reversible oxidation-reduction reactions provide a sort of an "on-off" switch in the process of metabolism regulation [Jones, 2008].

1.8. PERACIDS – A NOVEL CLASS OF PTPS INHIBITORS

Peracids are a class of chemical compounds belonging to organic peroxides characterized by the presence of the peroxy group (-O-O- bond). The organic peroxides are derivatives of hydrogen peroxide, in which the hydrogen atom is replaced with an organic group. The vast majority of peroxides are highly reactive compounds. The stability of organic peroxides increases with higher carbon content (longer hydrocarbon chain) [Klenk et al., 2012].

Peracids in their crystalline form associate into dimmers, whereas in solution they are likely to exist in a monomeric form. The short-chain peracids are soluble in water, whereas long-chain (> C6) peracids are increasingly less soluble in water, and more soluble in organic solvents [Patai, 1983]. Peracids are used mainly in the synthesis of organic compounds, such as epoxides (from the conversion of alkenes) or lactones (from the conversion of cyclic ketones). Peracids readily react with amines or sulfides, which is used in detergent manufacturing and pharmaceutical industry. Some of the peracids are utilized for disinfection. Peracetic acid is a popular disinfectant in medical practice and food industry [Block, 2000].

Peracids possess one of the highest oxidation potentials of all organic peroxides, and may be potent novel inhibitors of protein tyrosine phosphatases. Based on specific properties of the catalytic cysteine (described in Chapter 1.7.3.), peracids may induce inactivation of protein tyrosine phosphatases via oxidation reaction (Figure 17). During the realization of this project, other groups of scientists and researchers have been conducting parallel studies on PTP inhibitory properties of peroxides. It has been found that peroxidized arachidonic acid may induce oxidation of PTPs [Conrad et al., 2010]. Another study has shown that peracetic acid is a potent oxidative inhibitor of PTP1B phospahatse [Bhattacharya et al., 2008].

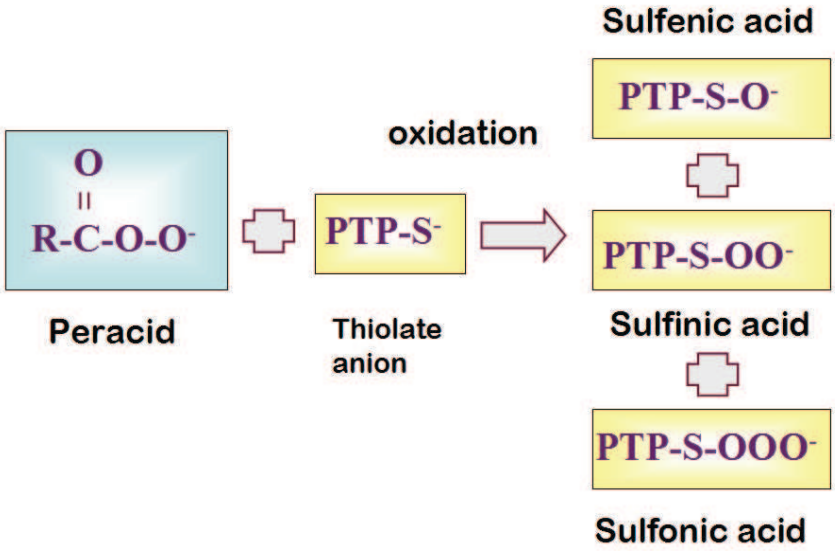


Figure 17. The hypothetical mechanizm of PTPs inactivation by peracids.

1.8.1. Peroxycarboxyl group

The peroxycarboxyl group (O=C-O-OH) in peracid substitutes the regular carboxyl group (O=C-OH) of an organic acid. In the peroxycarboxyl group a hydrogen atom is covalently bonded to the oxygen atom. The bond may be disrupted yielding an anion and proton. Peracids can also undergo decomposition to carboxylic acids, and simultaneously releasing active oxygen. The mechanism of peracids decomposition implies formation of singlet molecular oxygen. The stability of peracid aqueous solution depends on pH. Importantly, at alkaline pH peracids may undergo decomposition (Figure 18) [Klenk, 2012].

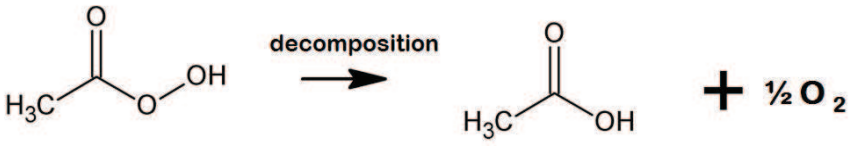


Figure 18. Decomposition of peracetic acid.

1.8.2. Peracid formation

Peracids may be formed in the reaction of hydrogen peroxide with carboxylic acids, e.g. peroxyacetic acid will be formed in the reaction of hydrogen peroxide with acetic acid

(Figure 19) [Rubio et al., 2005]. Hydrogen peroxide activation, with no metal ions involved, usuallyleads to peracid generation. In the mechanism, the activation of hydrogen peroxide caused by perhydrolysis of a carbonyl precursor [Strukul, 1992].

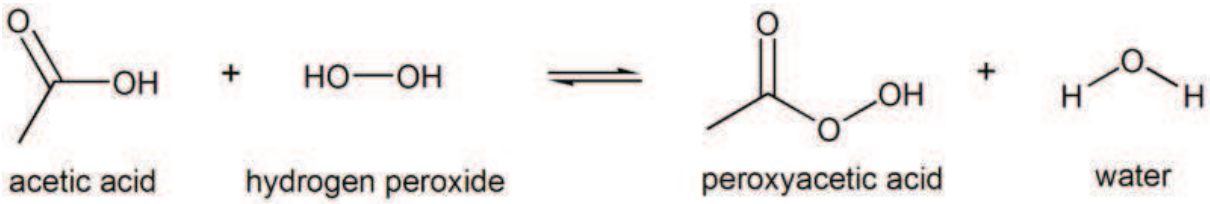


Figure 19. Peroxyacetic acid formation.

Hydrogen peroxide is naturally produced in various organisms (mitochondrial respiratory chain, oxidases, and secifically in the metabolism of white blood cells), and subsequently H_2O_2 can convert carboxylic acids (O=C-OH) into peracids (O=C-O-OH). The increased risk of peracid formation corresponds to increased production of carboxylic acids. Such increased synthesis of carboxylic acids has been reported in some liver disorders and psoriaris [Glasgow, Middleton, 2001; Khyshiktuev et al, 2008]. High amounts of free carboxylic acid, such as palmitic or stearic acids are generated in type 2 diabetes [Meng et al., 2004]. It has been shown that peracetic acid, likely formed in chronic alcoholics from acetic acid and hydrogen peroxide, may inhibit arachidonoyl-CoA: 1-palmitoyl-snglycero-3-phosphocholine acyl transferase localized in the erythrocyte membrane [Allen et al., 1991].

Because of its high reactivity, peracid is unlikely to accumulate up to a measurable amount and thus is sort of problematic to report under *in vivo* conditions.

2. AIM OF THE DOCTORAL THESIS PROJECT

The aim of the project can be focused on three main objectives:

1. Investigate the effect of hydrogen peroxide, selected peracids and their respective carboxylic acids on the enzymatic activity of protein tyrosine phosphatase CD45.

2. Explain the mechanism of protein tyrosine phophatase CD45 peracid-induced inactivation based on structural analysis and chemical reactivity of the inhibitor.

3. Discuss biological significance of potential peracid formation in the human body.

3. EXPERIMENTAL STUDY DESIGN

3.1. MATERIALS

3.1.1. Cell line

The human Jurkat T cell line, clone E.6-1, was obtained from European Collection of Cell Culture (ECACC, UK). The Jurkat cells, clone E.6-1 are human leukaemic T cell lymphoblasts (Figure 20). The Jurkat T cell line was selected for this study as CD45 being the protein tyrosine phosphatase of interest, is one of the major phosphatases in those cells, and is abundantly expressed therein.

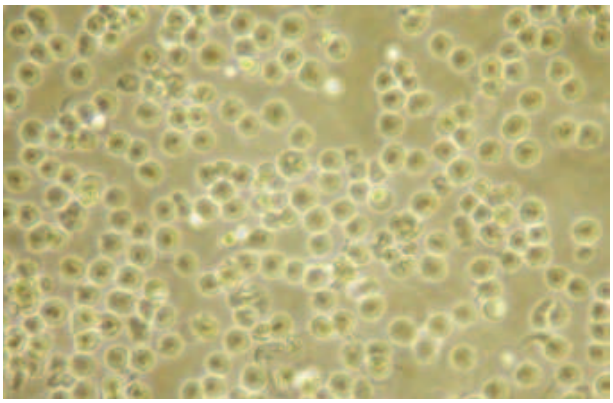


Figure 20. Jurkat T cell line, clone E6.1.

3.1.2. Reagents

Human recombinant CD45 protein tyrosine phosphatase (95-kDa molecular mass) was obtained from Calbiochem (San Diego, CA). Dithiotreitol (DTT), p-nitrophenyl phosphate (pNPP), Tris, HEPES, hydrogen peroxide and peracetic acid were obtained from Sigma-Aldrich. CD45 capture antibody and tyrosine phosphate substrate DADEY(PO_3)LIPQQG, malachite green and molybdic acid were purchased from R&D Systems. Detergent NP-40, EGTA, EDTA, NaCl, protease inhibitors (leupeptin, pepstatin, aprotinin) and phenylmethylsulfonylfluoride (PMSF), Trypan blue solution, RPMI 1640 medium and supplements were form Sigma-Aldrich.

3.1.3. Preparation of reagents

All solutions were prepared freshly right before every experiment. Tris HCl buffer was prepared with distilled water, and pH was set by adding 1M HCl with simultaneous reading on an electronic pH meter. Lysis buffer was prepared as 50 mM HEPES buffer containing 0.5% NP-40 detergent, 25 μg/mL leupeptin, 25 μg/mL pepstatin, 2 μg/mL aprotinin and 1mM PMSF, and kept on ice. The HEPES buffer pH 7.4 was prepared with distilled water and supplemented with 0.1 mM EGTA, 0.1 mM EDTA and 120 mM NaCl. The TBS solution pH 7.4 was drawn with 25 mM Tris and 150 mM NaCl in distilled water.

3.1.4. Laboratory equipment

In all of the experiments disposable pipette tips, test tubes, cell culture flasks and plates from Sarstedt were used. The cell culture plasticware was single use only and sterile packed. All the volumes were measured out with automatic pipettes and pipetus from Eppendorf. The enzymatic activity measurements of recombinant CD45 were taken in 96-well microplates with non-binding surface from Nunc in order to minimize molecular interactions between CD45 phosphatase and the surface of a plate. The enzymatic activity assessments of CD45 in cell lysates were performed in 96-well plates with high binding surface for efficient binding of antibodies.

3.2. METHODS

3.2.1. Assay of recombinant CD45 enzymatic activity

The solution of the recombinant protein tyrosine phosphatase CD45 was prepared in 50mM Tris HCl buffer, pH 7.4. The final concentration of phospahatse CD45 in reaction samples was 10 µg/mL (130 nM). The CD45 enzyme was untreated (control) or treated with selected peracids or hydrogen peroxide, and incubated for 15 minutes at 37ºC. The assay was performed in 96-well microplates, and the final volume of each sample was 100 µL. The enzymatic activity of CD45 was measured using 1mM chromogenic substrate *para*-nitrophenyl phosphate (*p*NPP) in 50mM Tris buffer, pH 7.4, at 37ºC. Phosphatase hydrolyzed *p*NPP to *para*-nitrophenol and inorganic phosphate (Figure 21). *Para*-nitrophenol is an intensely yellow colored soluble product under alkaline conditions. The increase in absorbance (due to *para*-nitrophenol formation) is linearly proportional to enzymic activity concentration (with excessive substrate, i.e. zero-order kinetics) and was assessed at 405 nm on a microplate reader Jupiter (Biogenet) using DigiRead Communication Software (Asys Hitech GmbH). The amount of phosphatase CD45 and time of incubation were optimized in order to obtain the absorbance reading of about 1.0 in the control sample. The enzymatic activity of CD45 was assessed in triplicate to correct for any nonspecific signal interference, and the same procedure was repeated without addition of enzyme to eliminate background noise. The data were affixed and analysed with GraphPad Prism (GraphPad Software v.4).

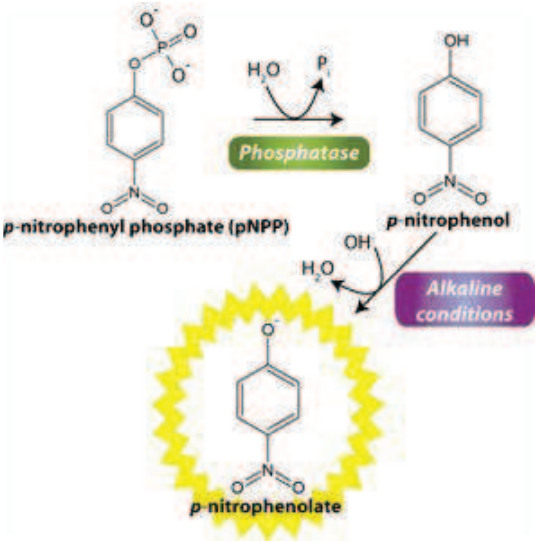


Figure 21. The formation of *p*-nitrophenol from *p*-nitrophenyl phosphate in the dephosphorylation reaction catalyzed by a phosphatase.

3.2.2. Recovery of enzymatic activity after inactivation of CD45

Subsequently, recombinant phosphatase CD45 that had been previously inactivated by a selected peracid or hydrogen peroxide, was then treated with 10mM dithiotreitol (DTT), and the samples were incubated for 15 minutes at 37ºC to reverse the inactivation, if possible. Restoration of CD45 enzymatic activity was measured as an increase of absorbance taken at 405 nm as previously described (see section 3.2.1).

