7

PLP-Dependent Enzymes: a Potent Therapeutic Approach for Cancer and Cardiovascular Diseases

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1. Introduction

The evolution on the field of biotechnology and proteomics create a novel therapeutic field (Enzymo-therapy) for treatment of various types of disease, particularly in the last fifty years. Exploitation of enzymes as anticancer, anti-cardiovascular, anticoagulants, antimicrobials and antioxidants (Vellard, 2003) was absolutely approved by Food and Drug Administration (FDA). Practically, almost of tumor cells were reported to be auxotrophs for L-methionine, glutamine, asparagine and arginine, due to the absence of intrinsic enzymatic systems synthesizing these amino acids, thus it depends for their growth and proliferation on the exogenous supply of these amino acids, that usually from diets (Hoffman and Erbe, 1976, Mecham et al., 1983, Pasut et al., 2007, El-Sayed, 2010). Consequently, L-methioninase, L-glutaminase, L-asparaginase and arginine deiminase were frequently used as common anticancer agents by sequestering their corresponding amino acids from the blood plasma (Hoffman, 1985, Klimberg and McClellan, 1996, Agrawal et al., 2003, Cheng et al., 2005).

Additionally, hyperaccumulation of certain metabolic intermediates as homocysteine and cystathionine usually associated with various cardiovascular diseases and complex disorders (Zou and Banerjee, 2005, Wang and Hegele, 2003). Cystathionine β-synthase and cystathionine γ-lyase are potential for sequestering homocysteine and cystathionine via transsulfuration metabolic pathways for production of glutathione and other antioxidants, so, these enzymes were described as anti-cardiovascular agents (Stipanuk, 2004, Zhu et al., 2008).

Unlike traditional approaches, Enzymo-therapy seems to be the promising therapeutic technology for their great specificity and affinity towards a clue substrate on specific metabolic pathway. However, the structural and conformational complexity of these enzymes makes it more vulnerable to extrinsic parameters, modulators, immunogenicity and proteolysis (Tan et al., 1996, Sun et al., 2003, Pasut et al., 2007). In addition, purification and formulation of these therapeutic enzymes, due to the economic expenses may add further complications.

Therefore, the various approaches for stabilization of these enzymes in situ and decreasing their immunogenicity were the objective of this context. The structural and catalytic identity of pyridoxal phosphate enzymes, molecular aspects to enhance their therapeutic potentiality, in addition to our prospectives will be exploited.

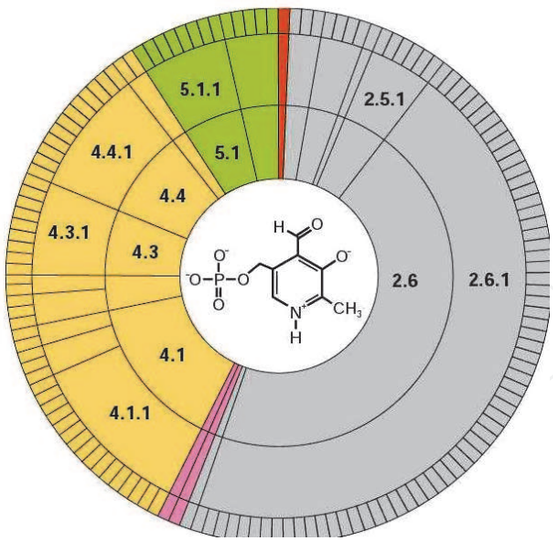
2. Pyridoxal 5'-phosphate dependent enzymes: classification and catalytic identities

Pyridoxal 5'-Phosphate (PLP) dependent enzymes, a group of versatile enzymes including about 145 distinct enzyme, 4% of the total cellular enzymatic activities (www.brenda.com) (Thornton et al., 2000, Percudani and Peracchi, 2003) which mainly uses PLP as covalent co-enzyme. PLP is an active phosphorylated derivative of vitamin B6 (pyridoxine) (Heyl et al., 1951). According to the international biochemical, molecular classification of enzymes (Enzyme Commission) (www.chem.qmul.ac.uk/iubmb), PLP-dependent enzymes were distributed in to five groups; 1- Oxido-reductase (EC 1; one enzyme), 2-Transferase (EC 2; eighty enzymes), 3- Hydrolase (EC 3; two enzymes), 4-Lyase (EC 4; Forty nine enzyme), and 5-Isomerase (EC 5; Thirteen enzyme) as totally 145 enzyme (Grishin et al., 1995, Percudani and Peracchi, 2003) as shown in Fig.1.

Practically, all the PLP-dependent-enzymes are mainly involved in amino acids transformation as decarboxylation, transamination, racemization, β, γ-elimination and β, γ-substitution (Percudani and Peracchi, 2003). The multiple functional catalytic modes of PLP-enzymes according to the position of net reaction were summarized in Table 1. PLP coenzyme has a pivotal role in catalysis of various enzymatic reactions. Pyridoxal phosphate can be synthesized de novo from preexisting precursor in bacteria (Brewke and Leistner, 2001), fungi (Bean et al., 2001, Ehrenshaft and Daub, 2001) and plants (Galperin and Koonin, 1997) from erythrose 4-phosphate, pyruvate and D-glyceraldhyde 3-phosphate. However, in human PLP cannot be synthesized de novo, it must be supplemented from the diet as pyridoxine, pyridoxamine and/or pyridoxal. PLP can be formed from pyridoxal via phosphorylation by action of kinase (PdxK; EC. 2.7.1.35), from oxidation of pyridoxamine/ pyridoxine by oxidase (EC. 1.4.3.5) (Choi et al., 1983, Bahn et al., 2002). Structurally, aldehyde group of PLP are bound covalently as internal aldimine (Schiff base)/ imine linkage to the Ɛ-amino group of lysine residues, close to *N*-terminus (Lopez et al., 2010) as in Fig. (2).

The role of PLP, during catalysis, is to reduce the energy for conversion of amino acids substrates to a zwitterionic carbonion (Richard and Amyes, 2004), substantially the apoenzyme catalyze the cleavage of substrate target bond yielding the product as reviewed by Richard et al. (2011). Regarding to the native internal aldimine of PLP-enzyme, the catalytic process started with formation of external aldimine linkage of amino group of substrate and pyridoxal phosphate coenzyme forming coenzyme-substrate Schiff base, replacing the Ɛ-amino group of lysine-enzyme (Schirch et al., 1993).This external aldimine of Co-enzyme-substrate Schiff base is the common intermediate for all PLP-dependent enzymes. Interestingly, pyridoxal phosphate without apo-enzyme, can slowly mediate many of reactions while the enzyme can ensure the substrate specificity and catalysis (Toney, 2005, Lopez et al., 2010), that justifies the lack of apoenzyme activity, with potentiality to recover its catalytic state upon supplementation by external PLP (El-Sayed, 2011). Since the formation of external aldimine, the pyridoxal phosphate is bounded tightly to the apoenzyme via nine hydrogen bonds (Johnson et al., 1990, Palm et al., 1990). The PLP extremely withdraw electrons, stabilizing the negative charge at α-carbon (carbanion) of the substrate as transition state that commonly referred as Quinonoid intermediates (Metha and Christen, 2000, Hult and Berglund, 2007). The catalytic promiscuity of PLP-dependent enzymes mainly depends on the mechanistic consequence of quinonoid intermediates (Alexander et al., 1994, Toney, 2005) as shown in Fig. (3).

