Reactivity-Based Drug Discovery Using Vitamin B_6-Derived Pharmacophores

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Abstract: Endogenous reactive intermediates including photoexcited states of tissue chromophores, reactive oxygen species (ROS), reactive carbonyl species (RCS), transition metal ions, and Schiff bases have been implicated in the initiation and progression of diverse human pathologies including tumorigenesis, atherosclerosis, diabetes, and neurodegenerative disease. In contrast to structure-based approaches that target macromolecules by selective ligands, reactivity-based drug discovery uses chemical reagents as therapeutics that target reactive chemical species involved in human pathology. Reactivity-based design of prototype agents that effectively antagonize, modulate, and potentially even reverse the chemistry underlying tissue damage from oxidative and carbonyl stress therefore holds great promise in delivering significant therapeutic benefit. Apart from its established role as an essential cofactor for numerous enzymes, a large body of evidence suggests that B_6-vitamers contain reactive pharmacophores that mediate therapeutically useful non-vitamin drug actions as potent antioxidants, metal chelators, carbonyl scavengers, Schiff base forming agents, and photosensitizers. Based on the fascinating chemical versatility of B_6-derived pharmacophores, B_6-vitamers are therefore promising lead compounds for reactivity-based drug design.

Key Words: Reactivity-based drug discovery, reactive pharmacophore, lead optimization, vitamin B_6, antioxidant, metal chelator, carbonyl scavenger, glycation.

INRODUCTION

The causative involvement of endogenous reactive intermediates including photoexcited states of tissue chromophores, reactive oxygen species (ROS), reactive carbonyl species (RCS), transition metal ions, and Schiff bases in various human pathologies is now firmly established. Endogenous reactive intermediates (also referred to as 'endoreactants') have been implicated in the initiation and progression of diverse human pathologies including tumorigenesis, atherosclerosis, diabetes, and neurodegenerative disease [1-7]. Importantly, the chemical nature of endoreactants suggests the feasibility of therapeutic intervention by specific molecular antagonists. Reactivity-based design of prototype agents that effectively antagonize, modulate, and potentially even reverse the chemistry underlying tissue damage from oxidative and carbonyl stress holds great promise in delivering significant therapeutic benefit. In contrast to structure-based approaches that target macromolecules by selective ligands, reactivity-based drug discovery uses chemical reagents as therapeutics that target reactive chemical species. A reactivity-based drug discovery strategy allows the rapid identification of promising leads because (I) the structure activity relationship (SAR) of active pharmacophores is exclusively based on chemical reactivity and therefore allows the construction of focused test compound libraries, and (II) hit identification occurs by simple chemical screening amenable to high throughput format.

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In contrast to most other vitamins, vitamin B_6 occurs as a family of closely related chemical derivatives comprising six vitamers as summarized in Fig. **1**: pyridoxine (**1**), pyridoxamine (**2**), and pyridoxal (**3**), all of which can be phosphorylated to the corresponding 5'-phosphates (**4**-**6**) [8]. Apart from its established role as an essential cofactor for numerous enzymes, a large body of evidence suggests that B_6-vitamers contain reactive pharmacophores that mediate therapeutically useful non-vitamin drug actions [9]. Based on the fascinating chemical versatility of B_6-derived pharmacophores, B_6-vitamers are therefore promising lead compounds for reactivity-based drug design. Importantly, in numerous preclinical studies discussed below no signs of adverse effects that could theoretically result from vitamin B_6-antagonistic activity of structural analogues were observed, suggesting the feasibility of designing B_6-derived drugs that do not interfere with essential vitamin B_6-coenzyme function and metabolism. In the following section, the design and refinement of B_6-derived therapeutic agents that act as potent antioxidants, metal chelators, carbonyl scavengers, Schiff base forming agents, and photosensitizers will be discussed as summarized in Fig. **1**. After discussing the SAR of vitamin B_6-associated spontaneous reactions under physiological conditions, current and emerging therapeutic applications that are based exclusively on this chemical reactivity of B_6-pharmacophores will be reviewed. Moreover, current drug development strategies based on lead refinement of pharmacophores contained in B_6-vitamers will be briefly introduced.

I. B_6-DERIVED ANTIOXIDANTS

A common mechanistic denominator of many B_6-dependent enzymatic reactions is resonance stabilization of anionic intermediates by the B_6-pyridinium moiety [10]. This

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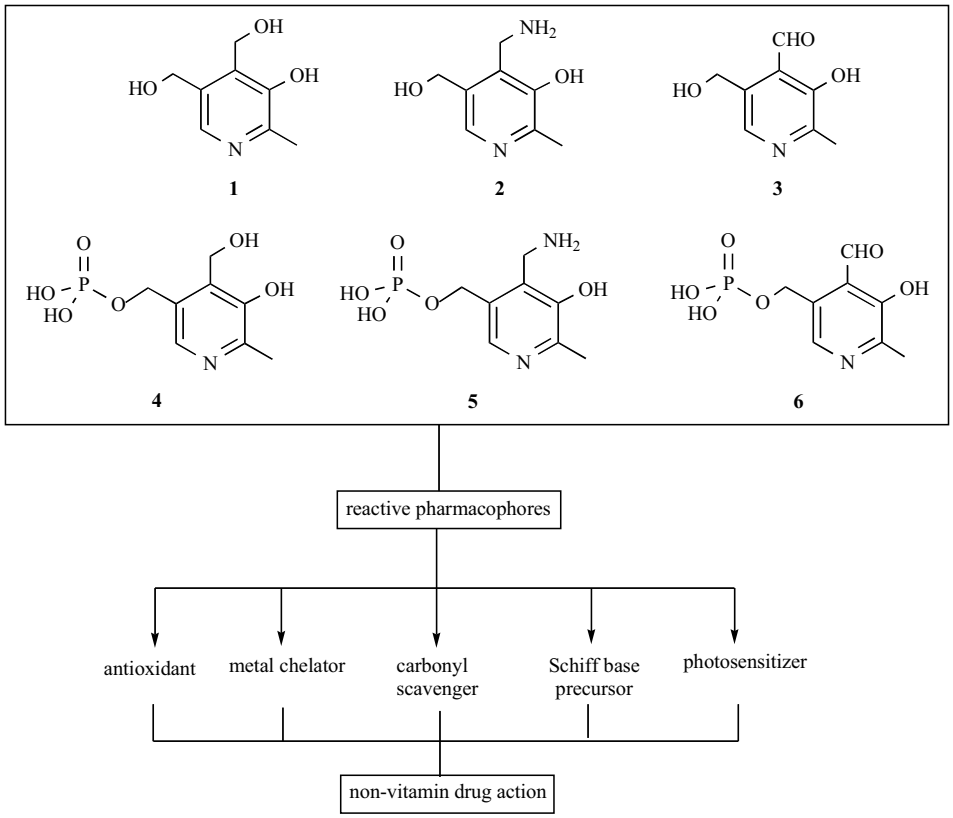