3.2.3. Cell culture procedures

The cells were cultured at 37ºC in RPMI 1640 medium suppplemented with 10% fetal bovine serum, 100µg/mL penicilin/streptomycin and 2mM L-glutamine. The culture was maintained at 37ºC and in an atmosphere containing 5% CO_2. The cell culture density kept at 1×10^6 cells/mL. At least every two days the medium was replaced with the fresh one, and the cells were counted and reseeded to maintain the recommended density. The cells were cultured in the plastic tissue culture flasks with filter caps, and stored in an incubator under the conditions secified above. The experiments were performed in a microbiological safety cabinet.

3.2.4. Determination of PTP CD45 activity in cell lysate

The Jurkat T cells were untreated (control) or treated with selected peracids or hydrogen peroxide and incubated for 1 hour at 37ºC. The cells were rinsed twice with TBS, suspended at the density of 1 x 10^7 cells/mL in Lysis buffer pH 7.4 with 0.5 % NP-40, 25 µg/mL leupeptin, 25 µg/mL pepstatin, 2 µg/mL aprotinin, 1mM PMSF, vortexed briefly and placed on ice for 15 minutes. The cells were then solubilized by forcing the lysates through a 19-gauge needle (0.686 mm inner diameter) 20 times and centrifuged at 12000 x g at 4ºC for 5 minutes. The supernatants were transferred to test tubes and assayed immediately.

The day prior to the assay, the 96-well microplates were coated with CD45 capture antibodies (8 µg/mL in PBS) and incubated overnight at room temperature. After washing the wells, cell lysate was added, and the plate was placed on a rocking platform at 30 rpm for 3 hours at room temperature. Lysates were aspirated from the wells and PTP activity was measured colorimetrically using 200 µM tyrosine phosphate substrate (phosphopeptide DADEY(PO_3)LIPQQG in a 10 mM HEPES buffer pH 7.4) and malachite green. The

phosphopeptide substrate was dephosphorylated by active CD45 to generate unphosphorylated peptide and free phosphate. The free phosphate was then detected by a sensitive dye binding assay using malachite green and molybdic acid. The increase in absorbance at 620 nm was measured with the microplate reader. The activity of CD45 was determined by calculating the rate of phosphate release.

3.2.5. Concentration of protein in cell lysate

Concentration of protein in Jurkat cell lysate was measured using the Bradford colorimetric method. The Bradford method is based on Coomassie Brilliant Blue G-250 absorbance shift in the presence of protein. Binding to the protein being assayed under acidic conditions, the red dye is converted into the blue derivative. The amount of protein in the sample is proportional to the amount of bound dye, and thus to increase of an absorbance at 595 nm. Based on prepared standard concentrations of bovine serum albumin, concentration of sample proteins was calculated.

3.2.6. Concentration of protein tyrosine phosphatase CD45 in cell lysate

The amount of protein tyrosine phosphatase CD45 in cell lysate was assayed using specific antibodies conjugated with horseradish peroxidase. The concentration of protein tyrosine phosphatase CD45 in a sample was measured spectrophotometrically at 492 nm with horseradish perixidase substrate *ortho*-phenylenediamine (OPD).

3.2.7. Cell viability test with Trypan blue

The assessment of Jurkat cells viability after treatment with selected peracids or hydrogen peroxide was performed using Trypan blue. This is a vital stain, which traverses the membrane of the dead cell and is not absorbed by the live cells. After dyeing with Trypan blue the dead cells are shown as a distinct blue colored spots under a microscope, and all the cells which exclude the dye are considered viable. In 0.5 mL of a cell suspension as much as 0.1 mL of 0.4% Trypan Blue solution was added. The suspension was mixed thoroughly and incubated for 5 min at room temperature. The viable and non-viable cells were observed and counted under the microscope.

3.2.8. Peracid synthesis

Peracids synthesis was performed in the Department of Medical Chemistry, Medical University of Gdansk, with the invaluable help from Dr Zbigniew Wypych. Peroxy acids were synthesized, according Parker's method [Parker *et al*., 1955] as a result of the reaction of a carboxylic acid of interest, and hydrogen peroxide (Figure 22). The carboxylic acid of interest was added into the solution of 50-60% hydrogen peroxide and 70% sulfuric acid (as a reaction medium), and the reaction mixture was incubated for 2 hrs at a room temperature, and then purified. The identity and purity of the synthesized peracids were tested with NMR and IR spectroscopy. Peroxy acids were deep frozen and stored in a powder form at -80°C.

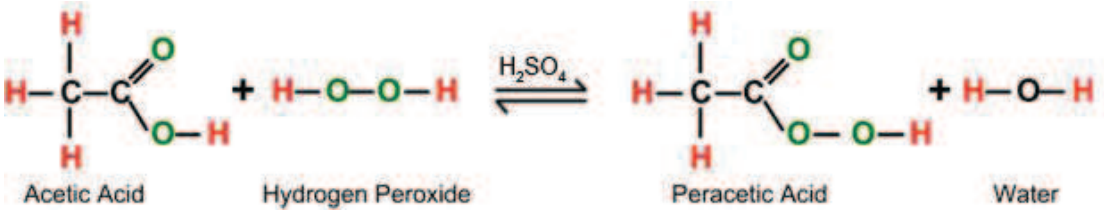


Figure 22. Peracetic acid synthesis from acetic acid and hydrogen peroxide.

3.2.9. Computational analysis

Computational analysis was performed in the Department of Oncology, University of Alberta in Edmonton, Canada with the invaluable help from Dr Philip Winter, and supervised by Professor Jack Tuszynski.

3.2.9.1. Receptor preparation

The PDB structure 1YGU of CD45 protein (gene name PTPRC) was selected as the target receptor for the molecular docking. The PDB file includes a bound phosphotyrosine peptide and is better for docking than the unbound conformation of the receptor. 1YGU is an X-ray diffraction structure with a resolution of 2.90 Å. 1YGU includes two domains of CD45: the D1 domain (which contains the PTP active site) and the D2 domain (which contains an inactive pseudo-phosphatase domain). In 1YGU the catalytic cysteine (Cys828) is substituted by serine and the methionine residues are substituted by selenomethionine. 1YGU includes no hydrogen atoms, water molecules or ions [Nam et al., 2005].

The 1YGU PDB file was downloaded from the Protein Data Bank (http://www.rcsb.org/). The chain A residues 608–890 were extracted, which corresponds to the D1 domain region. The RefSeq sequence NP_002829.3 was downloaded from the NCBI Protein database (http://www.ncbi.nlm.nih.gov/protein/). The sequence region 633–915 was extracted, which again corresponds to the D1 domain. The SWISS-MODEL webserver (http://swissmodel.expasy.org/) was used to generate a modeled structure using the 1YGU residues 608–890 as the template structure and the NP_002829.3 region 633–915 as the target sequence [Arnold et al., 2006]. SWISS-MODEL was run in Automatic Modeling Mode. This resulted in the catalytic cysteine (position 828 in 1YGU, position 853 in NP_002829.3) being restored, and the selenomethionine residues being restored to methionine. The QMEAN Z-Score, which is an estimate of the quality of the model, was reported by SWISS-MODEL to be -1.78 [Benkert et al., 2011].

Hydrogen atoms were added to the modeled structure using the PDB2PQR webserver version 1.8 (http://kryptonite.nbcr.net/pdb2pqr/) [Dolinsky et al., 2004]. The PARSE forcefield was selected, the internal naming scheme was selected, the option ensuring that new atoms are not rebuilt too closely to the existing atoms was selected, and finally the option optimizing the hydrogen bonding network was selected; the other options were unselected. The Asp189 residue was specified to be in the neutral (protonated), and the Cys221 residue was specified to be in the negative (unprotonated) state; the states of the remaining residues were not specified. The residue states were specified in order to ensure that these residues matched the expected states during the first step of the phosphatase reaction mechanism [Pannifer et al., 1998]. The resulting structure was visualized to ensure that the Asp189 and Cys221 residues were in the desired state.

3.2.9.2. Ligand library preparation

A library of ligands was generated for the molecular docking (Figure 23). Most of the structures were either drawn in ChemDraw (http://www.cambridgesoft.com/) or downloaded in 2-dimensional SDF format from PubChem (http://pubchem.ncbi.nlm.nih.gov/). The phosphotyrosine peptide (p-Tyr) was extracted from the 1YGU PDB structure.

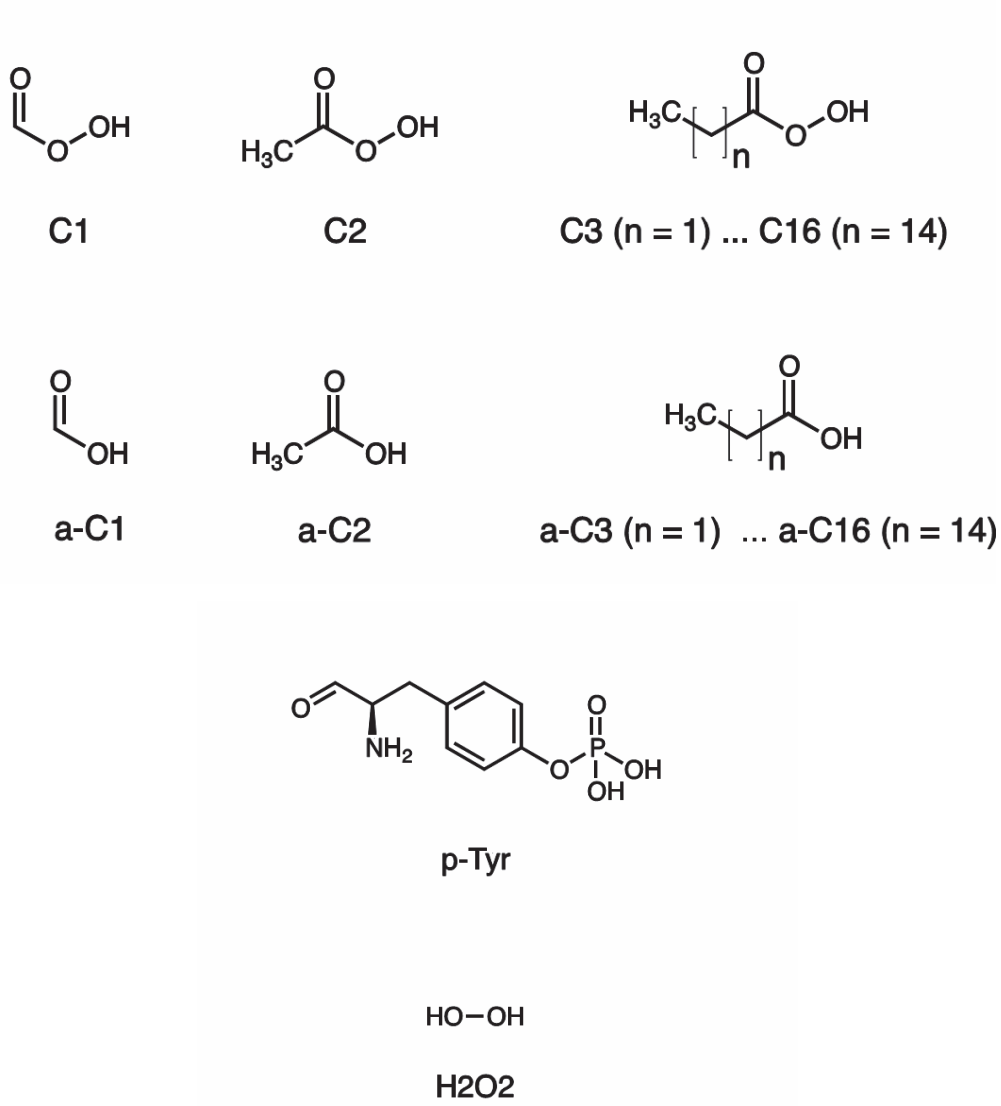


Figure 23. The structures of the ligands included in the chemical library for molecular docking analysis.

The following ligands were included in the library: the series of peroxy acids used in the laboratory experiments extended down to 1 carbon, and the missing members of the series were added and labeled C1 through C16. For each member of the preceding peroxy acid series, the corresponding carboxylic acid was also included, labeled a-C1 through a-C16. Additional compounds tested were included: hydrogen peroxide (H_20_2) and phosphotyrosine residue (p-Tyr).

The ligands were processed with Schrodinger LigPrep version 25111 (http://www.schrodinger.com/). The default LigPrep options were used. LigPrep added hydrogens and generated initial 3-dimensional conformations for each of the ligands.

3.2.9.3. Molecular docking

AutoDockTools version 1.5.4 was used to convert the receptor and ligand library to PDBQT format [Morris et al., 2009]. The molecular docking program AutoDock Vina version 1.1.1 was used to perform the docking [Trott, Olson, 2010]. A binding box was defined centered on the position of the phosphorus atom in the phosphotyrosine peptide in the bound complex; the center of the binding box coordinates was therefore set at (15, -7, 60). The largest ligand in a fully extended conformation was C16; the longest distance between atom coordinates for this ligand was calculated to be about 25 Å. The binding box for docking had to be large enough to contain this ligand, therefore the binding box was set at a cube with the side length of 25 Å.

Docking with a fully rigid receptor was performed. The Vina parameter exhaustiveness increases the time spent on the search; for the final docking runs an exhaustiveness parameter of 128 was used. Preliminary docking runs suggested that this parameter was more than sufficient; increasing the exhaustiveness is not expected to significantly change the results for the rigid docking. A set of 6 repetitions were performed with different random seeds. Since the search of ligand performed by Vina is stochastic, the results will be different depending on the random seed. The reported binding affinities are the means of all the repetitions, and the reported errors are the standard deviations. The best binding pose was defined as the pose with the strongest affinity in the largest cluster of poses, with poses clustered with a 1.5 Å RMSD thresholds.