Depending of crystallographic structures and topological studies, PLP-dependent enzymes were categorized in to five distinct folds (I-V) (Alexander et al., 1994, John, 1995, Jansonius, 1998) as illustrated in Table 2. The five independent fold types of PLP-enzymes were: Fold type I; Aspartate Aminotransferase (AAT) family; the most functionally diverse type containing α and γ-families of PLP-enzymes in which the internal aldimine was formed at lysine of *N*-terminus of the short helix at β-strand (Percudani and Peracchi, 2003). On AAT family, pyridine nitrogen is protonated and stabilized by interaction with enzyme aspartic and glutamic acid (Casanovas et al., 2009). Methionine γ-lyase, cystathionine β-synthase, cystathionine γ-lyase were belongs to this family. Fold type II; Tryptophan Synthase β-family, this family represented tryptophan synthase (Hyde et al., 1988), threonine deaminase (Gallagher et al., 1998) and *O*-acetylserine sulfhydrylase (Burkhard et al., 1998). The internal aldimine was at lysine residues of α-helix on the *N*-terminal domain. The apoenzyme was stabilized by interaction of its serine and threonine residues with pyridine ring of PLP (Percudani and peracchi, 2003). Fold type III; Alanine Racemase family; that represented by alanine racemase (Shaw et al., 2000), ornithine decarboxylase (Kern et al., 1999). Physically, the pyridine ring PLP, coenzyme, was interacted with NH moiety of arginine residues of apo-enzyme (Le-Magueres et al., 2005). Fold type IV; D-Amino Acid Aminotransferase family, that involve D-amino acid aminotransferase (Sugio, 1995), branched chain amino acid amino transferase (Okada et al., 1997) and 4-amino-4-deoxychorismate lyase (Nakai et al., 2000) as reviewed by Denesyuk et al. (2002). Like Type I, pyridine of PLP was interacted with glutamic acid of apoenzyme by hydrogen bonds (Sugio, 1995). Fold type V; Glycogen Phosphorylase Family; that contains glycogen phosphorylase (Sprang and Fletterick, 1979, Palm et al., 1990) and maltodextrin phosphorylase (Watson et al., 1999).



PLP-dependent enzymes were within five classes according to enzyme classification system (Enzyme Commission (EC) 1-Oxidoreductases, one enzyme (Red), 2-Transferase, 80 enzyme (faint blue), 3-Hydrolase, two enzymes (faint red), 4-Lyase, 49 enzyme (yellow) and 5-Isomearse, 13 enzyme (green). The catalytic diversity of PLP-dependent enzymes was described by Thornton et al. (2000) and Percudani and Peracchi (2003).

Fig. 1. Pyridoxal Phosphate Dependent Enzymes.

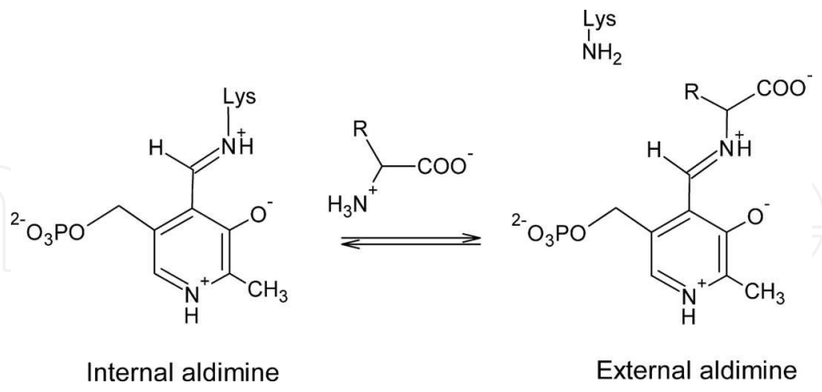


Fig. 2. Internal and External aldimine linkage of Pyridoxal Phosphate with enzyme and substrate, respectively

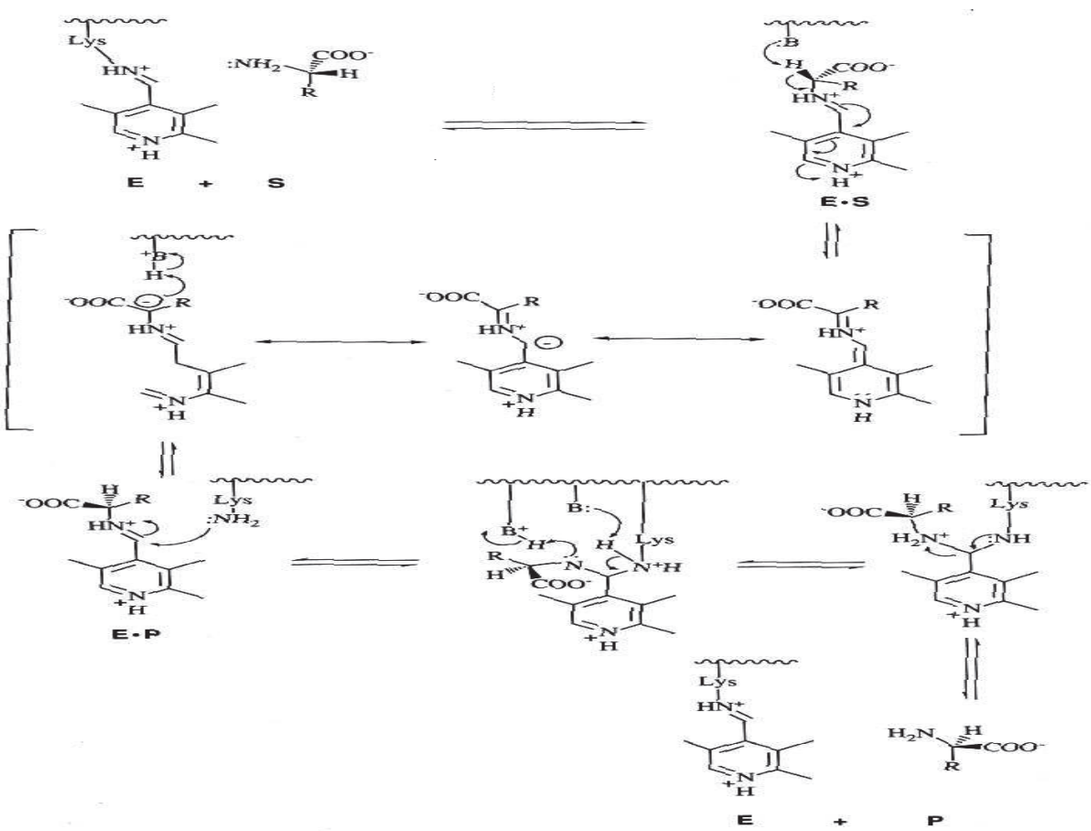


Fig. 3. Mechanism of PLP-enzyme catalysis via Quinonoid intermediates.

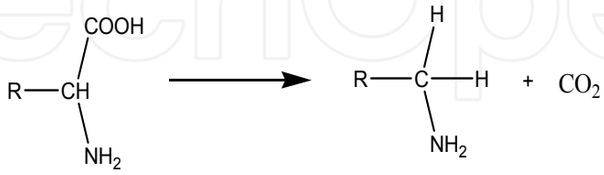
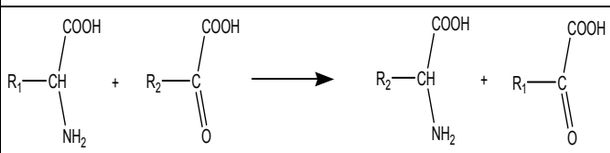
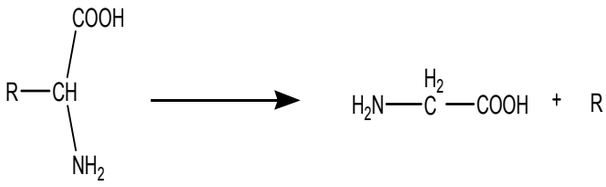
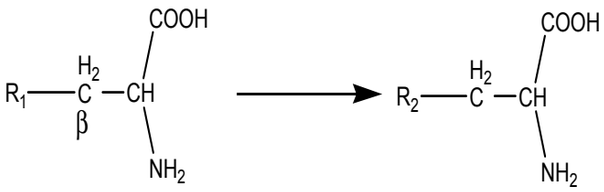
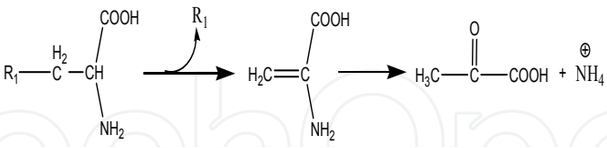
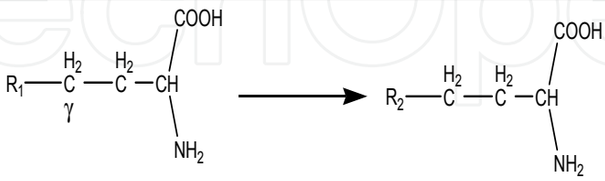
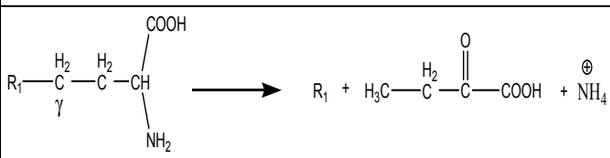
Reaction site	Reaction type	Mechanism of Reaction	Example of PLP-Enzyme
α-Position	Racemization		Alanine racemase
	Decarboxylation		Ornithine decarboxylase
	Transamination		Tyrosine aminotransferase
	Elimination		Serine hydroxyl-methyltransferase
β-Position	Replacement		Tryptophan synthase
	Elimination		Serine dehydratase
γ-Position	Replacement		Cystathionine γ-synthase
	Elimination		Methionine γ-lyase