Fig. (1). Vitamin B_6-derived reactive pharmacophores. Non-vitamin drug action of B_6-vitamers can result from the presence of reactive pharmacophores that impart activity as antioxidants, metal chelators, carbonyl scavengers, Schiff base precursors, and photosensitizers.. B_6-vitamers are therefore promising lead compounds for reactivity-based drug design.

mechanism of action implicates an intramolecular redox reactivity originating from electron transfer onto the pyridinium nitrogen. Indeed, coenzyme activity of B_6-vitamers includes redox reactions, e.g. the oxidative decarboxylation of α-amino acids. Independent of coenzyme function, B_6 vitamers display spontaneous redox reactivity under physiological conditions that is mainly attributable to the presence of a phenolic hydroxyl group in 3-position of the pyridine ring, a redox pharmacophore common to all B_6-vitamers [11]. Indeed, the reactions between pyridoxine and several oxygen radicals [hydroxyl (·OH), hydroperoxyl (·OOH), and superoxide ($O_2^{\cdot-}$)] have been investigated in much detail recently [12]. Based on density functional theory level calculations, two sets of reactions between pyridoxine and ROS can occur as follows: (*I*) addition reactions to the aromatic ring and (II) H-atom abstraction by radical mechanisms (reactions 1 to 3):

$$B_6 + {}^{\cdot}OH \rightarrow {}^{\cdot}B_6 + H_2O \quad (1)$$

$$B_6 + {}^{\cdot}OOH \rightarrow {}^{\cdot}B_6 + H_2O_2 \quad (2)$$

$$B_6 + O_2^{\cdot-} \rightarrow {}^{\cdot}B_6 + HOO^- \quad (3)$$

The sites of hydrogen abstraction and hydroxyl radical addition in pyridoxine can be summarized as shown in Fig. **2**.

The first set of reactions may be carried out by ·OH and ·OOH radicals with formation of addition products at the carbon atoms adjacent to the ring nitrogen (C^2 and C^6). However, these are unlikely to occur with $O_2^{\cdot-}$ due to electrostatic repulsion between the aromatic system and the negative charge of the radical. In contrast, homolytic hydrogen atom abstraction can occur with the methylene hydrogen atoms bonded to C^8 and C^9 and at the phenolic hydroxyl group (O^{10}). Removal of these hydrogens by the hydroxyl radical is essentially barrierless, and the reactions proceed with DG_{aq}^{298} values of approximately -40 kcal/mol, around seven times more exergonic than hydrogen atom abstraction by ·OOH radicals [12].

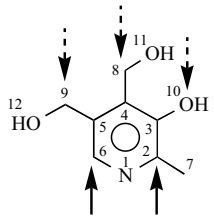
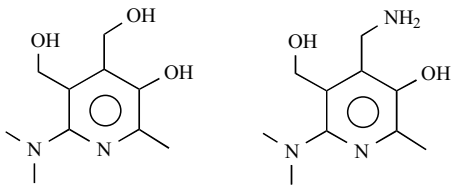


Fig. (2). Pyridoxine as sacrificial antioxidant. The formula indicates the sites of hydrogen abstraction (dashed arrows) and hydroxyl radical addition (full arrows).

Recently, pyridoxine has been shown to have highly effective antioxidant properties in chemical and cellular assays. Using a biological method that allows evaluation of

human plasma and red blood cell resistance against free radical-induced hemolysis by thermal decomposition of 2,2'-azobis (2-amidinopropane) hydrochloride (AAPH), the potent antioxidant activity of pyridoxine was demonstrated [13]. Moreover, pyridoxine exhibited antioxidant activity in several chemical assays including suppression of free radical enhanced luminol chemiluminescence and quenching of N,N-dimethyl-p-phenylenediamine radical cations. In human erythrocytes, both pyridoxine and pyridoxamine interfere with hyperglycemia-induced superoxide radical formation and prevent lipid peroxidation [14]. Strong antioxidant protection of U937 monocytes against H_2O_2-induced oxidative injury was demonstrated using pyridoxine, pyridoxal phosphate, or pyridoxamine [15]. In another study, pyridoxine and pyridoxamine suppressed hyperglycemia-induced crystalline oxidation in cultured lens cells [16]. These findings suggest an antioxidant mechanism of action for the beneficial effects of pyridoxine supplementation on clinical symptoms of neuropathy and retinopathy in diabetic patients. B_6-vitamers are now considered to be promising lead compounds for the development of novel antioxidant agents, and pyridoxamine analogues refined for enhanced free radical trapping activity have been designed recently [11]. In order to enhance the hydrogen atom donating activity of the 3-OH group contained in B_6-vitamers, electron donating substituents (including a dimethylamino-group) were introduced in para-position of the pyridine ring system reducing the bond dissociation enthalpy for the phenolic hydrogen, which theoretically enhances radical trapping rates. 6-Dimethylaminopyridoxine (**7**) and 6-dimethylaminopyridoxamine (**8**), B_6-derivatives refined for antioxidant potency formed by introduction of a 6-dimethylamino-substituent into pyridoxine and pyridoxamine, respectively, are shown in Fig. **3** [11].



7 **8**

Fig. (3). B_6-derivatives with enhanced antioxidant activity. Compounds 7 and 8 have been optimized for radical trapping activity by introduction of an electron-donating para-substituent (6-dimethylamino-group) that favors homolytic cleavage of the phenolic hydroxyl substituent during hydrogen abstraction by free radicals.

Both derivatives display increased radical trapping activity and peroxyl radical quenching in the allophycocyanin fluorescence assay and display an antioxidant potency that equals that of the water soluble α-tocopherol analogue trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid).