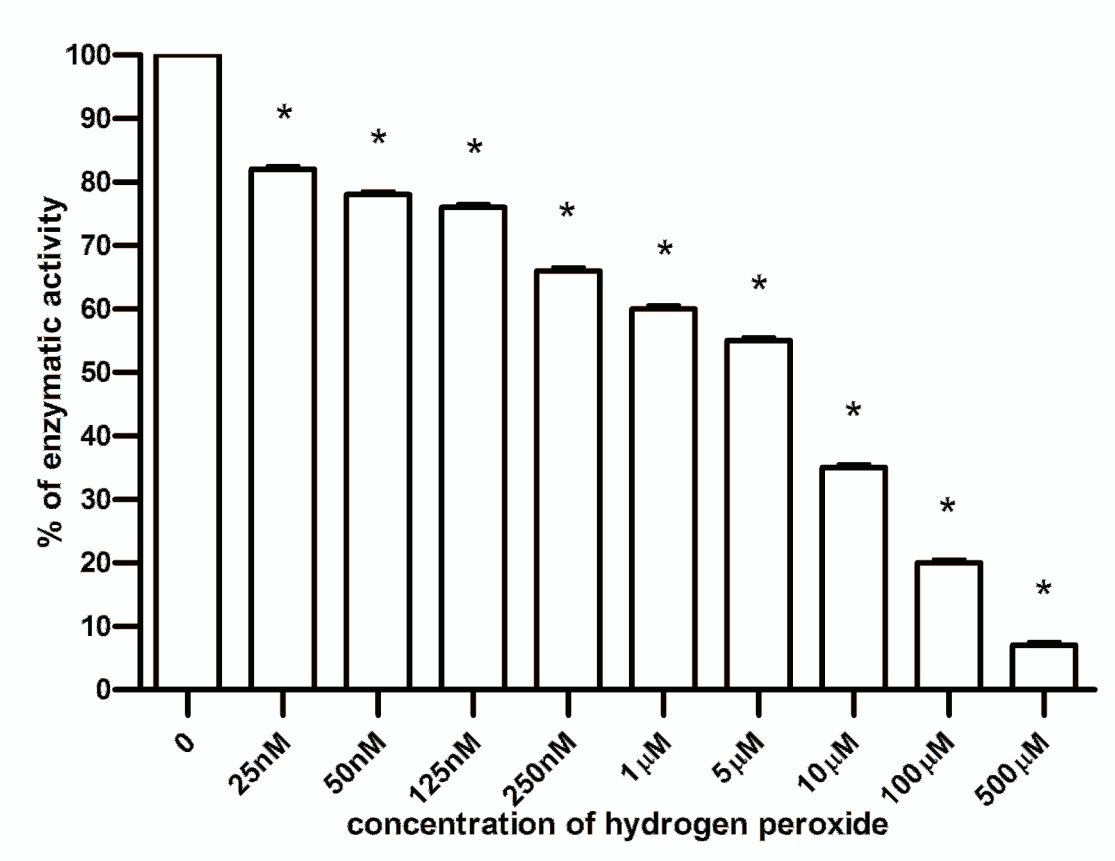
3.2.10. Statistical analysis

The non-computational experiments were performed at least three times. The data were applied and analyzed with GraphPad Prism (GraphPad Software v.4). Statistical analyses were performed using ANOVA combined with Tukey's test. The data were expressed as means ± SD. Differences between means were considered significant for $p < 0.05$. IC_{50} values were determined from a diagram of inhibitor concentration versus percentage of control enzymatic activity.

4. RESULTS

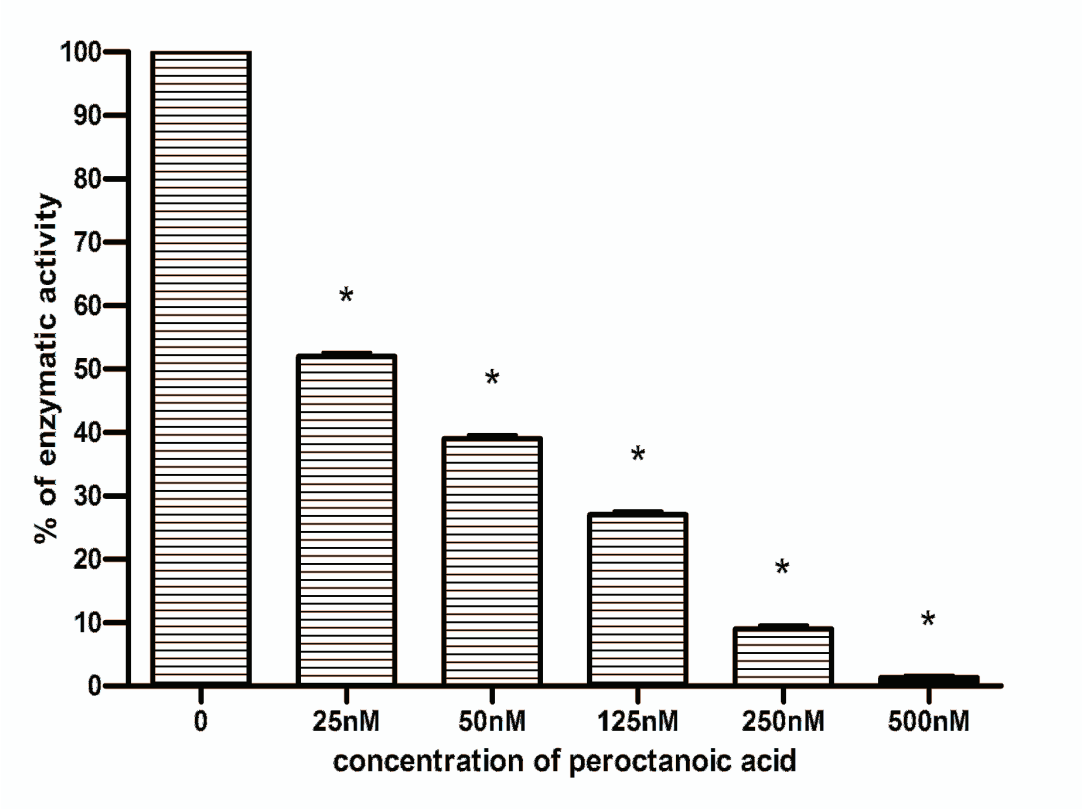
4.1. IMPACT OF HYDROGEN PEROXIDE AND SELECTED PERACIDS ON CD45 ENZYMATIC ACTIVITY

In this study recombinant protein tyrosine phosphatase CD45 was used to identify the effect of hydrogen peroxide on its enzymatic activity. We have treated protein tyrosine phosphatase CD45 with various concentrations of hydrogen peroxide. The percent of retained enzymatic activity of CD45 is presented in Graph 1. Hydrogen peroxide in concentration of 25 nM induces less than 20% loss of CD45 enzymatic activity. After incubation with 1 μM hydrogen peroxide the enzyme exhibits 60% activity of the control. The major loss of the activity (less than 10%) was observed after CD45 treatment with 500 μM hydrogen peroxide.



Graph 1. Impact of hydrogen peroxide on enzymatic activity of recombinant protein tyrosine phosphatase CD45. Data presented as means±SD (n=10). One-way Anova combined with Tukey test. * significantly different ($P < 0.001$) from control.

In a subsequent study recombinant protein tyrosine phosphatase CD45 was treated with different concentrations of peroctanoic acid. The percent of retained enzymatic activity of CD45 after incubation with peroctanoic acid is shown in Graph 2. The peroctanoic acid in concentration of 25 nM induces 50% inactivation of CD45 enzymatic activity as compared to the control. Nearly complete loss of activity of CD45 (less than 2%) was observed after treatment with 500 nM peroctanoic acid.



Graph 2. Impact of various concentrations of peroctanoic acid on enzymatic activity of recombinant phosphatase CD45. Data presented as means±SD (n=10). One-way Anova test. * significantly different ($P < 0.001$) from control.

4.2. LENGTH OF PERACID'S ACYL CHAIN AND THE INHIBITORY EFFECT

Recombinant phosphatase CD45 was treated with peracids of various acyl chain length. The inhibitory effect was proved to depend on the kind of peracid used. The IC_{50} values for

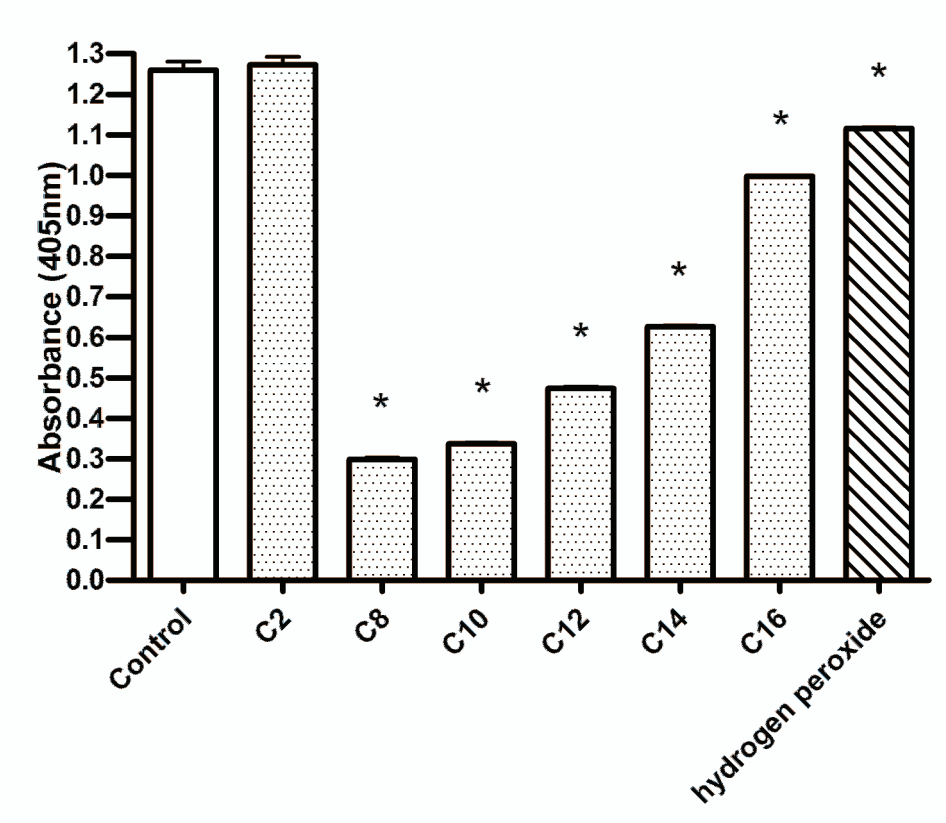
various peracids are presented in Table 1. IC_{50} values were determined based on diagram correlating peracid concentration versus percentage of CD45 control activity.

Name of inhibitor	Chemical formula of inhibitor	IC_{50} value
Peracetic acid (C2)	CH_3COOOH	5 μM
Peroctanoic acid (C8)	$CH_3(CH_2)_6COOOH$	25 nM
Perdecanoic acid (C10)	$CH_3(CH_2)_8COOOH$	40 nM
Perdodecanoic acid (C12)	$CH_3(CH_2)_{10}COOOH$	41 nM
Pertetradecanoic acid (C14)	$CH_3(CH_2)_{12}COOOH$	53 nM
Hexadecanoic acid (C16)	$CH_3(CH_2)_{14}COOOH$	140 nM
Hydrogen peroxide	H_2O_2	8 μM

Table 1. IC_{50} (CD45) values of peracids and hydrogen peroxide. IC_{50} values were determined from a diagram presenting peracid or hydrogen peroxide concentration versus percentage of the enzymatic activity of recombinant CD45.

4.2.1. PEROCTANOIC ACID HAS THE HIGHEST INHIBITORY EFFECT ON RECOMBINANT CD45

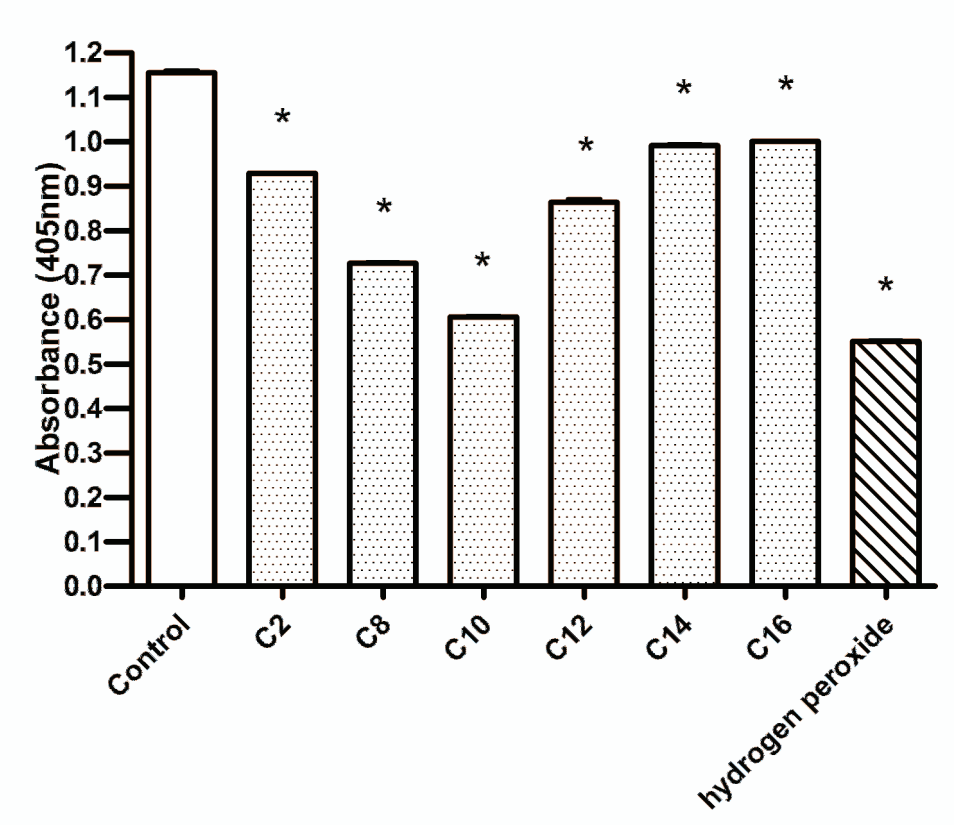
Recombinant CD45 phosphatase was treated with 50 nM peracids of different acyl chain length (from 2 to 16 carbons) or with hydrogen peroxide. The obtained results are presented in Graph 3. The peroctanoic acid (8 carbons in the acyl chain) proven to have the greatest impact on the enzymatic activity of CD45 phosphatase. The inhibitory effect decreases for peracids with longer acyl chain. The peracid containing less than 8 carbons turned out to have lower inhibitory impact on CD45 (Graph 3).