Table 1. Functional catalytic modes of PLP-enzymes according to the position of net reaction

No.	Fold Type I	No.	
1	Glycine dehydrogenase	50	Tyrosine decarboxylase
2	Glycine hydroxymethyltransferase	51	Glutamate decarboxylase b
3	Threonine aldolase	52	Histidine decarboxylase b
4	Trptophanase	53	Ornithine decarboxylase 1
5	Tyrosine phenol lyase	54	Arginine decarboxylase 1
6	Selenocysteine lyase	55	Lysine decarboxylase
7	Glyoxylate aminotransferase	56	Allinase
8	Serine aminotransferase		
9	Phosphoserine aminotransferase		
10	Amino ketobutyrate CoA ligase		Fold Type II
11	Aminolevulinate Synthase	1	Aminocyclopropane-carboxyl deaminase
12	Amino oxononanoate synthase	2	Tryptophan synthase β
13	Serine palmitoyltransferase	3	Cystathionine β-synthase
14	Kvnureninase	4	O-Acetylserine sulfhydrylase
15	Isopenicillin *N*-epimerase	5	D-Serine dehydratase
16	Cysteine desulfurase	6	L-serine dehydratase
17	Splicing protein SPL1	7	Threonine dehydratase
18	Cystathionine γ-Synthase	8	Threonine synthase 1
19	*O*-Succinylhomoserine sulhydrase	9	Diaminopropionate lyase
20	Methionine γ-Lyase	10	Threonine synthase 2
21	Cystathionine γ-Lyase	11	D-Alanine aminotransferase
22	Cystathionine β-Lyase	12	Amino deoxychorismate lyase
23	Aspartate aminotransferase a	13	Branched amino acid aminotransferase
24	Aromatic amino acid aminotransferase		
25	Tyrosine aminotransferase		
26	Alanine aminotransferase		Fold Type III
27	*malY* gene product	1	Ornithine decarboxylase 2
28	Phenylpuruvate aminotransferase	2	Diaminopimelate decarboxylase
29	Aminocyclopropane carboxylate synthase	3	Arginine decarboxylase
30	Rhizopine regulatory protein		alanine racemase
31	Aspartate aminotransferase b		
32	Aspartate aminotransferase c		
33	Histidinol phosphate aminotransferase		Fold Type IV
34	cobC gene product	1	D-amino acid aminotransferase
35	Valine-Pyruvate aminotransferase	2	branched chain amino acid amino transferase
36	Acetylornithine aminotransferase	3	4-amino-4-deoxychorismate lyase
37	Succinylornithine aminotransferase		
38	Ornithine aminotransferase		
39	Alanine glyoxylate aminotransferase		
40	Dialkylglycine decarboxylase		Fold type V

41	Alanine pyruvate aminotransferase	1	Glycogen phosphorylase
42	Aminooxononanoate aminotransferase	2	Maltodextrin phosphorylase
43	DNTP hexose aminotransferase		
44	Glutamate semialdehyde aminomutase		
45	Aminobutyrate aminotransferase		
46	Lysine Ɛ-aminotransferase		
47	Diaminobutanoate aminotransferase		
48	Glutamate decarboxylase a		
49	Histidine decarboxylase a		

Table 2. Families of Pyridoxal Phosphate dependent enzymes

No hydrogen bonds between pyridine nitrogen and apoenzyme (Percudani and Percacchi, 2003). The systematic and catalytic promiscuity of the five fold types PLP-dependent enzymes as adopted by Metha and Christen (2000) and Percudani and Peracchi (2003), were illustrated in Table 2.
Regarding to our previous work (Khalaf and El-Sayed, 2009, El-Sayed, 2009, 2010, 2011a, b), the subsequent section was focused on the metabolic interactions of sulfur containing amino acid by PLP-dependent enzymes of Aspartate Aminotransferase Family.

3. Metabolic interactions of sulfur-containing amino acids by PLP-dependent enzymes: Biochemical identity of normal and tumor cells

Sulfur-containing amino acids are pivotal compounds for almost of metabolic cellular process. In contrary to humans, plants and microorganisms can synthesize their sulfur containing amino acids from the inorganic precursor as sulfate, sulfite and sulfide via reduction or activation (Beinert, 2000, Kessler, 2006). In humans, methionine and cysteine were described as conditional essential amino acids for normal various biological aspects (Baylin et al., 1998, Davis and Uthus, 2004). The prominent sulfur containing amino acids on living cell are cysteine and methionine, that crucially incorporated on the synthesis of Co-enzyme, vitamins, epigenetic DNA modulators, DNA stabilizers and antioxidants (El-Sayed, 2010 and Cellarier et al., 2003). Additionally, cysteine is the essential source for H_2S biogenesis that critically acts as neuro-modulators, cardio-protector, relaxing muscle regulators, and vasodiolator for vascular system (Wang, 2002, Kimura, 2010, Predmore and Lefer, 2010 and Gadalla and Snyder, 2010). The general metabolic interactions of sulfur containing amino acids and their derivative by PLP-enzymes were illustrated in Fig. (3). Methionine can be metabolized via different ways that collectively regulated by PLP-dependent enzymes as methionine γ-lyase, cystathionine β, γ-synthases and cystathionine β, γ-lyases. In plants and microbes, methionine can be undergoes oxidative deamination/ demethiolation forming methanethiol, α-ketobutyrate and ammonia (Tanaka et al., 1977) via the action of L-methioninase. L-Methioninase was frequently reported as a substrate induced extracellular enzyme for various microbial cultures (Tanaka et al., 1977, Soda et al., 1983; Nikulin et al., 2008, Khalaf and El-Sayed, 2009), while it is absent in mammalian cells (Cooper, 1983). Biochemically, methionine was activated by methionine adenosyltransferase forming *S*-adenosylmethionine as a key intermediate for the following metabolic pathways;

4. Transmethylation pathway and methionine synthesis

S-Adenosylmethionine undergoes de-methylation by glycine-*N*-methyltransferase forming *S*-adenosyl homocysteine (Luka et al., 2006). This reaction is the source for more than 100 biological methylation reactions including epigenetic DNA regulation and protein posttranslational modifications, in living cell (Choi and Mason, 2002) Moreover, in microbial cells, the methyl group from adenosylmethionine can switch the formation of Siroheme via methylation followed by oxidation of Uroporphyrinogen II (Thomas et al., 1997, Hansen et