In addition to providing protection against ROS including $^{\cdot}OH$ and H_2O_2, B_6-vitamers are also effective singlet oxygen ($^1O_2$)-antagonists. $^1O_2$, a highly reactive form of molecular oxygen that forms by energy transfer upon photoexcitation of organic sensitizer molecules, oxidizes cellular targets with diffusion controlled reaction rates. Importantly, $^1O_2$ is a crucial reactive intermediate involved in cellular photodamage that results from photosensitization reactions relevant to skin solar damage and photocarcinogenesis [1]. Interestingly, pyridoxine-dependent resistance against self-sensitization and $^1O_2$ damage has been demonstrated in the parasitic fungus cercospora keidi [17]. Plant cell lysis by this microorganism involves secretion of cercosporin, a perylenequinone photosensitizer that generates $^1O_2$ in the presence of ground state $^3O_2$ and solar light. Pyridoxine has been identified as endogenous $^1O_2$-antagonist that protects this pathogenic organism against self-sensitization. In addition, various pyridoxine synthesis deficient mutants of fungi and yeast have been shown to be sensitive to ROS including $^1O_2$ and hydrogen peroxide suggesting a role of vitamin B_6 biosynthetic genes and their products in adaptation to oxidative stress [18]. Recent chemical studies have elucidated the reaction between $^1O_2$ and pyridoxine indicating the formation of hydroperoxide (**9**) and endoperoxide (**10**) intermediates leading to the complete sacrificial destruction of pyridoxine during $^1O_2$ detoxification as summarized in Fig. **4** [19].

The $^1O_2$-quenching rate for 3-methoxypyridine is reportedly 100 times slower than that for 3-hydroxypyridine, indicating that the phenolic hydroxyl substituent is required for efficient $^1O_2$-quenching by pyridoxine. $^1O_2$-antagonism by B_6-vitamers seems to depend on sacrificial reaction leading to irreversible chemical destruction, a mechanism of action that combined with the known activity of B_6-vitamers as weak UVA-photosensitizers excludes their use as skin photoprotective agents as discussed below.

II. B_6-DERIVED METAL CHELATORS

Pyridoxamine has long been known to be a moderately potent metal chelator that preferentially forms complexes with Cu^{2+} and Fe^{3+} ions [20]. The bidentate pharmacophore essential for metal ion binding involves the 4-aminomethyl- and the 3-hydroxyl-substituents of the pyridine ring. Chelation involves the phenolate form of the 3-hydroxyl-substituent that displays a high degree of acidity (pK_a 3.54) and therefore occurs in the anionic form under physiological conditions, where formation of a 2:1 complex between pyridoxamine and Cu^{2+} has been observed. Free and inadequately chelated transition metal ions are reactive species that contribute to cellular damage by participation in metal-catalyzed peroxide decay (Fenton reaction), lipid peroxidation, and other redox processes. Accumulative evidence suggests a causative involvement of free iron and copper ion-catalyzed reactions in the pathogenesis of various pathologies including ischemia reperfusion damage, neurodegenerative disease, secondary complications of diabetes, and atherosclerosis [21,22]. In skin photodamage, UVA-induced release of labile iron pools in human fibroblasts contributes to photo-oxidative stress and is a key factor in NFκB activation followed by expression of inflammatory cytokines [1]. The pathological role of free transition metal ions suggests the feasibility of therapeutic intervention using small molecule transition metal ion chelators including pyridoxamine that bind and neutralize these reactive species. The preventive and therapeutic efficacy of metal chelators against various human pathologies has been documented in many preclinical and clinical studies. Indeed, metal chelation with subsequent suppression of metal ion-catalyzed oxidative and glycoxida-

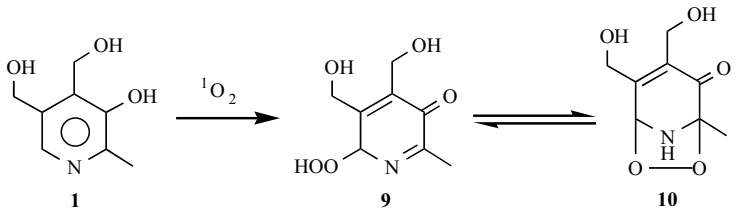


Fig. (4). Chemical quenching of $^1O_2$ by pyridoxine. Rapid inactivation of $^1O_2$ by pyridoxine occurs by sacrificial chemical quenching with formation of hydroperoxide (**9**) and endoperoxide (**10**) adducts.

tive tissue damage seems to contribute to the efficacy of using high doses of pyridoxamine in the prevention and treatment of type II diabetic nephropathy in mouse models of the disease, a therapeutic application currently evaluated in human phase II clinical studies as discussed below [23]. Based on these prototype observations pyridoxamine has been selected as a promising candidate for the development of improved metal chelators, and lead optimization studies have generated pyridoxamine-derivatives that display increases in metal ion binding potencies by up to three orders of magnitude as summarized in Fig. **5**.

Based on the known Fe^{3+}-binding properties of bidentate o-hydroxybenzaldehyde-amino acid Schiff base conjugates including N-(2-hydroxybenzyl)-glycine and N-(2-hydroxybenzyl)-serine, the reduced Schiff base conjugate formed from pyridoxal and L-serine, N-(4-pyridoxylmethylene)-L-serine (**11**), has recently been synthesized [24,25]. All of these Fe^{3+}-chelators suppressed ROS generation by inhibition of iron-catalyzed Fenton chemistry, where Fe^{2+} catalyzes the decay of peroxides and cycles between the Fe^{3+} and Fe^{2+} forms. In the absence of cytotoxic effects, generally observed with established iron chelators including desferrioxamine, **11** suppressed iron-induced hydroxyl radical formation and oxidative cell damage. Moreover, in skin photodamage studies using hairless mice exposed to chronic UVB-irradiation, topical administration of **11** protected against UVB-induced epidermal hypertrophy and lymphocytic infiltration [24]. Spontaneous hydrolytic cleavage of the initially formed Schiff base between pyridoxal and L-serine would lead to compound inactivation under physiological conditions and is prevented by $NaBH_4$-reduction of the imine-function leading to **11**.