Graph 3. Impact of peracids (50nM) of different hydrocarbon chain length or hydrogen peroxide (50nM) on the enzymatic activity of recombinant CD45. Data presented as mean±SD (n=10). One-way analysis of variance. * significantly different ($P < 0.001$) from control.

4.2.2. PERDECANOIC ACID INHIBITS THE ACTIVITY OF CD45 IN JURKAT CELLS AT THE HIGHEST LEVEL

Jurkat cells were treated with with 50 nM peracids of different acyl chain length (from 2 to 16 carbons) or hydrogen peroxide at room temperature for 1 hr. The enzymatic activity of CD45 was assessed in lysate and the obtained results are presented in Graph 4. Perdecanoic acid (10-carbon acyl chain) proved to have the strongest inhibitory effect on CD45 enzymatic activity in Jurkat cells. The inhibitory effect decreased along with both increasing and decreasing number of carbon atoms in the hydrocarbon chain of peracid.

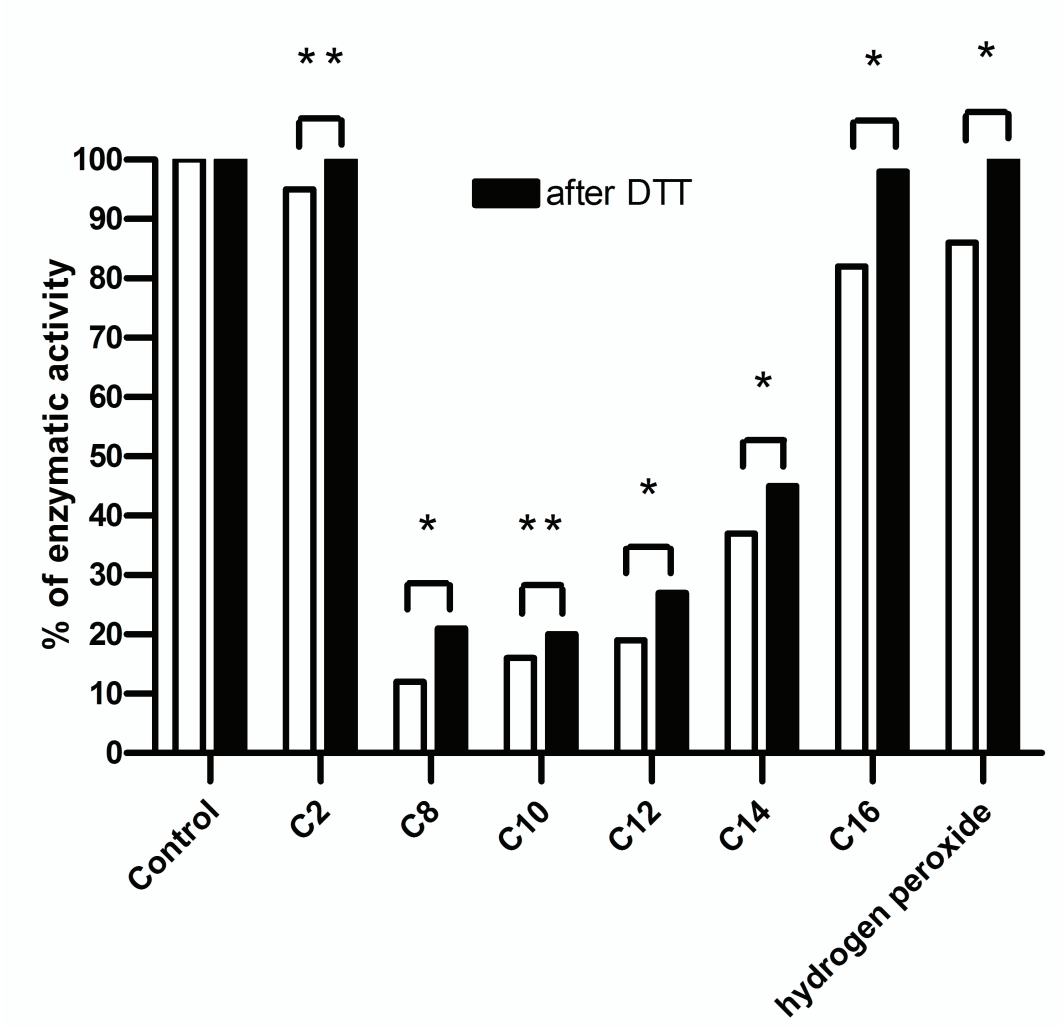


Graph 4. Infibitory effect of peracids (50nM) of different hydrocarbon chain lenght or hydrogen peroxide (50nM) on CD45 enzymatic activity in Jurkat cells. Cells were treated with peracids or hydrogen peroxide and then lysed. Activity of CD45 was measured using immunoprecipitation technique, and the specific substrate. Data presented as mean±SD (n=3). One-way analysis of variance. * significantly different ($P < 0.001$) from control.

4.3. REVERSIBILITY OF CD45 INACTIVATION

In this study recombinant tyrosine phosphatase CD45 was used to investigate a potential reversibility of enzymic inactivation induced by hydrogen peroxide or selected peracids. CD45 previously inactivated by hydrogen peroxide or peracids was subsequently incubated with 10 mM dithiotreitol (DTT) to reverse the inactivation, if possible. The restoration of enzymatic activity of CD45 is presented in Graph 5. The inactivation of CD45 by hydrogen peroxide was completely reversed by treatment with DTT. The inactivation caused by the shortest chain (C2) as well as longest chain (C16) peracids was also reversible. The enzymatic

activity of CD45 inactivated by treatment with medium chain peracids (C8 – C14) was restored only up to 40% at the maximum.



Graph 5. The restoration of CD45 enzymatic activity by 10 mM DTT. Inactivation of recombinant CD45 by selected peracids (50 nM) or hydrogen peroxide (50 nM), and the subsequent reversibility of the inactivation after treatment with 10 mM DTT. Data presented as mean±SD (n=10). One-way analysis of variance $P<0.0001$.

4.4. JURKAT CELLS VIABILITY AFTER TREATMENT WITH PERACIDS

Jurkat cells were treated with 50 μM hydrogen peroxide or selected peracids for 48 hrs, and cell viability was evaluated using Trypan blue staining technique. The results are presented in Table 2. Data are presented as the percentage of living cells compared to control (100%).

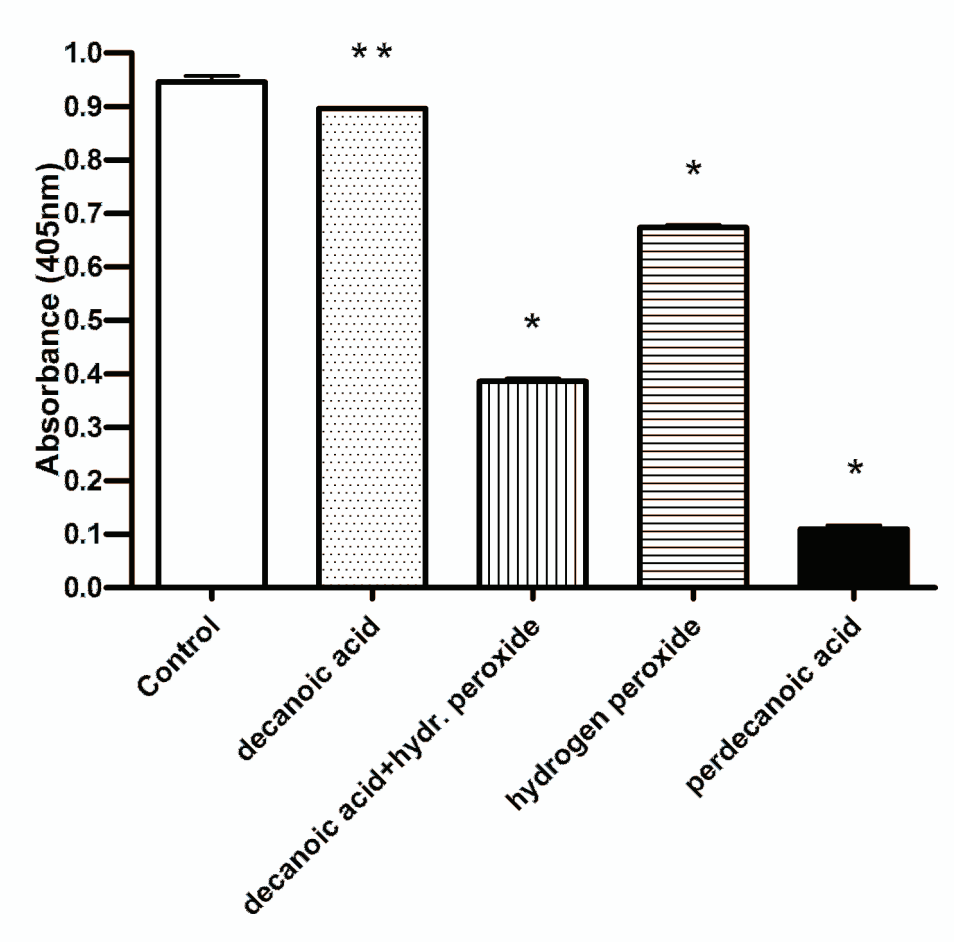
Treatment of Jurkat cells with different peracids had only minor effect or no effect at all on cell viability, as opposed to the incubation of the cells with the same concentration of hydrogen peroxide having reduced cell viability to as little as 36% of the control.

Substance (50 μM for 48h)	Peracid C2	Peracid C8	Peracid C10	Peracid C12	Peracid C14	Peracid C16	Hydrogen peroxide
Viability (%)	100	99	80	93	86	85	36

Table 2. Cell viability determined with Trypan blue staining. Data presented as the percentage of living cells after treatment with 50 μM peracids or 50 μM hydrogen peroxide for 48 hrs.

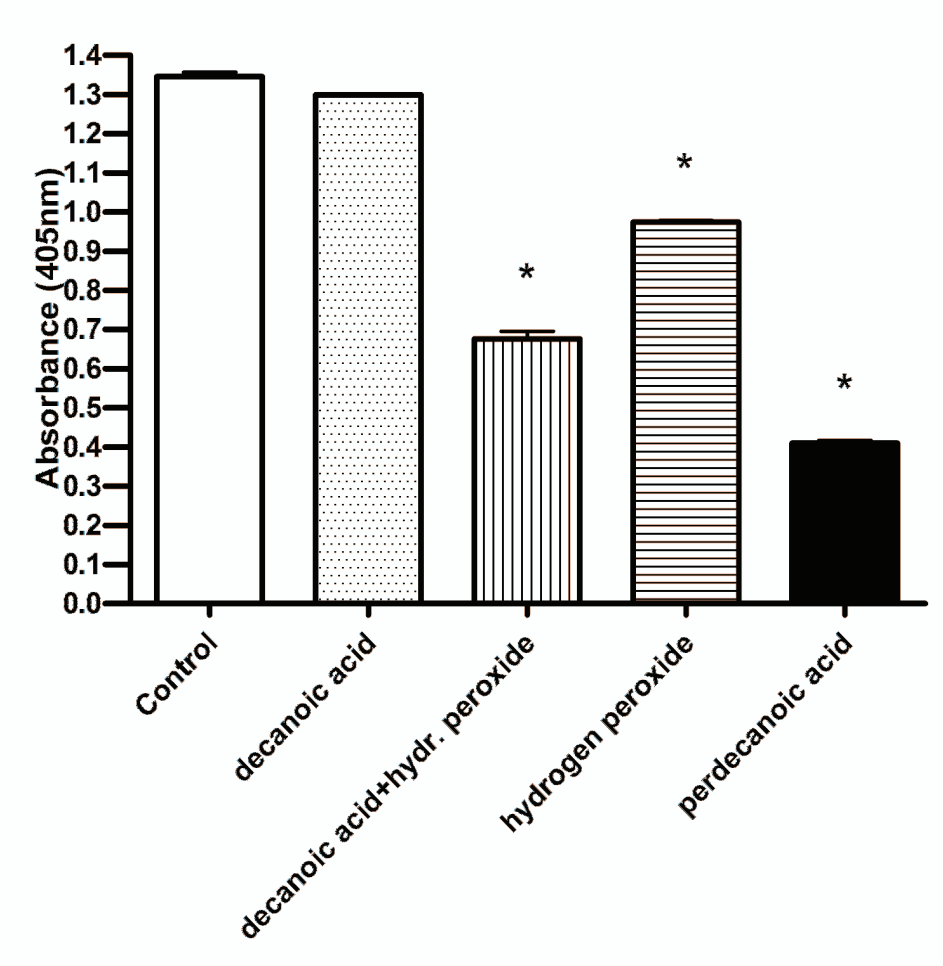
4.5. INHIBITORY EFFECTS OF CARBOXYLIC ACIDS AND THEIR RESPECTIVE PERACID ANALOGS

Recombined CD45 phosphatase was treated with selected peracids and their corresponding carboxylic acids to compare the inhibitory effect on enzymic activity. Graph 4 presents the inhibitory effect of decanoic acid and the corresponding perdecanoic acid on the enzymatic activity of phosphatase CD45. The effect of acids on CD45 enzymatic activity is relatively low or for some of the acids there is no effect. Recombinant CD45 phosphatase was also treated with a mixture of decanoic acid and hydrogen peroxide. There was only minimal inhibitory effect observed for decanoic acid as opposed to perdecanoic acid causing dramatic decrease of the enzymic activity. The inhibitory effect of hydrogen peroxide alone was lower than that observed for the mixture of hydrogen peroxide and decanoic acid.



Graph 6. Inhibitory effect of decanoic acid (50 nM), perdecanoic acids (50 nM), hydrogen peroxide (50 nM) or mixture of decanoic acid (50 nM) and hydrogen peroxide (50 nM) on the enzymatic activity of recombinant CD45 phosphatase. Data presented as means±SD (n=10). One-way Anova test. * significantly different ($P < 0.001$) from control. ** significantly different ($P < 0.01$) from control.