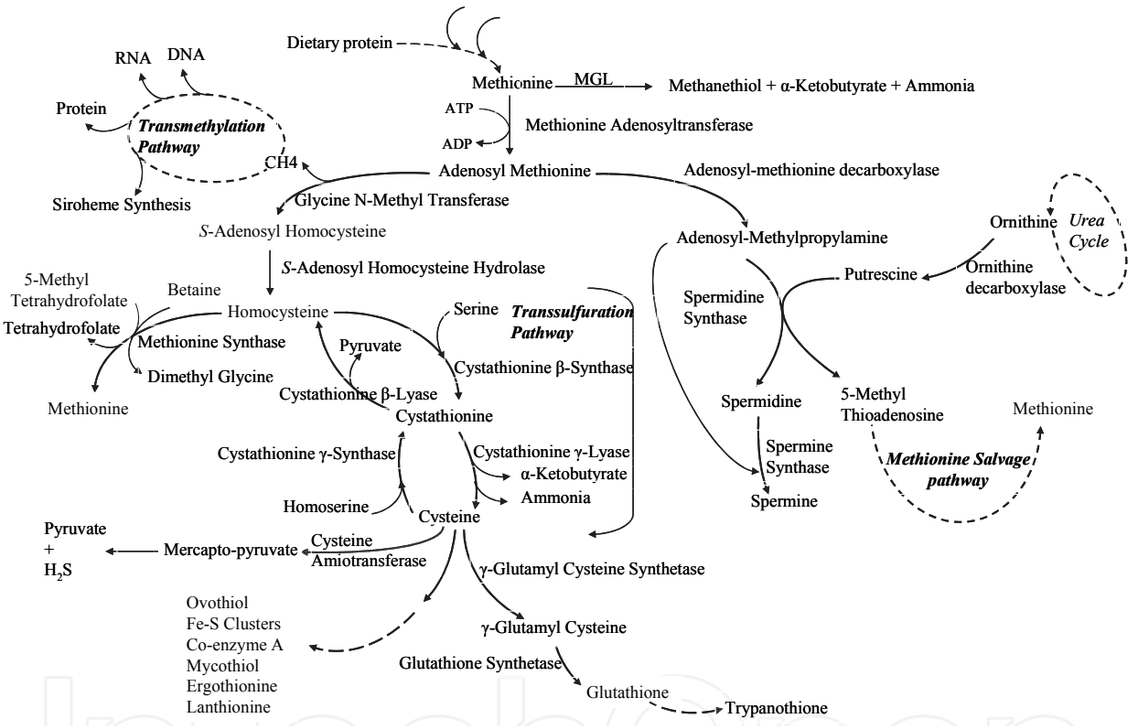


Fig. 3. Metabolic Interactions of sulfur-containing amino acids, as supplied from the diet or intracellular, via different cellular pathways. Except human, L-methionine was undergoes oxidative deamination and demethiolation by L-methioninase (EC 4.4.1.11) in various microorganisms and plants. In all living cells, adenosyl methionine, activated form of L-methionine, was implicated in various pathways: 1- Transmethylation, adenosylmethionine by glycine *N*-methyltransferase is demethylated to adenosyl-homocysteine that involved in methylation of DNA, RNA, and protein. 2- Transsulfuration pathway, forming glutathione and trypanothione as cellular antioxidants, cysteine was involved on formation of several S-containing compounds. 3- Polyamine synthesis, S-adenosylmethionine was decarboxylated by decarboxylase forming adenosyl methylpropylamine that condensate with putrescine forming spermidine by spermidine synthase, then forming spermine by spermine synthase.

al., 1997). Physiologically, tumors are genetically abnormal cells with uncontrolled rapidly growth and proliferation, for mutation on some genes encoding amino acids metabolizing enzymes, comparing to the corresponding normal cells (Hoffman, 1985). Elevated requirements of methionine are the common biochemical criteria by tumor cells comparing to corresponding normal ones, to fulfill their high protein synthesis and regulation of DNA expression (Stern et al., 1984, Swisher et al., 2009). Practically, hypermethylation of DNA is usually correlates with transcriptional silencing of many of tumor suppressor genes, P_{53} genes, thus disrupting DNA repairing systems and cell signaling modulators (Jones and Baylin, 2002, Santini et al., 2001) as reviewed by El-Sayed (2010). Also, methylation of tumor DNA change the identity of CpG islands, thus altering the expression of DNA repairing and apoptosis controlling genes (Sun et al., 1997, Matsukura et al, 2003) and changing of CpG islands (Swisher et al., 2009). On other hand, suppression of methionine synthase genes are a reliable cellular criteria in various cells as bladder, breast, kidney, lung and hematological tumors (Mecham et al., 1983, Hoffman, 1985, Kreis and Goodenow, 1978), thus it described as methionine dependent cells. In contrast, the normal cells have an active methionine synthase with the ability to form methionine from homocysteine in the presence of methyl tetrahydro folate and betaine as methyl group donors (Kenyon et al., 2002). Consequently, the tumor cells have no ability to grow on homocysteine, comparing to the normal growth of healthy cells. The different argumentations for methionine dependency of tumor cells and biochemical identity of L-methioninase as therapeutic agent were comprehensively interpreted by our previous studies (El-Sayed, 2010).

5. Polyamines synthesis

S-Adenosylmethionine was decarboxylated by adenosylmethionine decarboxylase forming *S*-adenosyl-methylpropylamine (Gilmour, 2007) that subsequently reacts with putrescine forming spermidine by spermidine synthase (Fig. 3). Subsequently, S-adenosyl-methyl propylamine react with spermidine forming spermine by action of spermine synthase (Martinez-Lopez et al., 2008). Putrescine residues are catabolically products of ornithine/ arginine as intermediates of urea cycle by action of decarboxylases. Polyamines are biogenic molecules that tightly bound to the poly-anionic macromolecules as DNA, RNA and phospholipids (Igarashi et al., 1982), regulating the gene expression and protein processing. Biochemically, it crucially involved in stabilization of DNA: Chromatin conformational structures (Davidson et al., 1999), reprogramming of some genes by translational frame-shifting mechanism and expression of the targeted overlapped open reading frames (such as nuclear phospholipids P_{53}) (Coffino, 2000). Also, it essentially interact with membrane phospholipids regulating the membrane linked enzyme transporters (Moruzzi et al., 1993), maintaining the proper membrane potentials. In contrast, high levels of polyamines usually associated with high carcinogenesis of skin, breast, liver, kidney (O'Brien, 1976, Pegg, 1988) and over apoptosis (Hoet and Nemery, 2000). Practically, elevated levels of ornithine decarboxylase a pyridoxal phosphate-dependent enzyme, is significantly correlates with high ratio of polyamines that usually used as relevant carcinogenesis marker (Persson et al., 1998).

6. Transsulfuration pathway

Transsulfuration is the main metabolic pathway for transformation of homocysteine to glutathione, and trypanothione (Cellarier et al., 2003), involving two pyridoxal phosphate

dependent enzymes, cystathionine β-synthase and γ-lyase (Fig. 3). By cystathionine β-synthase, homocysteine condensates with serine moiety forming cystathionine, that subsequently oxidized to cysteine, α-ketobutyrate and ammonia by cystathionine γ-lyase (Zou and Baerjee, 2005). Biochemically, transsulfuration pathway contributes in maintaining the cellular balance of cysteine-homocysteine pool that participates in about 50% of total antioxidant formation (Zhu et al., 2008). Practically, inactivation of cystathionine β-synthase causes hyperaccumulation of homocysteine that is a visual risk of cardiovascular diseases, damage to vascular endothelia (De Bree et al., 2002, Wald et al., 2002) and Alzheimer's disease (Morris, 2003). Moreover, the deficiency of cystathionine γ-lyase results in accumulation of cystathionine, known as Cystathioninuria that usually accompanied with diabetes, Down's syndrome, neuroblastoma (Wang and Hegele, 2003, Zhu et al., 2008). Glutathione was synthesized by γ-glutamylation (El-Sayed et al., 2010) of cysteine by γ-glutamyl cysteine synthetase, then complexation with glycine forming glutathione by glutathione synthetase (Cellarier et al., 2003) as reviewed by El-Sayed (2010). Trypanothione as dimmer of glutathione molecules joined by sperimidine linker, as powerful antioxidants against stress (Tover et al., 2003).