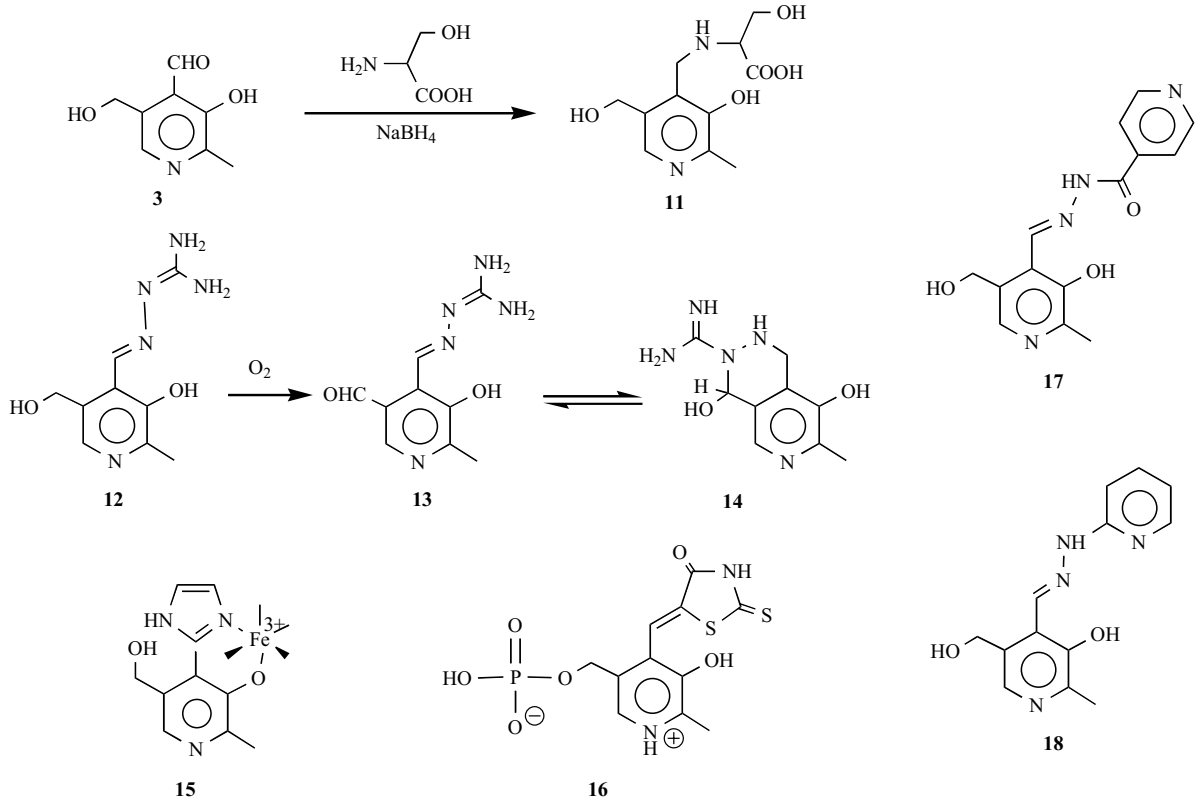


Fig. (5). Pyridoxamine as a lead compound for the design of therapeutic transition metal ion chelators. The bidentate 4-aminomethyl-3-hydroxyl-pyridine pharmacophore resonsible for metal ion binding has been optimized to enhance pharmacodynamic efficacy and pharmacokinetic profile of the experimental transition metal ion chelators **11-18**.

In contrast, when hydrazine-type amines are used for Schiff base formation with pyridoxal, stable hydrazone Schiff bases resistant to hydrolysis under physiological conditions are formed. Indeed, a prototype Schiff base chelator formed from reaction between aminoguanidine and pyridoxal (PL-AG, **12**) was stable upon oral administration and displayed potent metal chelation and antioxidant activities in an experimental mouse model of streptozotocin-induced diabetes [26]. Based on metabolite studies the antioxidant activity of the PL-AG hydrazone compound (**12**) was attributed to (*I*) transition metal (Cu^{2+}) chelation and (*II*) sacrifical ROS-induced oxidation of the 5-hydroxymethyl-subtituent of the pyridoxal moiety. The sacrificial oxidation to the carbaldehyde function (**13**) with subsequent ring closure by hemiacetal formation (**14**) is shown in Fig. **5**. BST-4997 (**15**, shown as the Fe^{3+} complex), a bidentate pyridoxamine-derivative that contains the Schiff base functionality as part of an imidazole ring system displays strongly enhanced Cu^{2+}- and Fe^{3} -ion affinity (1000 and threefold, respectively, as compared to pyridoxamine) and is currently in preclinical development for the treatment of diabetic neurovascular dysfunction [27]. Another bidentate pyridoxamine-derivative, 5'-O-phosphono-pyridoxylidenerhodanine (B6PR, **16**) with potent metal chelation (Fe^{2+}, Zn^{2+}) and antioxidant properties is currently undergoing preclinical assessment for immunomodulating and antiviral efficacy [28]. These trials are based on initial findings that demonstrated B6PR-protection of HIV-1-infected $CD4^{+}$ HUT 78 cells against HIV-1-mediated destruction and inhibition of HIV-1-induced syncytia formation. Finally, the tridentate iron chelator, pyridoxal isonicotinoyl hydrazone (PIH, **17**), has recently been shown to mobilize iron ions from both normal and neoplastic cells [29]. Based on the selective vulnerability of rapidly proliferating cancer cells to iron deficiency, PIH-compounds and similar agents including the 2-pyridyl-hydrazone-derivative **18** are under development as anti-proliferative therapeutics, particularly against neuroblastoma. Some of these agents deplete cellular iron with greater efficiency than desferrioxamine, and in a recent clinical trial in humans, **17** showed no evidence of toxicity and resulted in significant Fe excretion [30].

III. PYRIDOXAMINE AS CARBONYL SCAVENGER AND ANTI-GLYCATION AGENT

Among the various B_6-vitamers, only pyridoxamine contains the bis-nucleophilic 3-hydroxy-4-aminomethyl-pyridine pharmacophore known to impart activity as potent carbonyl scavenger and anti-glycation agent [23,31-33]. Carbonyl stress mediated by reactive carbonyl species (RCS) is an established source of endogenous tissue damage that occurs in various systemic pathologies, such as diabetes, atherosclerosis, Alzheimer's disease, and chronological aging [1,7,34-36]. Pyridoxamine is now in advanced clinical studies to test its therapeutic potential as a small molecule inhibitor of tissue carbonyl stress [27,37]. Chemical damage by RCS occurs by spontaneous amino-carbonyl reactions (referred to as 'glycation') between RCS and protein-bound amino groups leading to covalent protein adduction, crosslinking, and formation of protein-epitopes called advanced glycation end-products (AGEs). The chemistry of AGE-accumulation in human tissue proteins is complex and depends on the crucial involvement of various RCS including mono-, di-, and oligo-carbonyl intermediates (e.g. glyoxal, methylglyoxal, 3-deoxyglucosone, glucosone) that originate from sugar metabolism, sugar autoxidation, and lipid peroxidation [2]. AGEs accumulate on extracellular matrix proteins including collagen and elastin in age-related quantities inducing structural and functional alterations. Apart from protein crosslinking and matrix dysfunction, AGEs can stimulate pathological signaling through a receptor called RAGE involved in chronic inflammation and carcinogenesis [38,39]. Apart from this role of AGE-epitopes as potent protein crosslinkers and RAGE-ligands, some AGEs with extended heterocyclic chromophores are now established skin photosensitizers that produce ROS upon UVA-irradiation adding to the photooxidative burden of chronologically aged skin and thereby contributing to photocarcinogenesis [1].