Analogous experiments were performed for Jurkat cells. The cells were incubated for 1 hr with decanoic, perdecanoic acid and hydrogen peroxide, and also with a mixture of hydrogen peroxide and decanoic acid. The concentration of enzymatic activity of CD45 in lysate (as a function of measured absorbance) is presented in Graph 7. The impact of decanoic acid on enzymatic activity of CD45 in Jurkat cells is virtually negligible as compared to that of perdecanoic acid. Jurkat cells were treated for 1hr with a mixture of decanoic acid and hydrogen peroxide. Hydrogen peroxide as mixed with decanoic acid inactivated CD45 in Jurkat cells significantly more than hydrogen peroxide alone.



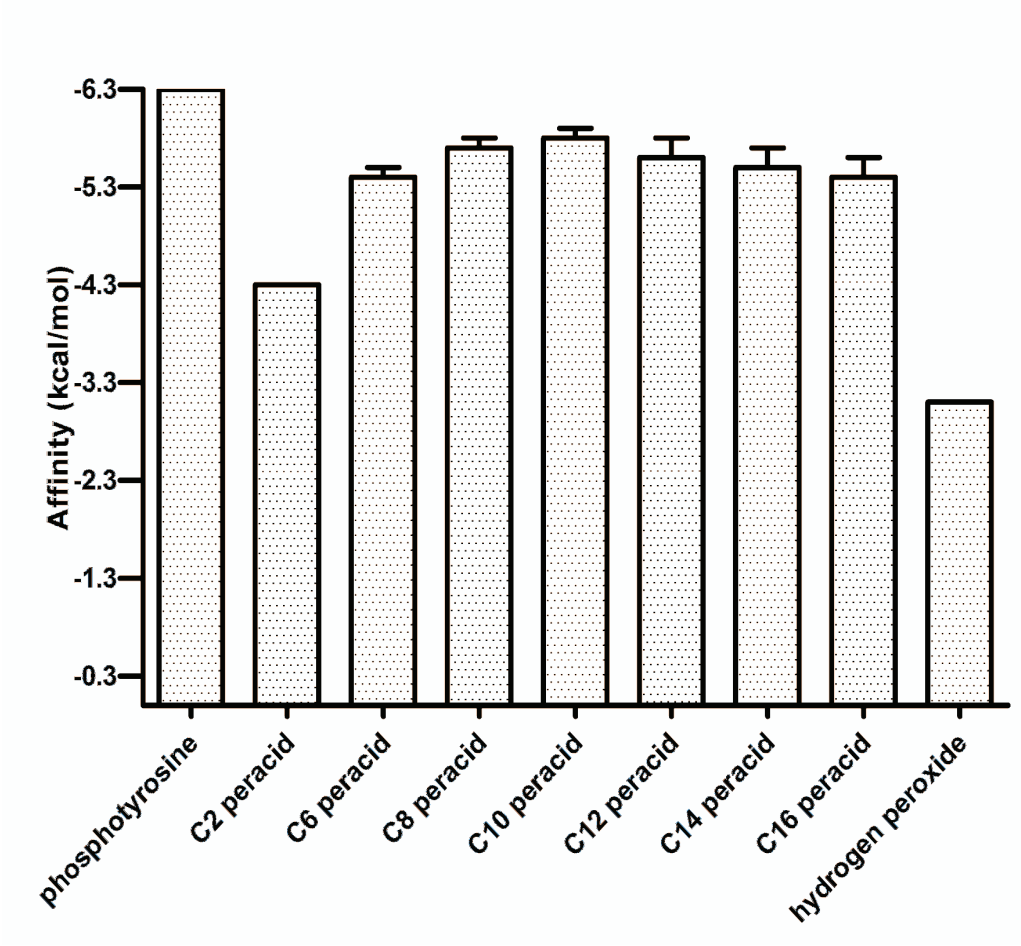
Graph 7. Inhibitory effect of decanoic acid (50 nM), perdecanoic acid (50 nM), hydrogen peroxide (50 nM) or mixture of decanoic acid (50 nM) and hydrogen peroxide (50 nM) on the enzymatic activity of CD45 in Jurkat cells. After treatment the cells lysed. Activity of CD45 was measured using immunoprecypitation technique, and the specific substrate. Data presented as mean±SD (n=3).
One-way analysis of variance. * significantly different ($P < 0.001$) from control.

4.6. COMPUTATIONAL ANALYSIS

Molecular docking computations were performed in order to study peroxy acids (peracids) as potential inhibitors of protein tyrosine phosphatases (PTPs) and help better understand the experimental findings obtained in the laboratory. The molecular docking computations determined the most likely binding coordinates (poses) and binding energies (affinities) of peroxy acids (ligands) interacting with the PTP active site (receptor).

4.6.1. Calculation of binding affinities

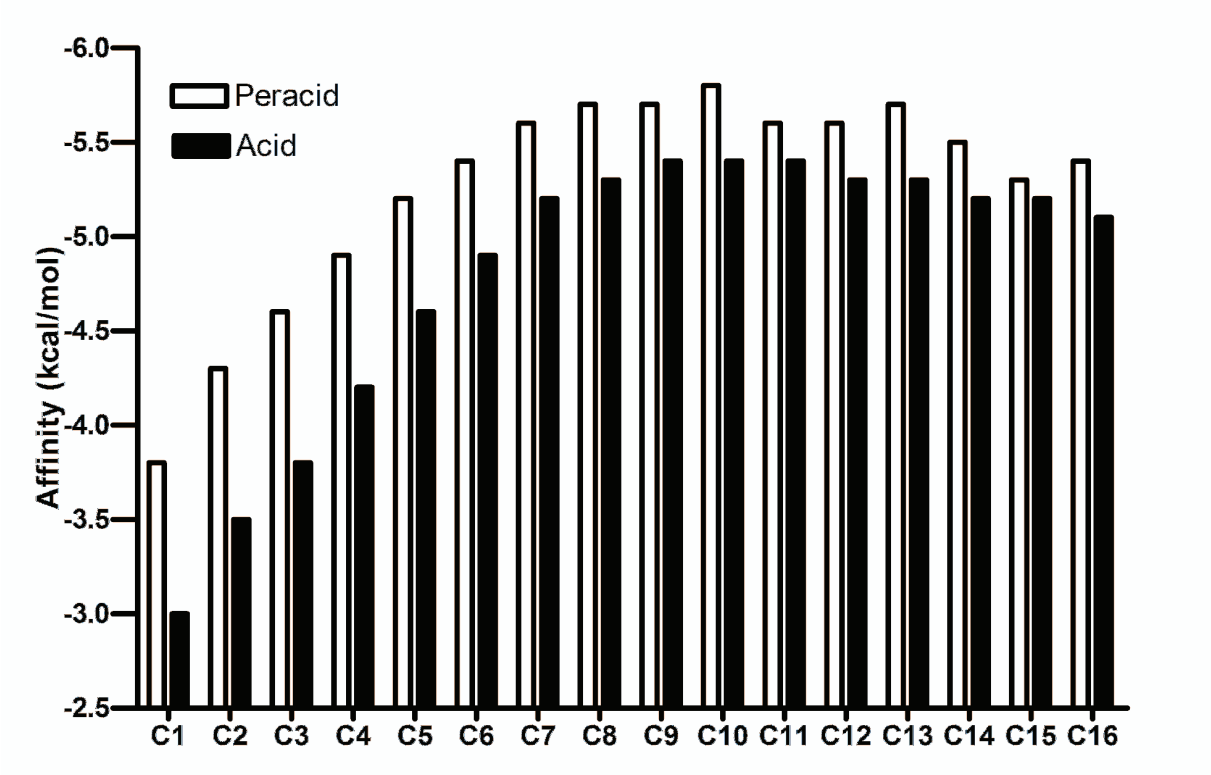
Binding affinities of virtual ligands (constructed as a series of peracids or hydrogen peroxide) to the catalytic center of CD45 phosphatase were determined *in silico*. The binding affinities calculated with AutoDock Vina are presented in Graph 8. The binding affinities of peracids proved to be higher (especially for medium chain peracids) as compared to hydrogen peroxide. The binding affinity for phosphotyrosine (as the natural substrate for tyrosine phosphatases) to CD45 active site was calculated for the control. The binding affinities for phosphotyrosine (-6.3) and for perdecanoic acid (-5.7) proved to be similar. The lowest binding affinity from the examined library of ligands was calculated for hydrogen peroxide (-3.1) (Graph 8).



Graph 8. The binding affinities (as calculated for the virtual model of CD45 catalytic center) of selected peroxy acids, hydrogen peroxide and phosphotyrosine. The binding affinity of phosphotyrosine (natural substrate) - control. The error bars are standard deviations of 6 repetitions with different random seeds. One-way Anova combined with Tukey test.

* significantly different ($p < 0.001$) from control.

The binding affinities for different peroxy acids and their corresponding carboxylic acids were calculated and compared in Graph 9. The peroxycarboxylic acids are a little bit more likely to bind to the catalytic center of CD45 than carboxylic acids. Among carboxylic acids, those with 9, 10 and 11 carbons in the acyl chain have the highest binding affinity.



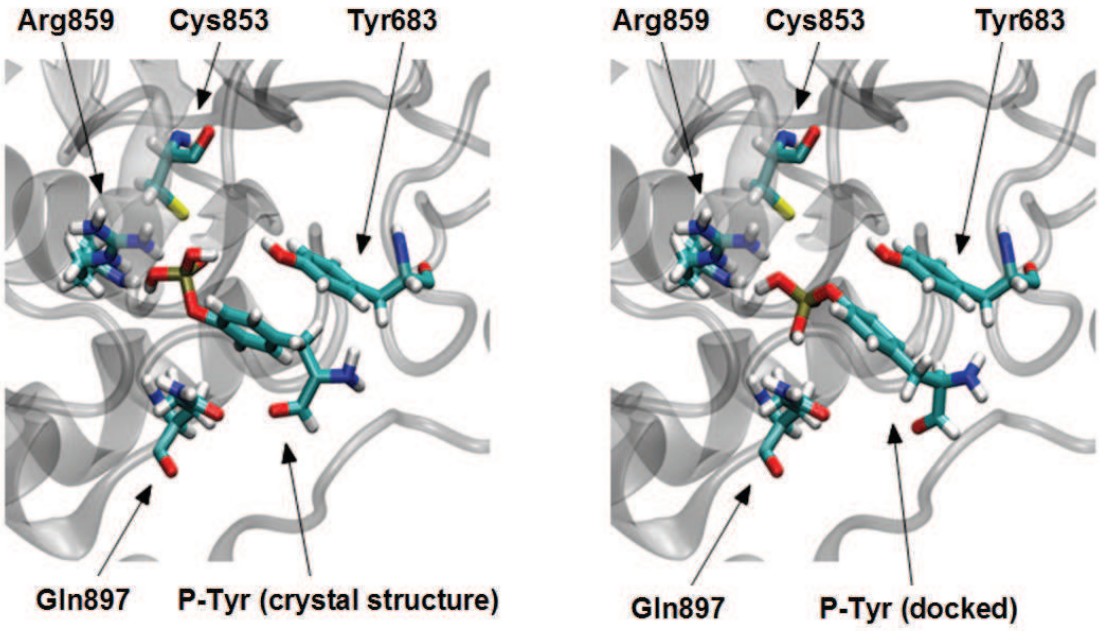
Graph 9. The calculated binding affinities of the peroxy acids and their corresponding carboxylic acids. The error bars are standard deviations of 6 repetitions with different random seeds.

4.6.2. Molecular docking

Molecular docking analysis was performed to investigate the binding conformations (poses) of compounds from the selected ligand library to the catalytic center of CD45. Searching for the binding poses of ligands within the given binding site of CD45 was performed with AutoDock Vina software. The binding pose figures (Graphs 10-12) show the single best predicted conformation of each ligand within the binding site determined using an energy scoring function.

4.6.2.1. Phosphotyrosine binding pose from docking analysis and from CD45 crystal structure

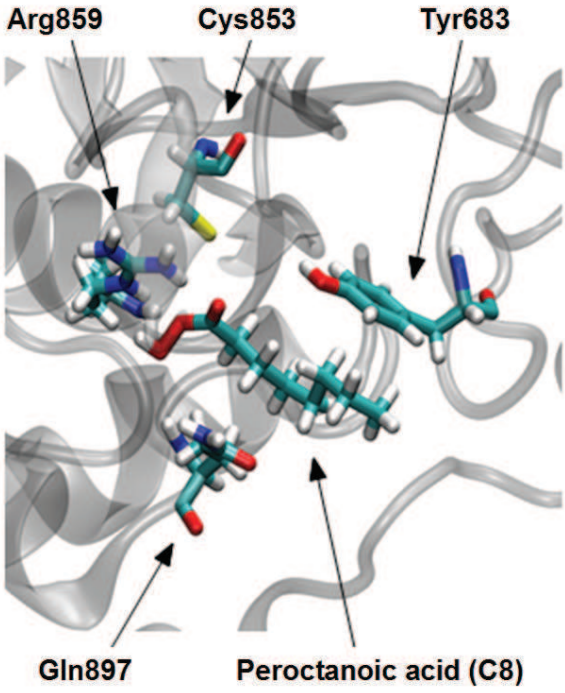
The most plausible binding pose of phosphotyrosine (p-Tyr) within the catalytic center of phosphatase CD45 was determined, and compared with the conformation obtained from the crystal structure as described by Nam and coworkers [Nam et al., 2005]. Graph 10 shows the extracted pose from 1YGU file and the docked pose for phosphotyrosine peptide in the catalytic center of tyrosine phosphatase CD45. The catalytic cysteine residue in the 1YGU structure was mutated to serine, but for the docking analysis the serine was restored to cysteine. This may lead to some differences between the binding poses obtained from the crystal structure and determined by docking analysis.