7. Rationality of PLP dependent enzymes as antitumor and anti-cardiovascular agents

The absolute dependence of tumor cells on the exogenous supply of plasma L-methionine, not homocysteine, due to their lacks to active methionine synthase is the biochemical target for many therapeutic strategies (Cellarier et al., 2003). Unlike the inactivity of methionine synthase in tumor cells, this enzyme was very active in normal cells ensuring their ability for synthesizing required methionine from homocysteine (Anderson, 1998). The argumentation of biochemical dependence of tumors on methionine synthase or their coenzymes for various cell lines corresponding to the normal cells was comprehensively documented (Liteplo et al., 1991; Fiskerstrand et al., 1994, El-Sayed, 2010). Several approaches were designed for triggering the methionine dependency of tumor cells, for example, starvation of the tumor cells from methionine using methionine-free diets that displays a reliable efficacy against various types of tumor cells (Goseki et al. 1992). However, this strategy is practically inefficient for many technical, therapeutical and economical considerations (Hoffman, 1985, Tan et al., 1996, Hoshiya et al., 1997). Consequently, application of L-methioninase for removal of plasma L-methionine is the potent justifiable strategy towards various methionine dependent tumor cells (Anderson, 1998 and Kokkinakis, 2006). Methionine depletions cause arresting of tumor cell growth on the late of S-G2 phase undergoing eventually apoptosis (Guo et al., 1993, Nagahama et al., 1998). Biochemically, nutrients with depleted or enriched specific amino acid affect on the growth of normal/tumor cells, according to Harper's concept of amino acid balance. Malnutrition of patient is closely associated with severe amino acid metabolic disorders, uncorrectable nitrogen balance with low activity of immune system (Nitenberg and Raynard, 2000). Interestingly, methionine/ valine depleted, tyrosine lowered and arginine enriched was the most rationalized form for inhibition of tumor growth (Chen et al., 2001, He et al., 2003). L-Methioninase was extensively tested a potent anti-proliferative enzyme towards Lewis lung (Yoshioka et al., 1998), human colon (Tan et al., 1998), glioblastoma (Kokkinakis et al., 2001), neuroblastoma (Hu and Cheung, 2009) as reviewed by Sato and Nozaki (2009) and El-Sayed (2010). Recently, Parenteral nutrition is a common co-supportive strategy for various aspects of tumor therapy (Buchman et al., 2006).

L-Methioninase was purified and characterized from different bacterial isolates (Hoffman and Erbe, 1976; Tanaka et al., 1977, Soda et al., 1983, Nikulin et al., 2008), particularly, *Pseudomonas putida* enzyme that extensively structurally studied via crystallographic studies (Ito et al., 1976, Tanaka et al., 1977, Nakayama et al., 1984, Lishko et al., 1993, Motoshima et al., 2000, Kudou et al., 2007). However, the therapeutic response of bacterial enzymes usually associated with high immunogenic reactions, rapid plasma clearance and proteolysis especially with multiple dosing (Tan et al., 1996; Sun et al., 2003), making the patient is more vulnerable to secondary immunogenic disorders by opportunistic pathogens. Also, as result of deaminating activity of this enzyme, extra amounts of ammonia (hyper-ammoniemia) was released during the course of tumor therapy, causing additional hazardous effects to the kidney and liver, as observed for many of anticancer deaminating enzymes (Balcao et al., 2001). Biochemically, hyperaccumulation of ammonia anticipates on formation of extra amounts of arginine and ornithine, via urea cycle, promoting the polyamines synthesis that indirectly induces tumorgenesis (Pegg, 1988, Gerner and Meyskens, 2004). The immunogenic reactions of bacterial enzyme could be attributed to their structural stereo-conformation during the enzyme posttranslational modifications, prokaryotic nature, making distinct epitopes immunogenic sites on the surface of the enzyme as assumed from the phylogenetic analysis of their amino acids (El-Sayed, 2010). Depending on database, the structural amino acid identity of *Arabidopsis thaliana* L-methioninase was similar to that of *Pseudomonas putida* by less than 25 %, suggesting the differences on the surface amino acid constitution (El-Sayed, 2010). Accordingly, L-methioninase was classified structurally in two forms; First, *Pseudomonas-Trichomonas* group that contain six subgroups namely; *Pseudomonas, Bacillus, Brevibacterium*, *Trichomonas*, *Rhizobium* and *Aeromonas.* Second; *Arabidopsis* group which represented by *A. thaliana.* Interestingly, the similarity of amino acid composition of L-methioninase is closely related to the systematic morphological and physiological position of the organism. Actually, the amino acid composition of the fungal L-methioninase open reading frame, nor crystallographic studies was not studied. Therefore, regarding to the therapeutic implications of currently applied L-methioninase, the searching for a novel enzyme with less immunogenic activity and high therapeutic potentiality or modifications of the currently used enzymes is a challenge for many biotechnologists.

8. Different approaches to increase the therapeutic potentiality of l-methioninase

8.1 PEGylation of L-methioninase

Immobilization of the therapeutic enzymes on polyethylene glycol (PEG), PEGylation, is one of the most successful strategies that originated in 1970 (Abuchowski et al., 1977). PEGylation of albumin and catalase was the milestone for the development and globalization of this technique, as efficient method for modification of the structural and immunogenic identities of proteins (Abuchowski et al., 1977). Polyethylene glycol is a neutral, water soluble, hydrophilic polyether with less immunogenicity that ensure various pharmacological properties as increasing half-life time, reducing the kidney clearance, protecting protein from proteolysis by via steric hindrance, reduce the immunogenicity of protein, increasing the solubility of target therapeutic agent (Reddy, 2000, Veronese and Pasut, 2005, Fee and Van Alsteine, 2004, Pasut and Veronese, 2009). The therapeutic behavior of PEGylated-enzyme was illustrated (Fig. 4). PEGylated-enzyme have a reduced

kidney excretion, with more plasma half-life time for the increasing of its molecular size (hydrodynamic radii), masking the sensitive amino to chemical modification, shielding the critical surface active sites from proteolysis, antibodies recognition and/or interaction with inhibitors (Harris, 1991; Zalipsky, 1995; Harris et al., 2001; Veronese and Harris, 2002), comparing to free enzyme. Also, PEGylation reduce the protein opsonization and adhesion to liposomes, microparticles (Fee and Van Alstine, 2004). Currently, PEGylation become a well developed technique for formulations of biopharmaceuticals for improving their solubility, bioavailability and decreasing their immunogenic properties as approved by FDA organization (Pasut et al., 2007). PEGylated forms of therapeutic enzymes as *Escherichia coli* asparaginase (Abuchowski et al., 1979), arginine deiminase (Izzo et al., 2007), *Bacillus subtilis* arginase (Cheng et al., 2005), *Aspergillus flavus* uricase (Bayol et al., 2002) displays affordable therapeutic potentialities comparing to corresponding free enzymes, as declared by FDA.

