FLAVOENZYMES AS DRUG TARGETS

R. Heiner Schirmer, Florian Lederbogen, R. Luise Krauth-Siegel, Gerhard Eisenbrand

Institut für Biochemie II der Universität und Deutsches Krebsforschungszentrum, Im Neuenheimer Feld 328/280, D-6900 Heidelberg, FRG

Georg Schulz

Institut für Organische Chemie und Biochemie der Universität, Albertstr. 21, D-7800 Freiburg, FRG

Albrecht Jung

Institut für Physiologische Chemie, Hoppe-Seyler-Str. 1, D-7400 Tübingen, FRG

Introduction

"For someone starting his career as a chemist, it was a distinct handicap in those years, viz. the 50' s, that B_2, in contrast to vitamins that were discovered later, was a 'breadless' vitamin, i.e. it had no lucrative pharmacological aspects to it. It was found ubiquitously, it was not expensive to produce, so that the producing company... could not realize much more out of it than chicken feed and an, in every respect, harmless food coloring... Pharmacologically active derivatives of B_2 were not known and have barely been developed until now, so that interest in B_2 was at a low point, particularly in medicine. This situation is only changing now... : we have an involvement of B_2 even in human medicine, namely in the oxygen metabolism of cells."

(From the memoirs of Peter Hemmerich dictated in August 1981)

Flavins and Flavoproteins

Flavins in practical and experimental medicine

The use of riboflavin and riboflavin analogues in clinical and experimental medicine - e.g. in hypertension research - have been reviewed recently (1,2). Another aspect, the structural similarity of flavin with drugs like tricyclic antidepressants has also attracted the attention of clinical pharmacologists (3).

Here we should like to comment on the enzymatic test for the detection of riboflavin (=B_2) deficiency (4,5). In our opinion, this test is compulsory for malnourished women prior to pregnancy. The B_2-status of a person is assessed by measuring the activity of glutathione reductase in hemolysate before and after addition of FAD; in this way the proportion of apoglutathione reductase lacking the flavin-containing cofactor is determined. The basic assumption underlying this test, namely the biological and physical stability of apoglutathione reductase of red blood cells (6,7), was recently confirmed by X-ray diffraction analysis. We found that the apoenzyme has a defined geometric structure; it even closely resembles the holoenzyme from which the structure of the prosthetic group has been subtracted (6,8). The occurrence of the stable apoenzyme *in vivo*, as well as the similarity of apo- and holoenzyme are points which have to be accounted for also in drug-designing studies (9).

Flavoproteins as targets of drugs and toxic compounds

As reviewed by Merrill et al. (1) inhibitors of flavoproteins play a prominent role in pharmacology. Points in case are allopurinol and other inhibitors of xanthine oxidase which are used in the treatment and prevention of gout, or inhibitors of monoamine oxidases which serve as antidepressant drugs.

A number of toxic compounds found in foodstuffs inhibit flavoenzyme activities. Examples are 3-nitropropionate, a suicide substrate of succinate dehydrogenase and hypoglycin A, the causing agent of Jamaican vomiting sickness; hypoglycin A is metabolized to methylene-cyclopropyl-acetyl-CoA, an inhibitor of acyl-CoA dehydrogenase. The interactions of dioxygen, the most abundant and possibly the most toxic foodstuff, with flavoenzymes is discussed below. The cited examples illustrate that flavin enzymologists have greatly contributed to the elucidation of the mode of drug action. Consequently one may hesitate to use the term 'flavin pharmacology' in a narrow sense as it is done in the next section.

Flavoenzymes in OSVAC processes, a challenge for drug research

An adult person consumes appr. 1000g O_2 per day. Some 3% to 5% of this oxygen, that is 30 to 50g O_2, is not directly reduced to water by cytochrome oxidase but metabolized to $^1O_2$, $\dot{O}_2^-$, H_2O_2 , $OH^{\cdot}$, OCl^- and other cytotoxic compounds. The sum of these reactive oxygen species represents the oxidant stress (10-16). Flavoenzymes play a predominant role in the production of oxidant stress (Table 1) . Points in case are the $\dot{O}_2^-$-producing NADPH-oxidase (16-19) of neutrophilic leucocytes and macrophages and the xanthine oxidase of endothelial and sinusoidal cells (20). These enzymes reduced O_2 directly; other flavoenzymes such as NADPH-cytochrome P-450 (c) oxidoreductase do so indirectly by reducing so-called redox-cyclers (=X in the Scheme), which in turn spontaneously react with O_2:

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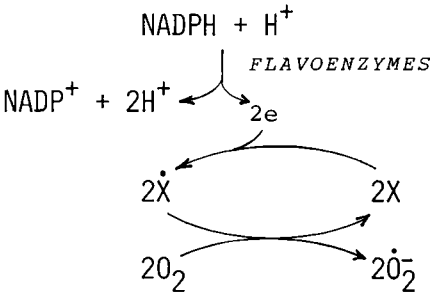
*OSVAC = Oxidant Stress versus Antioxidant Capacity



Scheme I

$\dot{O}_2^-$ is the educt for a number of other reactions (10-12,16) some of which are sketched in Fig.1

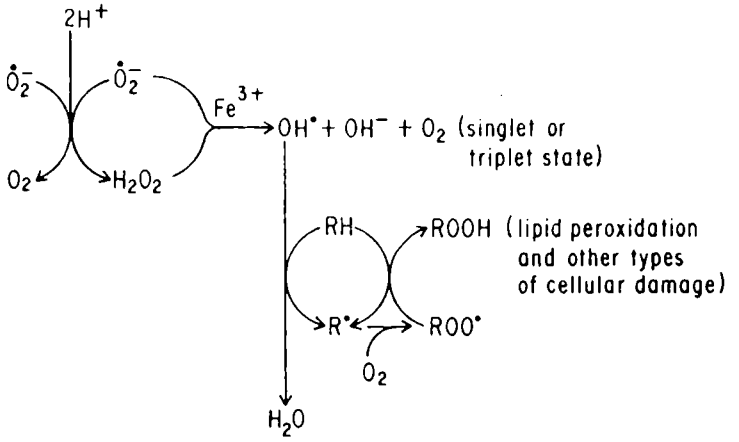


Fig. 1 Oxidant stress leading to damage of macromolecules(=R) As radical-initiated processes are conservative and propagative they may lead to the production of secondary and tertiary free radicals derived from lipids, amino acids, glutathione or nucleic acids (after Docampo and Moreno (12)).

Examples for the increasing number of redox-cycling compounds are catechols and related substances, iron chelators, aromatic nitrocompounds such as nitrofurantoin or nifurtimox, adriamycin and related cytostatic agents, the herbicide paraquat, and many naturally occurring pesticides (10-14). It should be noted that the reactions discussed do not only cause oxidant stress; at the same time they consume precious cellular compounds such as NADPH and O_2. The hypoxic condition induced by the redox-cycling of adriamycin, for instance, is believed to contribute to the cardiotoxicity of this compound (10).

As shown in Table 1, reactive oxygen species are not only harmful for the human organism; indeed they are vital for the destruction of pathogenic microorganisms and tumour cells.

There exist powerful defense systems against oxidant stress the sum of which represents the antioxidant capacity of a cell or tissue. A cornerstone of the antioxidant capacity is the FAD-enzyme glutathione reductase (6-9,21). Thus there are flavoenzymes on both sides in OSVAC processes. However, in the presence of trinitrobenzene sulfonate *in vitro* (22) and probably in the presence of paraquat *in vivo* (23), glutathione reductase turns into an NADPH oxidase which means that the enzyme can change sides in OSVAC processes. This behaviour is believed to contribute to the damaging effects of herbicides on plant cells as well as on lung cells or on fibres of the eye lens (23,24).

In conclusion, if OSVAC-processes are really as important in medicine as it is currently believed (Table 1 and ref. 10-20), the flavoenzymes involved will play a central role in drug research. As a rule, oxidative damage to cell constituents and tissues is preceded by a reduction step which is catalysed by a flavoenzyme.