Graph 10. The binding pose for phosphotyrosine (p-Tyr) extracted from the 1YGU structure (left panel) and the optimal binding pose determined by docking analysis (right panel). The docked pose was computed by AutoDock Vina using a rigid receptor and a binding box centered on the CD45 phosphatase active site. The CD45 binding site backbone is shown as a gray cartoon and four amino acid residues critical for the binding site are shown (the residue positions are numbered according to the UniProtKB sequence P08575).

4.6.2.2. The most plausible binding poses for peracids

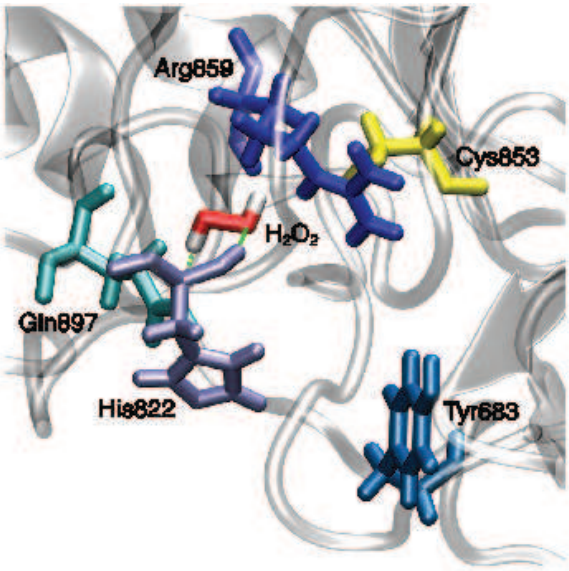
We have performed molecular docking experiments for series of peracids, and the results showed that peracids were not strerically precluded from binding in the catalytic center of CD45. Graph 11 shows the most plausible binding pose within CD45 active site for peroctanoic acid (peracid of the highest inhibitory effect on the enzymatic activity of CD45).
In the binding conformation peroxycarboxyl group of peroctanoic acid is in a relatively short distance from the catalytic cysteine residue and the arginine residue. Under such predefined sterical conditions there is a possibility of hydrogen bond formation between the arginine residue and peroxycarboxyl group. A relatively short distance between the acyl chain and the tyrosine residue may lead to hydrophobic interactions between the acyl chain and the tyrosine side chain.



Graph 11. Thermodynamically most plausible ligand binding poses for peroctanoic acid (C8) as determined by docking experiments prepared with AutoDock Vina using a rigid receptor and a binding box centered on the CD45 phosphatase active site. The CD45 binding site backbone is shown as a gray cartoon and the four amino acid residues critical for the binding site are shown (the residue positions are numbered according to the UniProtKB sequence P08575).

4.6.2.3. Hydrogen peroxide within catalytic center of CD45

The most plausible binding pose was determined for hydrogen peroxide. Hydrogen peroxide in the binding conformation is likely to form hydrogen bonds with histidine residue of CD45 active site. There are no interactions with tyrosine residue and the distance from the catalytic cysteine residue is greater than that calculated for the peroxycarboxyl group in a binding pose of the peracid.



Graph 12. Thermodynamically most plausible ligand binding pose for hydrogen peroxide as computed by AutoDock Vina using a rigid receptor and a binding box centered on the CD45 phosphatase active site. The CD45 binding site backbone is shown as a gray cartoon. The important residues involved in binding (Tyr683, His822, Arg859 and Gln897) and the catalytic cysteine (Cys853) are highlighted. The residues are numbered according to the P08575 sequence. Predicted hydrogen bonds with a 3.5 Å distance cut-off are shown as green dashed lines.

5. DISCUSSION

5.1. Peracids are more potent inhibitors of CD45 than hydrogen peroxide

Hydrogen peroxide is known to inhibit protein tyrosine phosphatases via oxidation of the catalytic cysteine [Ostman et al, 2011]. Hydrogen peroxide in the presence of carboxylic acids may lead to formation the respective peroxy acids, which are more potent oxidants. It was found that peracetic acid can induce inactivation of protein tyrosine phosphatase PTP1B [Bhattacharya et al, 2008]. In this project the inhibitory effect of hydrogen peroxide (Graph 1) and selected peracids (Graph 2) on enzymatic activity of recombinant CD45 phosphatase was determined. The results show that peroxy acids inhibit CD45 phosphatase several orders of magnitude more than hydrogen peroxide, with IC_{50} value equal to 25 nM for peroctanoic acid or 40 nM for perdecanoic acid, as compared to 8 μM for hydrogen peroxide (Table 1).
The peroxy acids induce inactivation of the recombinant protein tyrosine phosphatase CD45, as well as the native CD45 present in Jurkat cells. The results of this doctoral dissertation project show that treatment of Jurkat cells with 50 μM peracids for 48 hrs has no impact on cell viability, while incubation of Jurkat cells with the same concentration of hydrogen peroxide induces 64% loss of viability (Table 2).

5.2. Medium-chain peracids are stronger inhibitors of CD45

The results during the doctoral dissertation project show that not every kind of peracid inactivates CD45 phosphatase at the same level. The inhibitory impact is related to the length of the acyl chain of a peracid of analysis. Out of the selected peracids, the medium-chain peracids exhibit the highest impact on the enzymatic activity of CD45 phosphatase (Graph 3). The highly selective inhibition of phosphatase CD45 was observed for both: recombinant enzyme and the native one expressed in Jurkat cells (Graph 4). Out of the investigated peracids, peroctanoic acid (with 8 carbons in the acyl chain) proved to be the most potent inhibitor of the recombinant CD45, while perdecanoic acid (with 10 carbons in the chain) proved to have the strongest inhibitory effect on CD45 natively expressed in Jurkat cells. The inhibitory effect decreases along with both increasing or decreasing number of carbon atoms in the peracid hydrocarbon chain. This highly selective inactivation allows to assume that

only peroctanoic or perdecanoic acids with optimal size and structure can effectively penetrate and dock at the catalytic center of CD45.

5.2.1. Medium-chain peracids irreversibly inactivate CD45

In order to study the mechanism of CD45 inactivation by different peracids or hydrogen peroxide, potential reversibility of the inhibitiory effect was tested (Graph 5). The experimental results show that the inactivation caused by the medium chain peracids is virtually irreversible. The enzymatic activity of CD45 inactivated by treatment with medium-chain peracids may be restored only up to a few percentage points as compared to control. The treatment of CD45 phosphatase with short-chain or long-chain peracids induces reversible rather than irreversible oxidation, and the enzymatic activity may be almost completely recovered with DTT. The CD45 inhibition caused by hydrogen peroxide may be completely reversed (Figure 24). Howewer, previous studies indicate that organic peroxide, such as peracetic acid induces reversible inactivation of PTP1B phosphatase [Bhattacharya et al., 2008]. According to Bhattacharya et al., reversible PTPs oxidation may be a general mechanism of enzymic inactivation by organic peroxides. The results of this project clearly show that the reversible oxidation is not observed for the medium-chain peracids, whose optimal size and structure allows for effective penetration into the catalytic center of CD45 repeatedly, leading to further oxidation of the catalytic cysteine to the irreversible sulfinic and sulfonic acid derivatives.

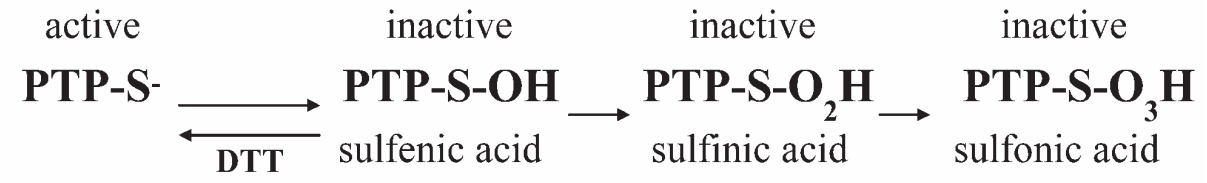


Figure 24. Inactivation of PTPs by oxidation.

5.3. Percarboxyl group as a triggering factor of CD45 inactivation

The only difference between peracid and homologous fatty acid is the peroxycarboxyl group (with the additional oxygen atom) instead of the carboxyl group typical for a regular fatty acid. In order to study the role of peroxycarboxyl group of peracid in PTP inactivation excluding a possible unspecific impact of hydrocarbon chain we treated CD45 with peracids and their corresponding carboxylic acids. We found that treatment of recombinant CD45 or native CD45 expressed in Jurkat cells with fatty acids had no impact on the enzymic activity, while the treatment with the respective homologous peracids significantly lowered the activity of CD45 (both the recombinant and native enzyme in the Jurkat cell line). These observations strongly suggest that peroxycarboxyl group is the one responsible for the inhibitory effect of peracids on the enzymatic activity of CD45 phosphatase. We propose that the peroxycarboxyl group induces oxidation of the catalytic cysteine, leading to inactivation of CD45 phosphatase.

5.4. Computational analysis with AutoDock Vina

Molecular docking analysis using dedicated software tools such as AutoDock [Morris et al., 2009] and AutoDock Vina [Trott et al., 2010] enabled to search through the possible poses of different ligands in a given binding site, and determine the most plausible pose applying an energy scoring function. AutoDock Vina program is a new docking program based on AutoDock. AutoDock Vina was selected as the docking program for this study due to its high performance and improved accuracy as compared with AutoDock. The poses determined by docking computation could be a good starting point for molecular dynamic analyses, and other computational or laboratory studies.

5.4.1. Molecular docking for phosphotyrosine

The calculations of phosphotyrosine binding affinity to the catalytic center of CD45 were performed as the control of the computational analysis, because p-Tyr is a natural substrate of tyrosine phosphatases. Our results show that phosphotyrosine ligand is predicted to have the highest binding affinity of -6.3 kcal/mol out of the whole ligand library (Graph 8).

The most plausible binding pose of p-Tyr in the CD45 binding site determined by docking analysis was not a good match to the experimentally determined coordinates from the phosphotyrosine ligand in 1YGU structure (Graph 10). However, in the 1YGU structure the catalytic cysteine residue was mutated to serine residue, which could explain the difference.

5.4.2. Peracids have a strong binding affinity to CD45 catalytic center

The molecular docking results show that peracids have higher binding affinity to CD45 active site than hydrogen peroxide (Graph 8). This corresponds to the experimental results obtained with the recombinant phosphatase, and may explain greater inhibitory impact of peracids on CD45 as compared to hydrogen peroxide (Graph 3). The examination of the binding pose for peroctanoic acid (Graph 11) shows, that in the predicted best binding pose, peroxycarboxyl group of the peracid is directed toward the catalytic cysteine residue. This conformation allows for the reaction between the peroxycarboxyl group and the cysteine residue being relatively susceptible to oxidation. It seems possible for the ligand to form hydrogen bonds with the arginine residue, and moreover some hydrophobic interactions between the acyl chain and the tyrosine residue may occur. These interactions contribute to the relatively strong calculated binding affinity of peroctanoic acid.

Hydrogen peroxide does not exhibit a strong binding affinity to CD45 active site, because it is a small molecule, and is not able to interact hydrophobically with the aromatic amino acid residues in the catalytic center (Graph 12). However, there may be hydrogen bonds formed between the histidine residue of CD45 active site and hydrogen peroxide. Taken together, these facts suggest that the inhibition of CD45 phosphatase by hydrogen peroxide may have a sort of unspecific character.

5.4.3. Medium-chain peracids have the strongest binding affinity to CD45 catalytic center

Both peroctanoic acid (with 8 carbons in the chain) and perdecanoic acid (with 10 carbons in the chain) were predicted to have the strongest binding affinity to the CD45 active site out of of the peroxy acid ligand library with perdecanoic acid having the binding affinity of -5.8 kcal/mol (Graph 8). These calculations are consistent with the experimental results showing that peroxy acids C8 and C10 have the strongest inhibitory effect on CD45 both the

recombinant and the native form. All in all, the obtained results support the hypothesis that the observed difference in the inhibitory effect depending on the chain length might be due to different binding affinity for the CD45 catalytic center.

5.4.4. Carboxylic acids and their respective peracid analogs have similar binding affinities to CD45 catalytic center

A comparison of the binding affinity of the peroxy acids with their homologous carboxylic acids (Graph 9) shows that the peroxy acids have slightly stronger binding affinity than the carboxylic acids (~1 kcal/mol stronger binding affinity). The overall trend of the binding affinity relative to the number of carbon atoms in the side chain is the same for both the peroxy acids and their homologous carboxylic acids. Importantly, according to the experimental results the carboxylic acids were not observed to have any inhibitory effect on the recombinant CD45 phosphatase nor the one natively expressed in Jurkat cells. Therefore, the fact that peroxy acids contain a hyperreactive peroxycarboxyl group, as opposed to their corresponding carboxylic acids, seems to be decisive in the mechanism of CD45 inhibition. This highly oxidizing group may interact with the catalytic cysteine leading to its oxidation.

6. CONCLUSIONS

1. Peracids are more potent inhibitors of protein tyrosine phosphatase CD45 than hydrogen peroxide inducing substantial enzymic inactivation at nanomolar concentrations.

2. Medium chain peracids, containing 8 or 10 carbon atoms in the acyl side chain, are the most potent inhibitors of, respectively, the recombinant CD45 phosphatase and the one natively expressed in Jurkat cells.

3. A plausible mechanism of the peracid-induced CD45 phosphatase inactivation seems to involve oxidation of the catalytic cysteine residue by the peroxycarboxyl group of peracid.

4. Molecular docking results confirm that peracids are not strerically precluded, and can be effectively bound in the catalytic center of CD45.

5. Computational analyses prove that peracids have higher binding affinities for the catalytic center of CD45 than hydrogen peroxide, and specifically the medium chain peracids have the highest binding affinities of all, which is in accordance with the obtained experimental results.

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LIST OF PUBLICATIONS AND CONFERENCE PRESENTATIONS BY THE AUTHOR

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1. **A. Kuban-Jankowska**, J. Tuszynski, P. Winter, M. Gorska, N. Knap, M. Wozniak (2012) Activation of hydrogen peroxide to peroxytetradecanoic acid is responsible for potent inhibition of protein tyrosine phosphatase CD45. PLoS One (accepted for publication on 22nd November 2012).