The role of carbonyl stress in the pathology of atherosclerosis, secondary diabetic complications associated with extracellular matrix protein damage including neuropathy, retinopathy, and nephropathy, and skin actinic and chronological damage is now well established [1,7,34-36,38]. RCS as key mediators of carbonyl stress are therefore potential molecular targets for therapeutic intervention, and experimental evidence suggests the possibility of therapeutic intervention by rational design of novel RCS-antagonists, called carbonyl scavengers [2,40]. Carbonyl scavengers are experimental small molecule nucleophilic agents that inactivate RCS by sacrificial covalent adduction. The SAR of carbonyl scavenging is well defined and depends on the presence of nucleophilic pharmacophores. Potent carbonyl scavengers contain bis-nucleophilic pharmacophores that irreversibly scavenge RCS with formation of rings or delocalized aromatic systems [2]. Screening assays for the identification of reactivity-based carbonyl scavengers have been described [2], prototype agents have been identified, and their efficacy has been tested in cellular and animal models of carbonyl stress [27,37,41-43].

Recently, pyridoxamine has been identified as a prototype agent for the therapeutic suppression of carbonyl stress and glycation reactions [23,31-33]. An abbreviated reaction scheme that illustrates the complex chemistry of carbonyl scavenging by pyridoxamine directed against RCS from lipid peroxidation and sugar autoxidation is given in Fig. **6**.

The scheme illustrates how RCS originating from lipid peroxidation, sugar autoxidation, and glycation reactions are trapped and detoxified by covalent pyridoxamine-adduction before they can react with tissue proteins and cause tissue damage and dysfunction. The complex chemistry of spontaneous trapping of pathophysiologically relevant reactive 1,4-dicarbonyls including the cyclooxygenase-derived arachidonic acid metabolites levuglandin (LG) D_2 and LGE_2 (see **19,** Fig. **6**) by pyridoxamine (**2**) involves formation of pyrroles (e.g. **22**) *via* spontaneous Paal-Knorr reaction (Fig. **6**, pathway A) and 2-oxopyrrolidinyl (lactam) derivatives [33,44]. Detailed mechanistic studies support a reaction pathway that depends on carbonyl group protonation by the acidic phenolic hydroxyl group contained in pyridoxamine that facilitates ring formation by the attack of the intermediate hemiaminal amine (**21**) as shown in Fig. **6**. Indeed, formation of pyridoxamine-pyrrole adducts has been observed in model reactions between pyridoxamine and 4-oxopen-

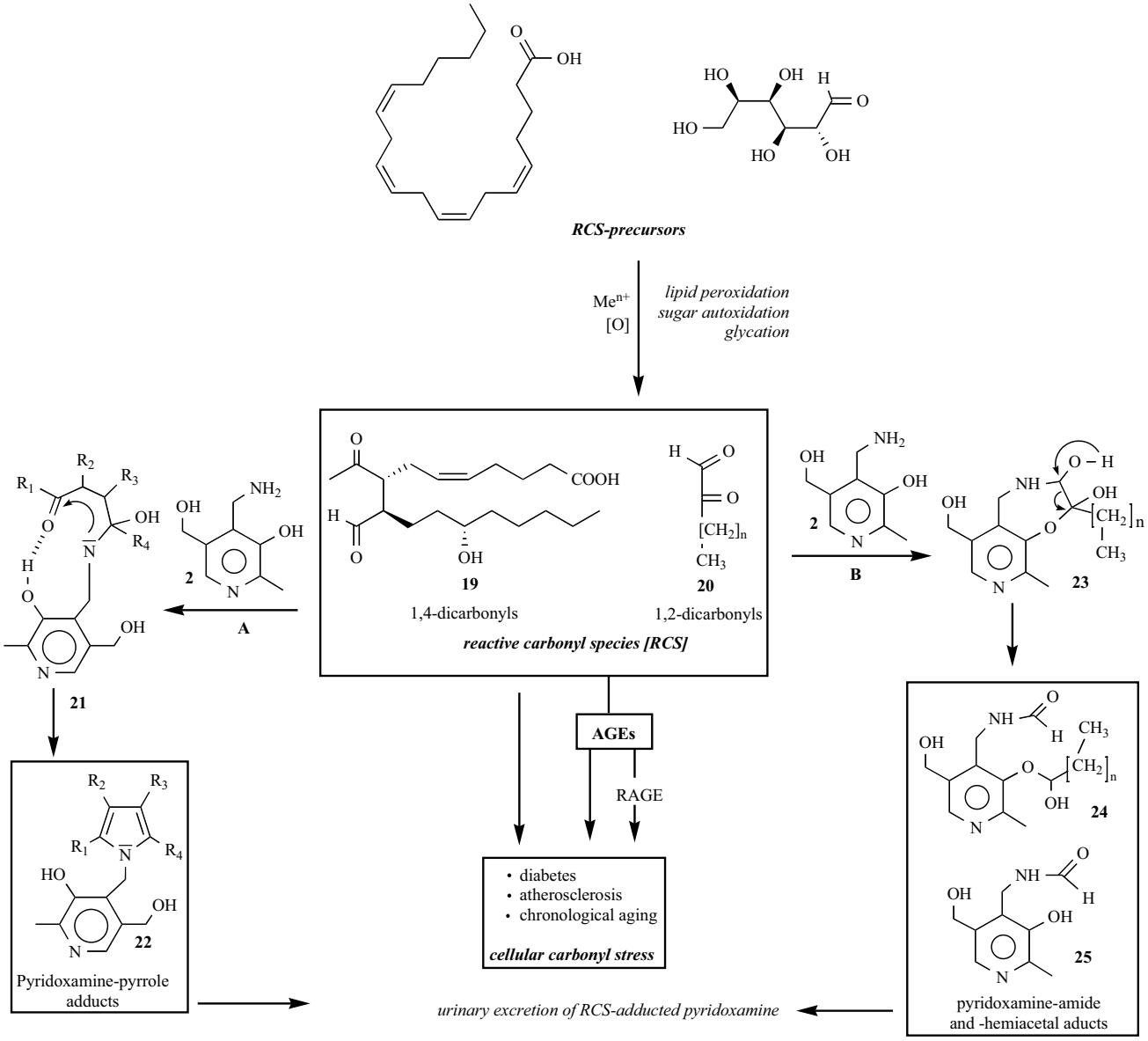


Fig. (6). Pyridoxamine as a lead compound for the design of therapeutic inhibitors of cellular carbonyl stress. Reactive carbonyl species (RCS) from spontaneous (transition metal ion-dependent, M^{n+}) oxidative and non-oxidative degradation of precursor molecules (e.g. reducing sugars and poly-unsaturated fatty acids) form under pathophysiological conditions including oxidative stress and hyperglycemia. RCS induce cellular carbonyl stress by direct interaction with critical targets and can also act via formation of advanced glycation endproduct epitopes on target proteins that may initiate pathological signaling through RAGE, the pro-inflammatory cell receptor for AGEs. Pyridoxamine (**2**) traps RCS of the 1,4-dicarbonyl and 1,2-dicarbonyl type by sacrificial covalent adduction (reaction pathway A and B, respectively), thereby interfering with tissue carbonyl stress involved in various human pathologies. Pyridoxamine-adducted RCS including N-formyl-pyridoxamine (FAPM, 25) are then eliminated via urinary excretion.