PEGylation of *Pseudomonas putida* L-methioninase was extensively studied (Tan et al., 1998 and Sun et al., 2003). The half-life time of PEG-L-methioninase was increased to 160 min, comparing to 80 min to the unmodified enzyme. However, the activity of L-methioninase was relatively not inhibited by PEGylation as appeared from the IC_{50} values against human lung and kidney tumors (Tan et al., 1998). In addition, PEGylation enhance the half-life times of the recombinant L-methioninase by about 20 fold and rate of serum methionine depletion by about 12 fold (Sun et al., 2003). Apparently, the degree of enzyme PEGylation usually correlate with the decreasing on enzyme immunogenicity and prolonged half-life time, in vivo. For example, the in vivo half-life times was increased from 12, 18 to 38 h upon degree of PEGylation of L-methioninase by 30/1, 60/1 and 120/1 molar ratios, respectively, comparing to the 2 h for the naked L-methioninase. PEGylation decreases the titer of IgG by about 10 and 10000 fold for PEG-rMETase 30/1 and 120/1 molar ratios, degree of PEGylation comparing to free enzyme (Sun et al., 2003). Also, administration of external pyridoxal phosphate remarkably confers the half-life times and activity of PEG-rMETase. The significant decrease on the enzyme immunogenicity was appeared from the titer of anti-PEG-METase as significantly reduced comparing to the native enzyme. As could be deduced, the more degree of PEGylation, the more masking of the superficial antigenic, epitopes, sites of the enzyme (Sun et al., 2003, Yang et al., 2004). The decrease on the activities of native of PEG-L-methioninase *in vivo* may attributed to dissociation of PLP Co-enzyme (Yang et al., 2004), that being confirmed by our results *in vitro* (El-Sayed, 2011). However, L-methioninase and co-enzyme being easily re-associate upon external supplementation of pyridoxal phosphate as appeared by A420/280 ratio parallel to spectroscopic analysis for determination of the enzyme activity (El-Sayed, 2011). Thus, the combination of PEG-L-methioninase and pyridoxal phosphate infusion, or periodical supplementation of PLP by external osmotic pump, may exhibit a new strategy for prolonging the half-life time, for maximum enzyme therapeutic exploitation. L-Methioninase displays two absorption maxima at 280 nm, as aromatic amino acids containing protein, and at 420 nm due to the internal aldimine linkage of the aldhyde group of pyridoxal phosphate and Ɛ-amino group of lysine N-terminal domain of PLP-enzyme. The absorption spectra of bacterial and fungal L-methioninase (Tanaka et al., 1977, Johnston et al., 1979, El-Sayed, 2011) are typically to other pyridoxal-dependent enzymes (Bertoldi et al., 2002; Saha et al., 2009). The pyridoxal phosphate co-enzyme was dissociate from the apo-enzyme by incubation with 10 mM hydroxylamine, giving one peak at 280nm, with disappearance of the 420 nm peak due to the dissociation of the PLP and hydrolysis of the internal aldimine bond. To evaluate the structural reconstitution potentiality of fungal L-

methioninase, different doses of PLP was supplemented to the apo-enzyme, the structural and catalytical efficiency was monitored. The ratio of A280/420 was decreased from 4.01 to 1.7, upon addition of pyridoxal phosphate (0.2 mM) to the apo-enzyme, indicating the full reconstitution of the active holo-enzyme (El-Sayed, 2011) (Fig.5). Johnston et al. (1979) reported that upon freezing and thawing the ratio of A280/420 of *Pseudomonas ovalis* L-methioninase was increased from 3.90 to 4.7, due to the dissociation of pyridoxal phosphate. *P. ovalis* L-methioninase lacks the ability to restore its original activity by dialysis against pyridoxal phosphate (Johnston et al., 1979), while that of *Trichomonas vaginalis* enzyme restore more than 90 % of its activity by 0.1mM PLP (Lockwood and Coombs, 1991). However, *A. flavipes* L-methioninase has the ability to reconstitute its fully structural catalytic state upon addition of pyridoxal phosphate (0.2mM), similarly to cystathionine γ-lyase (Zhu et al., 2008).

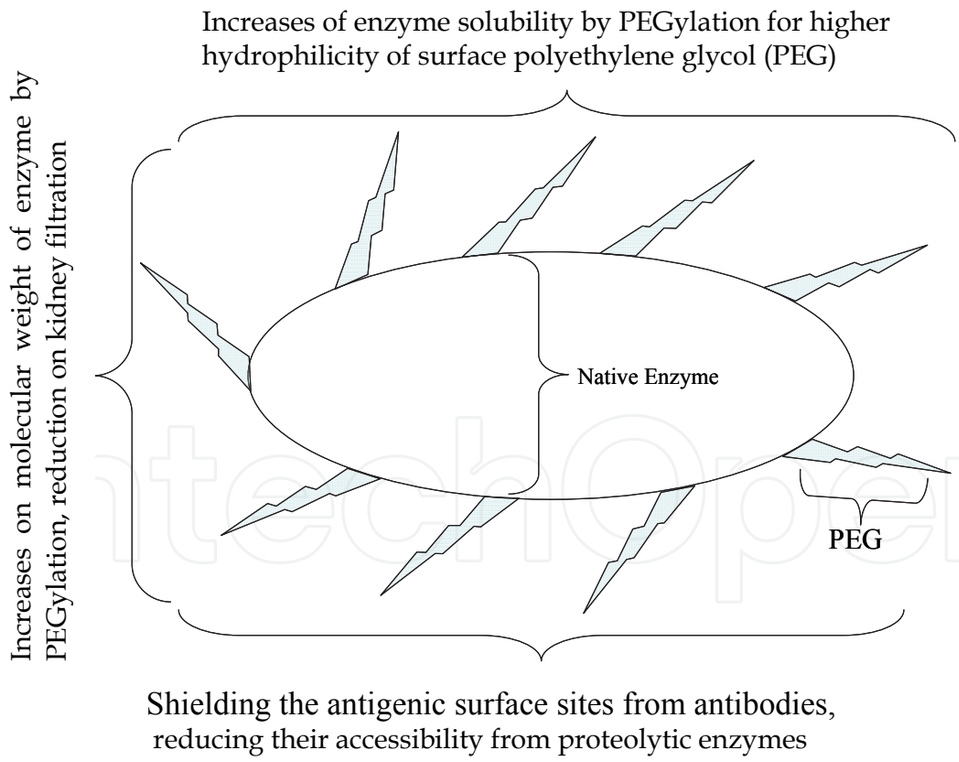


Fig. 4. Prospective of enzyme PEGylation

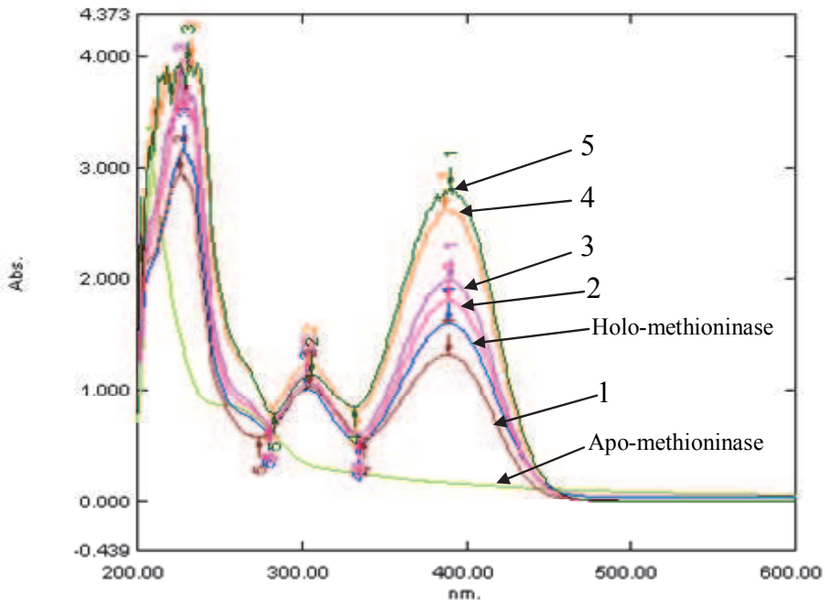


Fig. 5. Absorption spectra of holo and apo-methioninase from *A. flavipes* (El-Sayed, 2011). Apo-methioninase obtained by preincubation of the holo-enzyme (2.2 mg/ml) in potassium phosphate buffer (pH 7.8) with 10 mM hydroxylamine. Reconstruction of the holo-enzyme by incubation of the apo-methioninase with 0.04 mM PLP (1), 0.08 mM PLP (2), 0.1 mM PLP (3), 0.15 mM PLP (4) and 0.2 mM PLP (5).