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STRESZCZENIE PRACY W JĘZYKU POLSKIM

Rola nadtlenku wodoru i powstających w reakcji z nim nadkwasów w regulacji aktywności enzymatycznej białkowej fosfatazy tyrozynowej CD45

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WSTĘP TEORETYCZNY

Odwracalna fosforylacja białek jest uniwersalnym mechanizmem kontroli aktywności białek w komórkach eukariotycznych, a jej przebieg regulowany jest przeciwstawnym działaniem kinaz i fosfataz białkowych. Kinazy katalizują przyłączenie grupy fosforanowej do reszty aminokwasowej, podczas gdy fosfatazy hydrolitycznie tę grupę usuwają.

Białkowe fosfatazy tyrozynowe regulują szlaki sygnalizacji komórkowej kontrolujące rozliczne funkcje komórek i tkanek, np. proliferację, różnicowanie czy wzrost. Nieprawidłowe działanie tych enzymów związane jest z licznymi schorzeniami, w tym z procesem nowotworzenia. Zaobserwowano również związek białkowych fosfataz tyrozynowych z patogenezą chorób wirusowych oraz procesem infekcji w przypadku niektórych bakterii patogennych.

Białkowa fosfataza tyrozynowa CD45, zlokalizowana głównie w błonie komórkowej leukocytów, odpowiedzialna jest za prawidłowe funkcjonowanie systemu odpornościowego. Fosfataza CD45 bierze udział w transdukcji sygnału związanej z aktywacją limfocytów T i B. Nieprawidłowa ekspresja genów fosfatazy CD45 może być przyczyną zaburzeń immunologicznych, jak np. ciężki złożony niedobór odporności (SCID) lub autoimmunologicznych, np. obserwowanych u chorych na stwardnienie rozsiane (SM). Wykazano także udział fosfatazy CD45 w procesie odrzucenia przeszczepu.

W ludzkim genomie znajduje się ponad sto genów kodujących ok. 500 białkowych fosfataz tyrozynowych. Różnorodność białkowych fosfataz jest związana, m.in. z procesami alternatywnego splicingu mRNA oraz modyfikacjami potranslacyjnymi.

Ze względu na strukturę pierwszorzędową domen katalitycznych, białkowe fosfatazy tyrozynowe możemy podzielić na cztery klasy. Do klasy I należy najliczniejsza grupa klasycznych fosfataz tyrozynowych, które w zależności od lokalizacji komórkowej możemy podzielić na: fosfatazy receptorowe występujące w błonie komórkowej oraz niereceptorowe występujące w cytozolu.

Fosfataza CD45 należy do grupy klasycznych receptorowych fosfataz tyrozynowych i jak większość receptorowych fosfataz, posiada dwie cytoplazmatyczne domeny fosfatazowe. Domena zlokalizowana bliżej błony komórkowej wykazuje aktywność katalityczną, natomiast druga domena pełni wyłącznie rolę regulatorową. Enzymy należące do grupy fosfataz niereceptorowych charakteryzują się występowaniem pojedynczej domeny katalitycznej.

Domena katalityczna klasycznych fosfataz tyrozynowych składa się z ok. 280 reszt aminokwasowych i charakteryzuje się wysoką homologią sekwencji. W obrębie domeny fosfatazowej znajdują się zachowane ewolucyjnie motywy strukturalne, t.j. pętla wiążąca fosfotyrozynę substratu, pętla rozpoznająca fosfotyrozynę substratu, pętla WPD oraz pętla Q.

Wspólną cechą całej rodziny klasycznych fosfataz tyrozynowyh jest posiadanie w centrum katalitycznym konserwowanej ewolucyjnie sekwencji VHCSXGXGR tworzącej pętlę wiążącą fosfotyrozynę substratu, wewnątrz której znajduje się reszta katalitycznie aktywnej cysteiny oraz argininy o szczególnym znaczeniu dla aktywności enzymatycznej. Katalityczna reszta cysteiny pełni główną rolę w procesie katalizy (usuwaniu grupy fosforanowej). Szczególne znaczenie katalitycznej cysteiny podkreśla fakt, iż zamiana tego aminokwasu skutkuje całkowitą utratą aktywności katalitycznej enzymu.

Wszystkie klasyczne fosfatazy tyrozynowe wykorzystują ten sam mechanizm katalityczny, realizowany w dwóch etapach. W pierwszym etapie dochodzi do ataku nukleofilowego katalitycznej reszty cysteiny na fosfotyrozynę substratu i powstania przejściowej formy fosfo-cysteiny. W drugim etapie fosfo-cysteina hydrolizowana jest przez cząsteczkę wody, prowadząc do uwolnienia grupy fosforanowej i tym samym do reaktywacji czasteczki enzymu.

Aktywność białkowych fosfataz tyrozynowych, jako enzymów biorących udział w regulacji kluczowych ścieżek sygnałowych w komórce, jest ściśle kontrolowana poprzez modulację ekspresji ich genów oraz modyfikacje postranslacyjne białka, takie jak fosforylacja, defosforylacja, utlenienie oraz ograniczona proteoliza. Aktywność niektórych receptorowych fosfataz tyrozynowych jest dodatkowo regulowana poprzez dimeryzację.

Szczególne znaczenie ma kontrola aktywności fosfataz tyrozynowych przez utlenienie.

Reszta cysteiny w centrum katalitycznym enzymu występuje w postaci anionu tiolowego i jest wysoce podatna na utlenienie. Utlenienie katalitycznej cysteiny prowadzi do utraty aktywności katalitycznej enzymu. W zależności od stopnia utlenienia, katalityczna reszta cysteiny może przekształcić się w resztę kwasu sulfenowego (SOH), kwasu sulfinowego (SO_2H) lub sulfonowego (SO_3H). Utlenienie reszty cysteiny do reszty kwasu sulfenowego jest odwracalne, podczas gdy odpowiednio wysoki potencjał oksydacyjny środowiska powoduje przekształcenie reszty cysteiny do nieodwracalnych pochodnych kwasu sulfinowego i sulfonowego. Konwersja formy utlenionej do aktywnej formy zredukowanej możliwa jest poprzez powstanie przejściowej struktury sulfenylamidu. Przypuszcza się, że odwracalne utlenienie katalitycznej cysteiny może być uniwersalnym mechanizmem regulacji aktywności białkowych fosfataz tyrozynowych.

Wcześniejsze badania pokazują, że nadtlenek wodoru może hamować aktywność białkowych fosfataz tyrozynowych poprzez utlenienie katalitycznej reszty cysteiny. Nadtlenek wodoru w obecności kwasów karboksylowych może ulegać aktywacji do znacznie silniejszych utleniaczy - nadkwasów. Podczas reakcji tworzenia nadkwasu dochodzi do wprowadzenia atomu tlenu z nadtlenku wodoru do karboksylowego prekursora, a tym samym do powstania grupy nadkarboksylowej. Cząsteczka nadkwasu z racji posiadanej grupy nadkarboksylowej charakteryzującej się wysokim potencjałem oksydacyjnym jest silnym utleniaczem. Będąc relatywnie słabszymi kwasami od homologicznych kwasów karboksylowych, w fizjologicznym pH nadkwasy mogą ulegać dyspropionacji do odpowiedniego kwasu karboksylowego i wysoce reaktywnej cząsteczki tlenu singletowego.

Analiza potencjału elektrostatycznego na powierzchni białkowych fosfataz tyrozynowych wykazuje, że posiadają one stosunkowo wysoki ładunek dodatni w obrębie centrum katalitycznego, który mógłby być silnym atraktorem dla ujemnie naładowananej grupy nadkarboksylowej nadkwasu. Białkowe fosfatazy tyrozynowe, z racji wysokiej wrażliwości na utlenienie, mogłyby być potencjalnie inaktywowane przez powstające w wyniku aktywacji nadtlenku wodoru cząsteczki nadkwasów.

Nadtlenek wodoru jest obecny w organizmie ludzkim jako produkt uboczny szeregu aerobowych przemian metabolicznych. Sugeruje się, że do produkcji nadkwasów (w reakcji nadtlenku wodoru z kwasami karboksylowymi) może dochodzić w przebiegu niektórych chorób związanych z uwalnianiem w nadmiarze kwasów karboksylowych, takich jak zapalenie wątroby czy zespół Reye'a. Niezależne grupy badawcze stwierdziły także obecność zwiększonych steżeń kwasów kapronowego oraz arachidonowego w skórnych zmianach łuszczycowych.

Wykazano ponadto, że w przebiegu choroby alkoholowej może dochodzić do powstania nadkwasu octowego - prawdopodobnie wskutek reakcji nadtlenku wodoru z kwasem octowym jako produktem metabolizmu etanolu. Zaobserwowano również, że powstający nadkwas octowy może być inhibitorem enzymu błonowego biorącego udział w regeneracji fosfolipidów, tj. acylotransferazy arachidonylo-CoA:1-palmitoilo-sn-glicero-3-fosfocholinowej występującej w błonie komórkowej erytrocytów.

CELE PRACY

1. Zbadanie wpływu nadtlenku wodoru i powstających w reakcji z nim nadkwasów na aktywność enzymatyczną białkowej fosfatazy tyrozynowej CD45

2. Podjęcie próby wyjaśnienia mechanizmu inaktywacji fosfatazy CD45 indukowanej przez nadkwasy różniące się długością reszty alkilowej oraz wykazanie zależności pomiędzy strukturą i właściwościami chemicznymi inhibitora a stopniem i charakterem hamowania aktywności fosfatazowej CD45.

ZNACZENIE PROJEKTU

Niniejszy projekt naukowy podejmuje tematykę dotyczącą regulacji aktywności białkowej fosfatazy tyrozynowej CD45. Fosfataza tyrozynowa CD45 stała się w ostatnich latach potencjalnym celem terapeutycznym, z racji jej udziału w rozwoju licznych chorób immunologicznych lub autoimmunologicznych, oraz w procesie odrzucania przeszczepianych narządów. Ze względu na jej szczególne znaczenie i właściwości biologiczne, wyjaśnienie mechanizmu regulacji aktywności fosfatazy CD45 oraz próba poszukiwania potencjalnych substancji, z którymi mogłyby specyficznie oddziaływać (ligandów), wydają się być bardzo istotne. Próba poznania mechanizmów regulacji białkowych fosfataz tyrozynowych, bedących modulatorami sygnalizacji komórkowej, a tym samym rozlicznych funkcji komórek i tkanek, wydaje się ważnym celem badań naukowych.

Zagadnienia dotyczące nadkwasów były dotychczas rzadko opisywane w piśmiennictwie światowym. Nadkwasy, które traktowane były do tej pory głównie jedynie jako narzędzie badawcze, mogą mieć szczególne znaczenie w biologicznej regulacji białkowych fosfataz tyrozynowych. Białkowe fosfatazy tyrozynowe posiadają w centrum katalitycznym sekwencję zawierającą szczególnie wrażliwą na utlenienie cysteinę, która warunkuje aktywność enzymu jedynie w stanie zredukowanym. Aktywność białkowych fosfataz tyrozynowych może być hamowana przez nadtlenek wodoru. Nadtlenek wodoru w obecności kwasów karboksylowych może być aktywowany do znacznie silniejszych utleniaczy - nadkwasów. Nadkwasy, posiadające silne właściwości uteniające mogą być potencjalnymi inhibitorami białkowych fosfataz tyrozynowych. Nadkwasy powstają w reakcji nadtlenku wodoru z kwasami karboksylowymi. Od odpowiadającego im kwasu karboksylowego różnią

się posiadaniem grupy nadkarboksylowej, z dodatkowym atomem tlenu, nadającą nadkwasom silne właściwości utleniające. W niniejszej pracy po raz pierwszy nadkwasy zostały zaprezentowane jako cząsteczki o potencjalnym znaczeniu fizjologicznym. Badanie wpływu nadkwasów na aktywność enzymatyczną białkowych fosfataz tyrozynowych przedstawia nowatorskie podejście do poszukiwania nowych, selektywnych utleniaczy o znaczeniu regulacyjnym, które mogłyby stać się w przyszłości prototypami nowych leków stosowanych w terapii niektórych zaburzeń metabolicznych.

MATERIAŁY I METODY

Ocena aktywności rekombinowanej fosfatazy tyrozynowej CD45

Pomiar aktywności fosfatazy został wykonany z użyciem syntetycznego substratu *p*-nitrofenylofosforanu (*p*NPP) z wykorzystaniem metody kolorymetrycznej. Fosfataza hydrolizuje pNPP do *p*-nitrofenolu i nieorganicznego fosforanu. W środowisku zasadowym (pH 7,4) powstający *p*-nitrofenol jest intensywnie żółty, co umożliwia kolorymetryczną ocenę względnego stężenia produktu powstającego w próbie w określonym czasie. Wartość współczynnika absorbancji mierzona spektrofotometrycznie przy długości fali 405 nm jest wprost proporcjonalna do aktywności enzymatycznej fosfatazy przy nadmiarze substratu (kinetyka zerowego rzędu).

Ocena odwracalności inhibicji enzymatycznej

Pomiar odzyskania aktywności przez enzym po uprzedniej inaktywacji (a przez to, pośrednio ocena stopnia utlenienia katalitycznej cysteiny) była możliwa dzięki użyciu czynnika redukującego - ditiotreitolu (DTT). Umożliwiło to ustalenie, czy dany inhibitor inaktywuje fosfatazę CD45 w sposób odwracalny, czy też nieodwracalny.

Ocena aktywności fosfatazy tyrozynowej w komórkch Jurkat E6.1

W projekcie została wykorzystana linia komórkowa Jurkat, Clone E6-1 (białaczka limfoblastyczna) ze względu na wysoki poziom ekspresji fosfatazy CD45. Hodowla komórek była prowadzona z wykorzystaniem medium RPMI 1640 przy gęstości zawiesiny 1×10^6 komórek/ml.

Aktywność fosfatazy CD45 była mierzona w lizatach komórek Jurkat E6.1. wykorzystując metodę immunoprecypitacji ze specyficznymi dla fosfatazy CD45 przeciwciałami oraz substrat specyficzny dla fosfataz tyrozynowych. Ocena aktywności enzymatycznej była możliwa dzięki detekcji kolorymetrycznej (przy długości fali 620 nm) powstającego wolnego fosforanu za pomocą reakcji z zielenią malachitową.