tanal. In contrast, trapping and inactivation of pathophysiologically relevant 1,2-dicarbonyls such as the glycolytic by-product methylglyoxal (**20**, n=0) and the lipid peroxidation product 2-ketoheptanal (**20**, n=4) by pyridoxamine involves bis-nucleophilic adduction *via* the 4-aminomethyl- and 3-hydroxyl-functionalities with formation of a seven-membered ring (intermediate **23**), followed by electronic rearrangement, ring cleavage, and formation of amide- (N-formyl-pyridoxamine, FAPM, **25**) and hemiacetal-adducts (**24**) (Fig. **6**, pathway B). Indeed, FAPM is the major pyridoxamine-adduct detected in urine of diabetic and obese rats treated with pyridoxamine [32]. It is important to note that pyridoxamine antagonism of glycation damage by 1,2-dicarbonyl trapping as depicted in Fig. **6** (reaction pathway B) involves chemical cleavage of the α-dicarbonyl group. Based on this mechanism of action it has been suggested that

pyridoxamine may also be useful as AGE-breaker [32]. AGE-breakers are a class of innovative experimental therapeutics that reverses tissue crosslinks by facilitating the cleavage of existing AGEs known to contain α-dicarbonyl functional groups [45].

Feasibility of using AGE-breakers to restore tissue function has recently been demonstrated using the bidentate nucleophilic experimental AGE-breaker ALT-711 [4,5-dimethyl-3-(2-oxo-2-phenylethyl)-thiazolium chloride, Alagebrium™, Alteon] that reversed the diabetes-induced increase of large artery stiffness as measured by systemic arterial compliance, aortic impedance, and carotid artery distensibility [46]. However, future experimentation is required to address the question whether similar therapeutic effects can be achieved using pyridoxamine as AGE-breaker. Importantly, in addition to reactivity-based activity as carbonyl scavenger and potential AGE-breaker, the impressive antiglycation efficacy of pyridoxamine may also result from the antioxidant and metal-chelation effects of this compound already described above, since oxidative reaction steps are involved in RCS- and AGE-formation during glycation reactions [47]. Indeed, in an attempt to dissociate metal chelation from carbonyl scavenger activity, a pyridoxamine-derivative with reduced bidentate nucleophilicity and enhanced metal chelation potency has been designed and tested in an animal model of glycation damage **(**Fig. **5,** formula **15)** [27]. Importantly, no adverse clinical effects that could theoretically result from vitamin B_6-antagonistic activity of this close structural analogue were observed in this study consistent with the general safety of other B_6-analogues that do not interfere with vitamin B_6-coenzyme function [26-28]. Numerous preclinical and some early clinical studies suggest that pyridoxamine is a potent inhibitor of carbonyl stress and tissue glycation damage *in vivo* [27,37]. Pyridoxamine inhibited the progression of renal disease, decreased hyperlipidemia, and protected against a range of pathological changes in the retinas of streptozotocin-diabetic rats [48]. Pyridoxamine also inhibited AGE-formation in skin collagen and in the retinas of diabetic animals. In the Zucker rat model of diabetes and hyperlipidemia, characterized by elevated levels of plasma α-dicarbonyl compounds and protein AGEs, pyridoxamine treatment had significant renoprotective effects and effectively decreased plasma levels of RCS including glyoxal and methylglyoxal [49]. Moreover, RCS-adducts of pyridoxamine were detected in urine of pyridoxamine-treated animals, indicating that the carbonyl-trapping mechanism is operative *in vivo* [50]. In human clinical studies, pyridoxamine has shown a favorable safety profile, and phase 2 clinical studies of this agent (Pyridorin™, Biostratum Inc.) in patients with diabetic nephropathy have been conducted [37].

IV. B_6-MEDIATED FORMATION OF SCHIFF BASES AND OTHER CARBONYL ADDUCTS: TOXICOLOGICAL AND THERAPEUTIC IMPLICATIONS

The B_6-vitamer pyridoxal contains an aromatic ortho-hydroxyl carbaldehyde-pharmacophore that can act as a pharmacologically relevant amine-trap. This occurs by spontaneous formation of stable conjugated Schiff base adducts of primary amines contained in many biomolecules and drugs. Generally, formation of the carbon-nitrogen double bond (imine-group) that constitutes a Schiff base occurs by condensation of an aldehyde- and a primary amino-group with water elimination. Under physiological conditions, Schiff base formation is completely reversible due to spontaneous hydrolytic cleavage. Interestingly, Schiff bases derived from pyridoxal are stable under physiological conditions and their formation is kinetically favored [20]. Spontaneous adduction by pyridoxal can therefore lead to irreversible covalent modification of various amino-group containing target molecules. This particular reactivity of pyridoxal results from the chemistry associated with the 3-hydroxy-4-carbaldehyde-pyridinium-pharmacophore contained in pyridoxal: First, the electron withdrawing effect of the pyridinium ring nitrogen increases the electrophilicity of the aromatic aldehyde thereby enhancing its reactivity towards the attacking amino-group. Secondly, the ortho-hydroxyl substituent can act as a hydrogen bond donor that facilitates the amino-carbonyl condensation reaction. Third, the newly formed Schiff base (imine functional group) is in conjugation with an aromatic ring system and its resonance stabilization will therefore interfere with nucleophilic attack of water that would initiate the backward reaction with hydrolytic bond cleavage.

Spontaneous Schiff base formation is an important determinant of vitamin B_6-drug interactions as exemplified in Fig. **7**. It has long been known that that drug molecules that contain nucleophilic amino groups can form Schiff base adducts with pyridoxal leading to pharmacotherapy-induced vitamin B_6 depletion [51,52]. This effect is expected to occur with all B_6-vitamers due to their metabolic conversion into pyridoxal 5'-phosphate (PLP, **6**), the active form of vitamin B_6 that contains the Schiff base forming pharmacophore. It is well established that levodopa (3,4-dihydroxy-L-phenylalanine) treatment in Parkinson's disease induces B_6-deficiency.