9. Combination between L-methioninase and chemotherapeutic agents

Synergism between L-methioninase and chemotherapeutic agents present a new strategy against various types of tumors (Yoshioka et al., 1998). Biochemically, starvation of the tumor cells to L-methionine, by action of L-methioninase or L-methionine depleted diets usually make the tumor cells more vulnerable to any biochemical modulator as reviewed by El-Sayed (2010). Methionine starvation and simultaneous phase-specific chemotherapeutic agent is an overall concept for all therapeutic strategies. 5-Fluorouracil, common biochemical modulator, an analogue to thiamine, that competitively bind to thymidylate synthetase, causing prompted suppression to DNA synthesis of tumor cells (Poirson-Bichat et al., 1997). The sensitivity of Lewis lung carcinoma to L-methioninase was increased by about 4.5 fold by addition of 5-fluorouracil (Hoshiya et al., 1997, Tan et al., 1998, Yoshioka et al., 1998). Similarly, doxorubicin as intercalating agent blocks the cell cycle, greatly improves the activity of L-methioninase against human lung carcinoma H460 (Gupta et al., 2003). Ethionine, as methionine analogue, in combination with methionine starved diets display a potent activity against human colon and lung tumors, that attributed to inhibition of methyltransferase leading to DNA, RNA, protein hypomethylation (Razin and Riggs, 1980 and Poirson-Bichat et al., 2000). Also, methionine free diets potentiality against human prostate and glioma was strongly augmented by supplementation of ethionine that attributed to reduction of ATP pool and glutathione synthesis (Guo et al., 1996, Poirson-Bichat et al., 1997). Also, methionine deprivation induce the hypomethylation reactions that lowers the glutathione and alters folate metabolism causing selective arresting to the cell cycle in the late of S/G2 phase, strongly modulate the efficiency of Cisplatin against human

breast carcinoma (Hoshiya et al., 1996). However, continuous starvation of human serum to methionine may exert hazardous implications to liver (Kokkinakis, 2006). Biochemically, combination of methioninase with various therapeutic agents, for methionine starvation, as doxorubicin (Stern et al., 1984), 5-fluoruracil, mitomycin C (Goseki et al., 1992), nitrosoureas (Kokkinakis et al., 2001) and BCNU (Kokkinakis et al. 1997), display a new approach for treatment of methionine dependent tumor cells.

10. L-Methioninase gene therapy and selenomethionine as prodrugs

Introduction of the L-methioninase encoding genes to tumor cells with regulating their expression one of the recent challenges for treating of tumor cells as reviewed by El-Sayed (2010). Transduction of bacterial L-methioninase gene via developed adenoviral vector, with exogenous L-methioninase, display a powerful activity towards human ovarian cancer cells (Miki et al., 2000a). The potentiality of gene therapy for methionine dependent tumors based on deprivation of the tumor cells from the intrinsic L-methionine, so this technique significantly intensified by combination with external L-methioninase, to remove the serum L-methionine. A plausible anti-proliferative activity towards human lung cancer by transduction of *P. putida* L-methioninase gene via retroviral vectors, in combination with methioninase treatment, was observed (Miki et al., 2000b). Transduction of *P. putida* L-methioninase gene to human lung carcinoma using retroviral victors and their therapeutic implications were extensively studied by Tan et al., 1997, Miki et al., 2000a,b, 2001). In contrary to hypomethylation by extrinsic starvation of L-methionine, introduction of p53 genes using retroviral, adenoviral, lipid based delivery, displays an efficient strategy against various types of p53 regulated tumors (Miki et al., 2001). Unfortunately, different genetic therapeutical trials is rarely occurred without signs of biochemical implications as low clinical efficiency and lethal toxicity (Fox, 1999), due to the production of some anti-apoptic mitochondrial proteins that counteract the introduced methioninase gene (Yamamoto et al., 2003). Mitochondrial protein (Bc1-2) is the common released anti-apoptotic proteins that hinder the release of mitochondrial cytochrome *c* to cytosol thus counteracting different gene therapeutic approaches (Hamel et al., 1996, Carsten et al., 2000). Additionally, the low transduction efficiency by retroviral L-methioninase gene hinders their therapeutic potentiality. Recently, a novel strategy for reduction the clinical hazardous and augmentation of the pharmacokinetic impact of this enzyme via combination of gene therapy and selenomethionine as prodrugs as reported by Miki et al. (2001) and Yamamoto et al. (2003). In addition to intracellular and extracellular depletion of L-methionine, introduction of selenomethionine, as non-toxic prodrug, plausibly maximize the therapeutic potentiality of this approach, against tumor cells. Selenomethionine, methionine analogue, is a prodrug that under γ-elimination forming a powerful toxic methylselenol, α-ketobutyrate and ammonia (El-Sayed, 2010). Subsequently methylselenol catalyzes thiols oxidation generating reactive oxygen species as superoxide causing mitochondrial swelling, releasing cytochrome *c*, activation of *caspase* inducing prompt cell apoptosis (Green and Reed, 1998, Miki et al., 2001, Yamamoto et al., 2003). Methylselenol released from L-methioninase gene transduced tumor cell can easily adsorbed by surrounding cells, bystander effect, generating the same toxicity on mitochondrial system (Miki et al., 2001) as reviewed by El-Sayed (2010). Unlike the sensitivity of tumor cells to methylselenol, the normal human cells were not being affected for their lack to L-methioninase (Hoffman, 1984). The sensitivity of tumor cells to transduction by adenovirus methioninase gene and selenomethionine as prodrug was increased by 1000 fold (Miki et al., 2000a) comparing to only gene transduced cells.

Currently, in a revolution of biotechnology in the fight against cancer, directing of the enzyme via antibodies to the target tumor cells for removal of extracellular methionine in addition to transduced enzyme gene and selenomethionine as prodrug is the promising strategy against various types of tumors (Napier et al., 2000, Zhao et al., 2006). This novel approach referred as Antibody Directed Enzyme Prodrug Therapy (ADEPT) that is reliable strategy to avoid the systemic clinical and therapeutical limitations of the traditional approaches (Sharma et al., 2005). However, several trials for construction of enzyme antibodies models (Bagshawe, 1987, Stener and Springer, 2001, Bagshawe et al., 2004) were experimented. However, the design of enzyme-antibody without interactions on the enzyme catalytic sites and specific to receptors on the surface of tumor cells is main challenge by biotechnologists, for approving this strategy. After transduction of L-methioninase gene to the target tumor cells, the enzyme-antibody was introduced to the human plasma followed by injection of selenomethionine as planned by several trials as reviewed by El-Sayed (2010).

11. Exploitation of L-methioninase as a target for antimicrobial drugs

Uniqueness distribution of L-methioninase as intracellular enzyme among all microbial pathogens, but not humans makes this enzyme is a novel target for antibacterial, antifungal and anti-protozoal therapies (Ali and Nozaki, 2007, Sato and Nozaki, 2009). Trifluoromethionine (TFM), a fluorinated methionine that undergoes β, γ-elimination forming trifluoro-methanethiol (CF_3SH) that converted to carbonothionic difluoride which subsequently interacted with primary amino groups of the enzymes lysine moieties, causing cellular toxicities (Alston and Bright, 1983, Sato et al., 2008). TFM was reported as a potent antibacterial agent towards the growth of *Mycobacterium smegmatis*, *M. phlei* and *Candida lipolytica* (Zygmunt and Tavormina, 1966, Sato and Nozaki, 2009), *P. gingivalis, F. nucleatum* (Yoshimura et al., 2002) and antiprotozoal agent *Entaemoba histolytica* (Coombs and Mottram, 2001). Also, TFM is highly effective against many anaerobic bacteria as *Clostridium botulinum* (botulism), *C. difficile* (colitis), *Porphyromonas* sp (tooth decay) and *Bacteroides* sp (intra-abdominal infections) (Finegold and Wexler, 1996). Methionine and TFM was activated by methionine adenosyltransferase for further metabolic biochemical pathways. Mammalian cystathionine γ-lyase doesn't affected by the presence of TFM (Alston and Bright, 1983). Myrsinoic acid B and terpeno-benzoic acid extracted from *Myrisine seguinii,* were reported as potent anti-methioninase, anti-inflammatory and anti-edema agents (Hirota et al., 2002, Ito et al., 2008). Myrsinoic acid exhibits a significant activities against *F. nucleatum* (IC_{50} 0.39 μm), *T. denticoal* (IC_{50} 30.3 μM) and *P. gingivalis* (IC_{50} 82.4 μM), it used as powerful anti-malodor and periodontal disease (Sato and Nozaki, 2009). Propargylglycine as cysteine analogues, was widely reported as suicide L-methioninase catalytic inhibitor, *in vitro* (El-Sayed, 2011), with relatively low deleterious effect on whole growth of amoebic trophozites, *E. histolytica* (Ali and Nozaki, 2007), suggesting alternative shunt for sulfur amino acid metabolism other than L-methioninase (Coombs and Mottram, 2001). Thus, for unique distribution of L-methioninase as intracellular enzyme among pathogenic microbes, the searching for various inhibitors with reliable specificity to this enzyme will be a justifiable new trend of antimicrobials agents.