Oznaczanie zawartości białka i fosfatazy CD45 w lizacie

Pomiar ilości białka w lizacie komórek Jurkat przeprowadzono za pomocą metody Bradford, wykorzystującej zmianę barwy błękitu kumasyny po związaniu z białkiem. Ilość białka mierzona była kolorymetrycznie przy długości fali 595 nm. Zawartość białkowej fosfatazy tyrozynowej CD45 w lizacie oznaczono za pomocą immunoprecypitacji, z użyciem

specyficznych przeciwciał sprzężonych z peroksydazą chrzanową. Ocenę ilości fosfatazy umożliwiła detekcja kolorymetryczna (przy długości fali 492 nm) z zastosowaniem substratu dla peroksydazy chrzanowej ortofenylenodiaminy (OPD).

Ocena żywotności komórek

Ocena żywotności komórek po traktowaniu nadtlenkiem wodoru i wybranymi nadkwasami została przeprowadzona z wykorzystaniem błękitu trypanu. Komórki żywe i martwe (zabarwione na niebiesko-fioletowo) były zliczane pod mikroskopem świetlnym, a następnie obliczano procent żywych komórek w preparacie.

Synteza nadkwasów

Synteza nadkwasów została przeprowadzona w Katedrze i Zakładzie Chemii Medycznej Gdańskiego Uniwersytetu Medycznego dzięki uprzejmości i nieocenionej pomocy dr Zbigniewa Wypycha. Do syntezy została wykorzystana metoda Parker'a, polegająca na reakcji kwasów karboksylowych z 50-60% nadtlenkiem wodoru w obecności 70% kwasu siarkowego jako katalizatora. Czystość otrzymanego produktu została sprawdzona za pomocą spektroskopii NMR i IR. Nadkwasy były przechowywane w postaci proszku w temp. -80°C.

Modelowanie komputerowe odziaływania białkowej fosfatazy tyrozynowej CD45 z nadtlenkiem wodoru i nadkwasami

Modelowanie komputerowe zostało przeprowadzone w Katedrze Onkologii, Uniwersytetu Alberta w Edmonton w Kanadzie dzięki uprzejmości i nieocenionej pomocy dr Philipa Winter pod nadzorem profesora Jacka Tuszynskiego. Wykonano symulację komputerową oddziaływania nadkwasów z centrum katalitycznym białkowej fosfatazy CD45 przy pomocy programu AutoDock Vina.

Analiza komputerowa pozwoliła na ocenę powinowactwa nadkwasów oraz nadtlenku wodoru do centrum katalitycznego fosfatazy CD45. Modelowanie komputerowe umożliwiło ustalenie najbardziej prawdopodobnych konformacji, w jakich cząsteczka inhibitora może związać się i oddziaływać z resztami aminokwasowymi wewnątrz centrum katalitycznego fosfatazy CD45.

Przygotowanie receptora (białkowej fosfatazy tyrozynowej) oraz ligandów

Z pobranej struktury pdb z ProteinDataBank, możliwe było wyekstrahowanie domeny katalitycznej. Poprawność sekwencji aminokwasowej została sprawdzona przy użyciu

bazy UniProt. Do wygenerowania modelowej struktury posłużył serwer SWISS-MODEL (http://swissmodel.expasy.org/). Struktury ligandów (nadkwasów i nadtlenku wodoru) zostały rozrysowane za pomocą ChemDraw i sformatowane za pomocą oprogramowania Schroedinger LigPrep wersja 25111 (dodanie atomów wodoru, wygenerowanie trójwymiarowej konformacji). Program AutoDockTools wersja 1.5.4 został wykorzystany do konwersji receptora oraz biblioteki ligandów do formatu PDBQT.

Analiza statystyczna

Wszystkie eksperymenty zostały wykonane przynajmniej trzykrotnie. Analiza statystyczna została przeprowadzona z użyciem oprogramowania GraphPad Prism wersja 4 z zastosowaniem testu ANOVA oraz pomocniczego testu Tukey'a. Dane prezentują średnią ± SD. Dla obserwowanych różnic przyjęto poziom istotności statystycznej $p < 0,05$.

WYNIKI I DYSKUSJA

Nadkwasy są silniejszymi inhibitorami fosfatazy CD45 niż nadtlenek wodoru

Wcześniejsze badania wykazały, że nadtlenek wodoru może hamować aktywność białkowych fosfataz tyrozynowych poprzez utlenienie katalitycznej cysteiny. Nadtlenek wodoru w obecności kwasów karboksylowych może prowadzić do powstania nadkwasów, które są znacznie silniejszymi utleniaczami. W czasie realizacji projektu, ukazała się praca Bhattacharya i wsp. (2008) pokazująca, że nadkwas octowy może powodować inaktywację białkowej fosfatazy tyrozynowej PTP1B.

Wyniki doświadczeń przeprowadzonych w ramach pracy doktorskiej wykazały, że wybrane nadkwasy są znacznie silniejszymi inhibitorami białkowej fosfatazy CD45 niż nadtlenek wodoru (IC_{50}=8 μM), z wartościami wynoszacymi odpowiednio IC_{50}=25 nM dla nadkwasu oktanowego oraz IC_{50}=40 nM dla nadkwasu dekanowego. Nadkwasy powodowały inaktywację zarówno rekombinowanej CD45, jak i fosfatazy CD45 występującej natywnie w komórkach Jurkat. Badania wykazały również, że traktowanie komórek Jurkat 50 μM nadkwasami przez 48 godzin nie miało wpływu na żywotność komórek, podczas gdy inkubacja komórek z nadtlenku wodoru w tym samym stężeniu powodowała ok. 60-procentową utratę żywotności komórek.

Wyniki symulacji komputerowej wykazały, że nadkwasy miały znacząco wyższe powinowactwo do centrum katalitycznego fosfatazy tyrozynowej CD45 niż nadtlenek wodoru. Brak silnego powinowactwa nadtlenku wodoru tłumaczyć może niewielki rozmiar cząsteczki oraz brak zdolności do hydrofobowej interakcji z aromatycznymi resztami aminokwasów zlokalizowanych w centrum katalitycznym. Wyniki te pozwalają przypuszczać, że inaktywacja aktywności fosfatazy CD45 powodowana przez nadtlenek wodoru ma charakter niespecyficzny.

Średniołańcuchowe nadkwasy inaktywują fosfatazę CD45 w najwyższym stopniu

Wyniki przeprowadzonych doświadczeń z rekombinowanym enzymem oraz na linii komórek Jurkat ukazały wysoce selektywne hamujące działanie niektórych nadkwasów na aktywność enzymatyczną fosfatazy CD45. Okazało się, że wybiórcza inhibicja związana była z różną długością łańcucha acylowego nadkwasów. Spośród krótko-, średnio- oraz długo-łańcuchowych nadkwasów, średnio-łańcuchowe nadkwasy najsilniej hamowały aktywność fosfatazy CD45. Enzym rekombinowany najsilniej hamowany był przez nadkwas oktanowy

(8-węglowy), natomiast nadkwas dekanowy (10-węglowy) był nadkwasem powodującym największy spadek aktywności fosfatazy CD45 w komórkach Jurkat.
W celu wyjaśnienia owej wybiórczej inhibicji, przeprowadzone zostało modelowanie komputerowe, którego wyniki wykazały, że nadkwasy średnio-łańcuchowe posiadają największą zdolność przyłączenia się do centrum katalitycznego fosfatazy CD45.
Nadkwasy 8, 9 i 10-węglowy wykazują największą wartość powinowactwa (-5.8 kcal/mol dla nadkwasu C10), prawie dwukrotnie silniejszą niż nadtlenek wodoru (-3.1 kcal/mol).
Można by wysunąć hipotezę, że optymalna wielkość średnio-łańcuchowych nadkwasów pozwala na ich preferencyjną interakcję steryczną z centrum katalitycznym fosfatazy CD45.

Nadkwasy średnio-łańcuchowe powodują nieodwracalną inaktywację fosfatazy CD45

W celu wyjaśnienia mechanizmu inhibicji fosfatazy CD45 przez nadkwasy, testowano odwracalność reakcji inaktywacji za pomocą reduktora tioli (DTT). Wyniki doświadczeń wykazały, że inaktywacja fosfatazy CD45 przez średnio-łańcuchowe nadkwasy jest nieodwracalna. Aktywność enzymatyczna CD45 utracona przez działanie średnio-łańcuchowego nadkwasu mogła być przywrócona jedynie w kilku procentach w stosunku do kontroli. Traktowanie fosfatazy CD45 krótko-, i długo-łańcuchowymi nadkwasami oraz nadtlenkiem wodoru powodowało odwracalną inaktywację enzymu; aktywność enzymatyczna powracała prawie w 100% po inkubacji z DTT.
Prace innej grupy badaczy (Bhattacharya i wsp., 2008) pokazują, że nadkwas octowy inaktywuje białkową fosfatazę tyrozynową PTP1B odwracalnie. We wspomnianym artykule, badacze sugerują, że odwracalna inaktywacja fosfataz tyrozynowych może być powodowana przez wszystkie nadkwasy. Wyniki niniejszej pracy wskazują jednak, że mechanizm ten może nie dotyczyć średnio-łańcuchowych nadkwasów. Ze względu na optymalną wielkość oraz strukturę średnio-łańcuchowych nadkwasów, mogą one stosunkowo łatwo wnikać do centrum katalitycznego fosfatazy CD45 prowadząc do sekwencyjnego utlenienia katalitycznej reszty cysteiny do odwracalnej pochodnej kwasu sulfenowego, i dalej do fizjologicznie nieodwracalnych reszt kwasu sulfinowego lub sulfonowego.

Grupa nadkarboksylowa warunkuje zdolność nadkwasu do inaktywacji fosfatazy CD45

Aby wykluczyć niespecyficzny wpływ hamujący węglowodorowego łańcucha nadkwasów, porównano wpływ nadkwasu i odpowiadającego mu homologicznego kwasu karboksylowego na aktywność enzymatyczną fosfatazy CD45 rekombinowanej oraz występującej natywnie

w komórkach Jurkat. Zastosowane nadkwasy zawierające grupę nadkarboksylową o wysokim potencjale oksydacyjnym, skutecznie inaktywowały fosfatazę CD45, podczas gdy prawie identyczne strukturalnie kwasy karboksylowe (w takim samym stężeniu) pozostawały zasadniczo bez wpływu na aktywność fosfatazową CD45.

Zaobserwowano także, że kwas dekanowy w mieszaninie z nadtlenkiem wodoru wykazywał silniejsze działanie hamujące niż sam nadtlenek wodoru. Nasuwa to przypuszczenie, że w wyniku reakcji nadtlenku wodoru z kwasem dekanowym mógł powstać nadkwas dekanowy.

Analiza komputerowa powinowactwa kwasów karboksylowych i odpowiadających im homologicznych nadkwasów do centrum katalitycznego fosfatazy CD45 nie wykazała znaczących różnic między tymi związkami. Pomimo tego, że kwasy karboksylowe posiadają zbliżone powinowactwo wiązania w centrum katalitycznym fosfatazy CD45, nie powodowały one hamowania jej aktywności. Różnica pomiędzy grupą nadkarboksylową nadkwasu a grupą karboksylową homologicznego kwasu okazuje się być decydująca w kontekście zdolności hamowania aktywności fosfatazy CD45. Na podstawie otrzymanych wyników, można wysnuć hipotezę, że za inaktywację białkowej fosfatazy CD45 powodowanej przez nadkwasy odpowiedzialne jest utlenienie katalitycznej cysteiny przez grupę nadkarboksylową nadkwasu, a tym samym można wykluczyć mechanizm inhibicji powodowanej przez steryczne zablokowanie centrum katalitycznym enzymu.

Modelowanie komputerowe interakcji nadkwasu z fosfatazą CD45 wykazało, że cząsteczka nadkwasu może związać się w centrum katalitycznym fosfatazy, a przyjęta po związaniu konformacja pozwala na wprowadzenie silnie utleniajacej grupy nadkarboksylowej w okolicę reszty cysteiny, mającej kluczowe znaczenie dla aktywności fosfatazowej CD45. Wyniki symulacji komputerowej pozwalają przypuszczać, że dochodzi do oddziaływania elektrostatycznego między ujemnie naładowaną grupą nadkarboksylową nadkwasu oraz resztą argininy, wchodzącą w skład konserwowanej sekwencji centrum katalitycznego fosfatazy CD45. Możliwe również, że dochodzi do stabilizacji cząsteczki nadkwasu w centrum katalitycznym enzymu dzięki oddziaływaniom hydrofobowym pomiędzy łańcuchem acylowym nadkwasu a resztą tyrozyny znajdującą się w pętli rozpoznającej fosfotyrozynę substratu.

WNIOSKI

1. Nadkwasy wykazują znacznie silniejsze niż nadtlenek wodoru działanie hamujące aktywność enzymatyczną białkowej fosfatazy tyrozynowej CD45 mierzone w zakresie stężeń nanomolowych.

2. Nadkwasy średnio-łańcuchowe, zawierające 8 i 10 atomów węgla, wykazują największą zdolność do inaktywacji fosfatazy CD45 zarówno rekombinowanej oraz występującej natywnie w komórkach Jurkat.

3. Mechanizm inaktywacji białkowej fosfatazy tyrozynowej CD45 przez nadkwasy wydaje się być związany z utlenieniem katalitycznej cysteiny przez grupę nadkarboksylową nadkwasu.

4. Wyniki symulacji komputerowej pokazują, że pod względem sterycznym, nadkwasy mogą zostać związane w centrum katalitycznym fosfatazy CD45.

5. Molekularna analiza komputerowa wykazała, że nadkwasy charakteryzują się większym niż nadtlenek wodoru powinowactwem wiązania do centrum katalitycznego fosfatazy CD45, a także, że powinowactwo to jest największe dla średnio-łańcuchowych nadkwasów, co pozostaje w zgodności z uzyskanymi wynikami badań doświadczalnych.

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