Vice versa, levodopa therapeutic and dyskinetic side effects are abolished when large doses of pyridoxine are simultaneously administered. The spontaneous formation of a stable Schiff base between levodopa and PLP is a well established explanation for these antagonistic effects [51]. Similarly, isoniazid (isonicotinic acid hydrazide) therapy has been long recognized as responsible for inducing the symptomatology of vitamin B_6 deficiency, particularly neuropathy and has been linked to reduction of PLP availability by formation of a pyridoxal-isonicotinyl hydrazone adduct (**26**) excreted in the urine [52,53]. Again, life-threatening toxicity of isoniazid overdose may be antagonized by intravenous administration of very high doses of pyridoxine (up to 70 mg/kg) and drug detoxification by spontaneous Schiff base adduction/hydrazone formation may contribute to the therapeutic efficacy of this antidote [53]. Other drugs known to undergo covalent pyridoxal-adduction include the diuretic muzolimine and the antibiotics thiamphenicol glycinate and cycloserine [52]. Long-term therapy with the copper ion chelator D-penicillamine (3,3-dimethyl-D-cysteine) increased xanthurenic acid and kynurenine excretion after tryptophan load indicating induction of a B_6-deficient state [54]. Therefore, supplemental administration of pyridoxine to patients receiving D-penicillamine has been recommended. D-penicillamine is a multifunctional agent that also contains a potent carbonyl scavenger pharmacophore [2]. Bis-nucleophilic

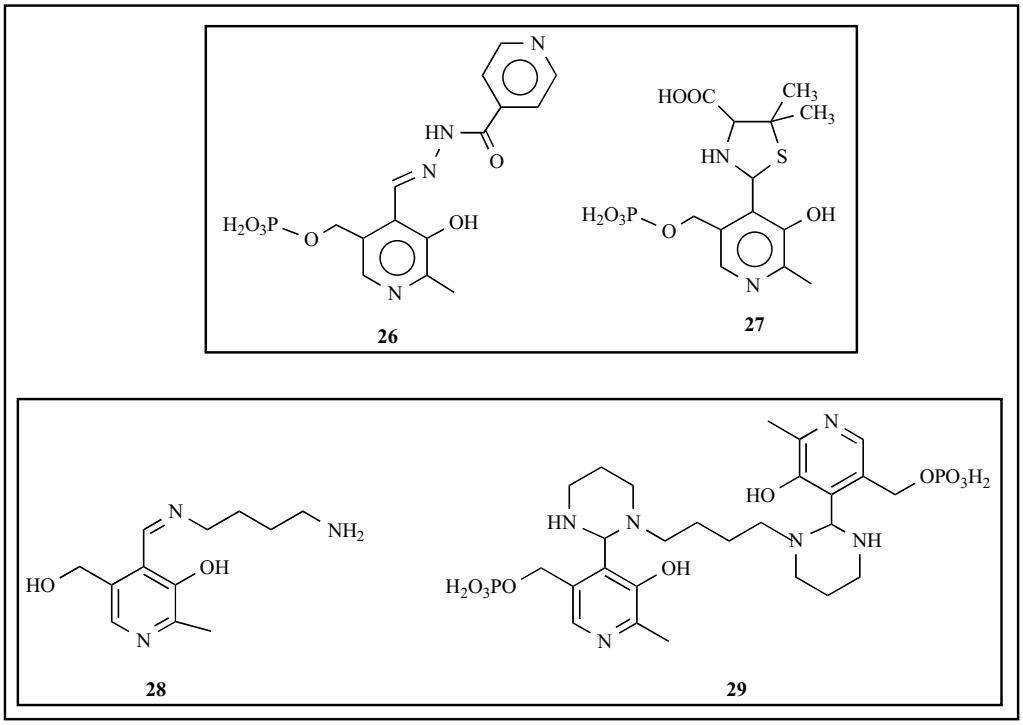


Fig. (7). Pharmacologically relevant pyridoxal-derived Schiff bases. Spontaneous Schiff base formation occurs under physiological conditions between pyridoxal and amino-group containing drugs (22, 23), endogenous amino-reactants including polyamines (24, 25), and protein-bound amino groups in tissue.

carbonyl adduction aided by a geminal dimethyl-effect leads to irreversible pyridoxal-trapping by D-penicillamine with conversion of the aldehyde function into a cyclic thiazolidine-derivative (**27**) [52]. Due to this unique chemical trapping mechanism that depends on spontaneous formation of conjugated Schiff bases and other covalent adducts including hydrazones and cyanohydrin, pyridoxine and pyridoxal 5'-phosphate have also been used as antidotes for dopamine, polyamine (spermine), gentamicin, hydrazine, and even cyanide poisoning [55]. However, the necessity for enzymatic conversion of pyridoxine to its active Schiff base forming metabolite pyridoxal 5'-phosphate seems to favor direct intravenous administration of the latter in emergency situations, where antidote action by covalent adduction and neutralization of the poison must occur in minutes [53]. Interestingly, polyamine-pyridoxal-(phosphate) Schiff base adducts (e.g. **28** derived from putrescine) occur constitutively in human urine, suggesting the physiological relevance of spontaneous B_6-Schiff base adducts with endogenous amines [56]. More complex cyclic polyamine-B_6 adducts (**29**), derived from nucleophilic attack of the secondary amine group contained in spermidine and spermine on the initially formed Schiff base, form spontaneously under physiological conditions [57]. It has been hypothesized that spontaneous B_6-adduction by polyamines, known to be elevated under conditions of malignant cell proliferation, contributes to B_6-deficiency during tumorigenesis [56]. Vice versa, pyridoxal and pyridoxal-derived Schiff base forming agents could be clinically useful for the therapeutic depletion of cellular polyamines in various human malignancies including colon carcinoma where elevated polyamine levels seem to be essential for rapid cell proliferation and viability [58].

In addition to covalent drug-pyridoxal interactions, the pharmacological significance of pyridoxal-mediated Schiff base formation has also been evaluated in an attempt to use this B_6-vitamer as a prototype agent for the therapeutic inhibition of the sickling behaviour of hemoglobin S [59]. Among various test compounds, the o-hydroxybenzaldehyde analogues salicylaldehyde, 2,3-dihydroxy-benzaldehyde, o-vanillin, and pyridoxal were most effective in spontaneously adducting and increasing the oxygen affinity of hemoglobin, and the antisickling effects of these aromatic aldehydes were demonstrated in human erythrocytes. Finally, other pharmacological activities of pyridoxal that are based on nonenzymatic formation of Schiff base adducts have been described including pyridoxal 5'-phosphate inhibition of ADP-induced platelet aggregation by Schiff base formation with platelet surface amino groups [60]. However, it is important to note that apart from pyridoxal itself, no pyridoxal-derived pharmacological agents that would facilitate potential clinical applications associated with spontaneous Schiff base formation are currently in drug use or development.