12. Pyridoxal phosphate-dependent enzymes deficiency and cardiovascular diseases

Biochemically, transsulfuration and reverse-transsulfuration pathways are the pivotal mechanisms for proper accumulation of homocysteine and cystathionine. As shown in Fig.

(3), these pathways are controlled by four PLP-dependent enzymes namely; cystathionine β, γ-synthases and cystathionine γ, β-lyases. Inactivation of transsulfuration enzymes as cystathionine β-synthase causes hyperaccumulation of homocysteine (homocysteinuria) that is a visual risk of cardiovascular diseases, damage to vascular endothelia, increases to the risk of abnormal blood clots and skeletal abnormalities (De Bree et al., 2002; Wald et al., 2002) and Alzheimer's disease (Morris, 2003). Also, homocysteinuria usually associated with intellectual disability, seizures and megaloblastic anemia (Mudd et al., 1985). Practically, it commonly reported as genetic or metabolic disorders that affect on the metabolism of protein, generally one to 200,000 babies are born with genetic disorders. Homocysteinuria is a biochemically disorder due to the deficiency of cystathionine β-synthase, owing to the genetically inborn error of the transsulfuration pathway that increases the homocysteine and methionine, with crucial decreasing to the cysteine pool (Mudd et al., 1985). The major clinical implication of homocysteinuria includes mental retardation, dislocation of the optic lenses, skeletal abnormalities (Schuh et al., 1984). Dietary therapy relying of deprivation of methionine and supplementation of cysteine display a reliable positive result in delaying the clinical manifestations (Pullon, 1980). However, supplementation of cystathionine β-synthase was the potent relevant therapy for dramatically conversion of homocysteine to cystathionine (Zhu et al., 2008). Since cystathionine β-synthase is a PLP-dependent enzyme and the rapid dissociation of PLP as coenzyme from the apoenzyme is a common structural and catalytic criteria (El-Sayed, 2010, 2011), external supplementation of pyridoxal phosphate, vitamin B6 gave a plausible results for decreasing the amount of homocysteine, assuming the reassociation of apo-cystathionine β-synthase forming the holo-enzyme (Barber and Spaeth, 1967). Moreover, the deficiency of cystathionine γ-lyase results in accumulation of cystathionine (Cystathioninuria) that usually accompanied with diabetes, Down's syndrome, neuroblastoma (Wang and Hegele, 2003) as reviewed by Zhu et al. (2008).

13. Industrial application of l-methioninase: methanethiol production

Methanethiol has enormous biotechnological applications such as key gradient in gas odorants, jet fuel additives and coke formation in steel mill furnaces (Welirnan 1966). Recently, it is used for preparation of modified gold biosensors, manufacturing of plastics and pesticides (Jin et al. 2005, Nakamura et al. 2006). Also, it is the precursor for dimethyltrisulfide, *S*-methylthio-esters and 2,4-dithiapentane production (Chin and Lindsay 1994, Amarita *et al*. 2004), as presulfiding, hydrocracking catalysts, extracting solvents, manufacturing of ion-exchange resins and as modifier of Nylon fibers (Herschler 1962; Stewart and Lasis 1965) (www.gaylardchemical.com).

Chemically, methanethiol can be synthesized by reaction of methanol with hydrogen sulfide (Scott et al., 1955). The reaction was preceded at 380°C (716°F) in the presence of K_2MoSO_4/SiO_2 as catalyst and 2MPa. By chemical methods usually methanethiol accompanied with formaldehyde, requiring more steps for purification, especially with higher solubility of methanethiol in water (Yang et al., 1998). Commercially, the price of methanethiol salt is about eighteen fold higher than L-methionine, also one gram of sodium methanethiolate is 3.6 folds more than one liter of methanol (Sigma-Aldrich Co 2010).

Consequently, the enzymatic method, using L-methioninase seems to be a plausible process for large scale production of methanethiol from the technical and economical point of views. For the high expense of enzyme purification from the microbial cultures, immobilization is a

promising technique for enzyme stabilization and continuous production of methanethiol (El-Sayed and Shindia, 2011). Among the tested methods, polyacrylamide (42.2%), Ca-alginate (40.9%) and chitin (40.8%) displaying the highest *A. flavipes* L-methioninase immobilization efficiency. Chitin-enzyme gave a plausible stability till 4th cycle for production of methanethiol under controlled system. Applying GC and HNMR analysis, methanethiol has identical chemical structure to the standard compound. A new method for continuous production of pure methanethiol using a simple low expense enzymatic method was developed (El-Sayed and Shindia, 2011).

14. Prospectives for improving the therapeutic potentiality of PLP-dependent enzymes

Regarding to publications describing the structural and catalytic identities of PLP-dependent enzymes and their wide therapeutic and pharmaceutical applications, the immunogenicity and relative instability were the common limitations from pharmacokinetic point of view. Actually, all of these enzymes that receive considerable attention as therapeutic agent were of bacterial sources as reviewed by El-Sayed (2010). However, the crystallographic and biochemical studies for various therapeutic enzymes reveal a reliable distinction on the enzyme conformational structural of surface amino acids and immunogenic sites, from prokaryotes to eukaryotes (Kusakabe et al., 1979). Unlike the extensive studies of bacterial PLP-dependent enzymes, no more publications for biochemical characterization and crystal structures of these enzymes from eukaryotes, in spite of their distinct structural identity from eukaryotes as revealed from amino acid constitution (El-Sayed, 2010). Thus, further biochemical and crystallographic studies to elucidate the catalytic identity and tertiary structure of eukaryotic PLP-enzymes for the maximum therapeutic exploitation of these enzymes need to be resolved. The plausible re-association of PLP coenzyme with the apo-L-methioninase with full activity, comparing to lower to inability to re-association of the corresponding enzymes from prokaryotes, is an obscure, and their authorization with greatly participates in explanation of their catalytic and immunogenic identities. In addition, immobilization and co-immobilization studies of these enzymes will significantly maximize their biotechnological and therapeutical applications. Regarding to gene therapy, PEGylation and directing of these enzymes to specific tumors via targeting by modified antibodies or nao-particles is the main challenge for our ongoing research.

15. References

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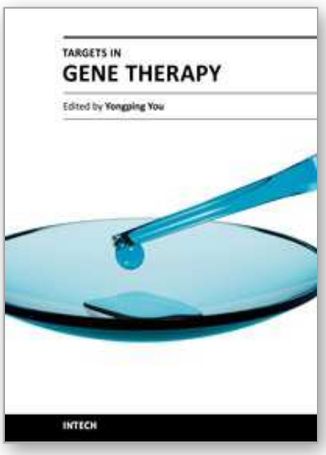

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This book aims at providing an up-to-date report to cover key aspects of existing problems in the emerging field of targets in gene therapy. With the contributions in various disciplines of gene therapy, the book brings together major approaches: Target Strategy in Gene Therapy, Gene Therapy of Cancer and Gene Therapy of Other Diseases. This source enables clinicians and researchers to select and effectively utilize new translational approaches in gene therapy and analyze the developments in target strategy in gene therapy.

How to reference

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