V. B_6-DERIVED PHOTODYNAMIC PHARMACOPHORES: MEDIATORS OF SKIN PHOTOSENSITIZATION AND POTENTIAL PHOTODYNAMIC THERAPEUTIC AGENTS

All B_6-vitamers share the 3-hydroxypyridine (3-HP) core moiety known to act as a potent photodynamic pharmacophore as recently revealed by a detailed structure activity

relationship study of skin cell photosensitization by various endogenous pyridinium derivatives including B_6-vitamers [61]. Photosensitization occurs as a consequence of initial formation of excited states of chromophores and their subsequent interaction with substrate molecules (type I photoreaction) or molecular oxygen (type II photoreaction) through energy and/or electron transfer [1,62]. Members of the extended 3-HP class of endogenous photosensitizers comprise all B_6 vitamers, enzymatic collagen crosslinks of the pyridinoline type, and AGEs contained in human chronologically and photoaged skin collagen and elastin. UVA photosensitization of human skin cells by B_6-vitamers occurred in the lower micromolar range with dose-dependent inhibition of proliferation, cell cycle arrest in G_2/M, induction of apoptosis, intracellular oxidative stress, and p38 MAPkinase activation, all of which were reversible by thiol antioxidant intervention [61]. Obviously, the presence of a phenolic 3-OH substituent is an essential structural requirement for sensitizer activity of B_6-vitamers, since 2-HP and 4-HP, which occur predominantly as the tautomeric pyridone structures in aqueous solutions at neutral pH [63], display no photosensitizer activity. The phenolic character of 3-HP-derivatives may be of crucial importance for the observed sensitization effects: It is well documented that upon photoexcitation phenolic substances release electrons into aqueous solutions by electron ejection with formation of phenoxyl-type organic free radicals from the excited triplet state, but conversely act as potent electron scavengers in the ground state [64]. Moreover, single electron transfer reactions leading to the formation of 3-hydroxypyridinium [65] and pyridoxine [66] free radicals have been reported previously, and free radical polymerization of synthetic N-substituted 3-oxypyridinium betaines can be initiated by UV-irradiation [67]. This is consistent with a free radical mechanism of phototoxicity of biogenic 3-HP-derivatives observed in a recent study using cultured human skin cells [61]. In simple *in vitro* models of protein photooxidation, B_6-vitamers were moderately potent photosensitizers, effecting protein photo-crosslinking and photo-oxidation with incorporation of molecular oxygen. Based on these results, it was concluded that B_6-vitamers constitute a novel class of UVA-photosensitizers capable of mediating skin photooxidative damage. Indeed, increased photosensitivity is a known consequence of B_6-overdosing in humans [68], and phototoxicity of UV-irradiated pyridoxamine was reported as early as 1947 by Shwartzman and Fisher [69] followed by other reports thereafter [70,71]. B_6-vitamers may therefore act as endogenous skin photosensitizers with relevance to human skin *in vivo*, since human skin contains various B_6-vitamer forms in significant amounts (approximately 100 nmol per g protein [72]), with pyridoxal-5'-phosphate and pyridoxal being the predominant vitamers *in vivo*. However, it is important to note that the potential phototoxic activity of B_6-vitamers in human skin may be balanced *in vivo* by their antioxidant and singlet oxygen quenching activity above mentioned, suggesting a complex role in prevention and mediation of skin photodamage to be addressed by future studies. Apart from this potential role as endogenous photosensitizers in human skin photodamage, recent work has demonstrated that 3-HP- and N-alkyl-substituted 3-HP-derivatives may serve as photodynamic pharmacophores for the targeted elimination of pre-malignant and cancer cells [73]. When cultured human G361 melanoma cells were exposed to the combined action of UVA irradiation and a covalent albumin-vitamin B_6-conjugate, formed by spontaneous pyridoxal-adduction of protein-lysine residues followed by reductive stabilization using $NaCNBH_3$, pronounced induction of melanoma cell apoptosis was achieved. Apoptosis was not observed after treatment with either B_6-conjugate or irradiation only, confirming the photodynamic nature of the observed effects. These preliminary data demonstrate feasibility of using B_6-derived phototoxic 3-HP-chromophores including B_6-vitamers as experimental photodynamic pharmacophores for anti-proliferative intervention. Moreover, 3-HP-derivatization of experimental anticancer agents that contain a pyridine moiety, including the mitochondriotoxic N-methylpyridinium-derivative F16 {4-[(1E)-2-(1H-indol-3-yl)ethenyl]-1-methyl-pyridinium iodide}, may represent a valid strategy for photodynamic functionalization of interesting lead compounds [74].

CONCLUSIONS

The chemical versatility of B_6-vitamers is remarkable and has been the subject of many detailed studies. Early findings obtained in the 1940s in the laboratory of Esmond Snell already indicated that pyridoxal phosphate dependent enzyme reactions can be catalyzed by pyridoxal in the complete absence of any enzyme, demonstrating for the first time the potential of B_6-vitamers to participate in spontaneous reactions under physiological conditions [75]. More recent studies have confirmed that B_6-reactivity under physiological conditions is not only essential for coenzyme function during enzymatic transformations including transamination, decarboxylation, racemization, and β-elimination, but also allows participation in various non-enzymatic reactions as antioxidant, carbonyl scavenger, and metal chelator. This spontaneous reactivity may therefore contribute to physiologically and pharmacologically relevant non-vitamin actions of B_6-vitamers. Importantly, recent studies strongly suggest that B_6-derived reactive pharmacophores can achieve therapeutic effects in a variety of human pathologies that depend on the causative involvement of ROS, RCS, and transition metal ions including atherosclerosis, diabetes, and other age-related diseases. B_6-vitamers and their reactive pharmacophores are therefore promising lead structures for the future design and development of novel reactivity-based therapeutics.

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ABBREVIATIONS

AGEs = Advanced glycation endproducts

AAPH = 2,2'-azobis (2-amidinopropane) hydrochloride

3-HP = 3-hydroxypyridine

PLP = Pyridoxal 5'-phosphate

ROS = Reactive oxygen species

RCS = Reactive carbonyl species

SAR = Structure activity relationship

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