TABLE 1
ROLE OF FLAVOENZYMES IN OSVAC-PROCESSES (OSVAC = OXIDANT STRESS VERSUS ANTIOXIDANT CAPACITY)

SYSTEM	OXIDANT STRESS	REASON FOR IMPAIRED ANTIOXIDANT CAPACITY	CONSEQUENCES
TUMOURS TREATED WITH THE CYTOSTATIC AGENT BCNU (25,28)	RESPIRATORY BURST OF MACROPHAGES, CATALYZED BY THE FAD-ENZYME NADPH-OXIDASE ($NADPH+2O_2 \rightleftharpoons NADP^+ + H^+ + 2O_2^{\cdot-}$)	INHIBITION OF THE FAD-ENZYME GSSG REDUCTASE BY BCNU IN THE MALIGNANT CELLS	DEATH OF THE TUMOUR CELLS
HEART MUSCLE CELLS SUFFERING FROM THE SIDE EFFECTS OF CYTOSTATIC DRUGS(26,27)	$O_2^{\cdot-}$-PRODUCTION CATALYZED BY FLAVOENZYMES AND ADRIAMYCIN(10)	A) LOW ACTIVITY OF ANTIOXIDANT ENZYME SYSTEMS IN HEART MYOCYTES (27) B) INHIBITION OF GSSG-REDUCTASE BY BCNU (28)	CARDIOMYOPATHY
BACTERIAL INFECTIONS (16-19)	RESPIRATORY BURST OF NEUTROPHILIC LEUCOCYTES CATALYZED BY THE FAD-ENZYME NADPH-OXIDASE	LACK OF ANTIOXIDANT ENZYME SYSTEMS IN THE BACTERIAL COATS	DEATH OF THE MICROORGANISMS
INFECTIONS IN PATIENTS HAVING CHRONIC GRANULOMATOUS DISEASE (CGD) (31)	NO $O_2^{\cdot-}$-PRODUCTION BY LEUCOCYTES BECAUSE OF NADPH-OXIDASE DEFICIENCY		FREQUENT AND SERIOUS BACTERIAL INFECTIONS
CHRONIC RHEUMATIC DISEASES (10,11)	RESPIRATORY BURST OF NEUTROPHILIC LEUCOCYTES IN JOINT CAVITIES	LACK OF ANTIOXIDANT ENZYME SYSTEMS IN EXTRACELLULAR SPACES	CHRONIC DISEASE
CHAGAS DISEASE (12)	$O_2^{\cdot-}$-PRODUCTION CATALYZED BY FLAVOENZYMES PLUS DRUGS	LOW ACTIVITY OF ANTIOXIDANT ENZYME SYSTEMS IN *TRYPANOSOMA CRUZI*	DEATH OF TRYPANOSOMES
ALVEOLAR CELLS IN THE LUNG OF PATIENTS TREATED WITH NITROAROMATIC DRUGS (10-12,29)	A) HIGH O_2-TENSION IN THE LUNG B) $O_2^{\cdot-}$-PRODUCTION, CATALYZED BY FLAVOPROTEINS PLUS DRUGS	THE FAD-ENZYME GLUTATHIONE REDUCTASE IS INHIBITED OR CHANGES SIDES UNDER THE INFLUENCE OF THE DRUG	LUNG FIBROSIS
MALARIA-INFECTED ERYTHROCYTES IN PATIENTS HAVING FAVISM * (30,32-35)	H_2O_2 PRODUCTION, CATALYZED BY ISOURAMIL AND DIVICINE, TWO ACTIVE PRINCIPLES OF FAVA-BEANS (FIG.2)	INBORN DEFICIENCY OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE	DEATH OF PLASMODIUM FALCIPARUM

* THIS SYSTEM IS LISTED HERE BECAUSE IN CAN BE IMITATED BY INHIBITING THE FAD-ENZYME GSSG-REDUCTASE OF PARASITIZED ERYTHROCYTES (SEE TEXT)

Inhibitors of glutathione reductase as potential antimalarial drugs

Recent biochemical studies (32) corroborate the hypothesis that favism confers some protection against malaria, and suggested to us that the biochemistry of favism might be mimicked by inhibiting the enzyme glutathione reductase in human erythrocytes (Fig.2) The threedimensional structure of this flavoenzyme which catalyzes the reaction NADPH + GSSG + H^+ $\rightleftharpoons$ $NADP^+$ + 2GSH is known (8,9,21,36), so that the interactions of glutathione reductase and inhibitors can be studied in atomic detail and the design of new inhibitors with desirable properties is facilitated. -We have studied two compounds which inhibit glutathione reductase as antimalarials: the clinically used antineoplastic agent carmustine (BCNU) and its newly developed water-soluble and less toxic analogue, 1-(2-chloroethyl)-1-nitroso-3-(2-hydroxyethyl)urea = HECNU (37). Both drugs inhibited the growth of Plasmodium falciparum in culture. In addition, HECNU was shown to have a curative effect on ro-

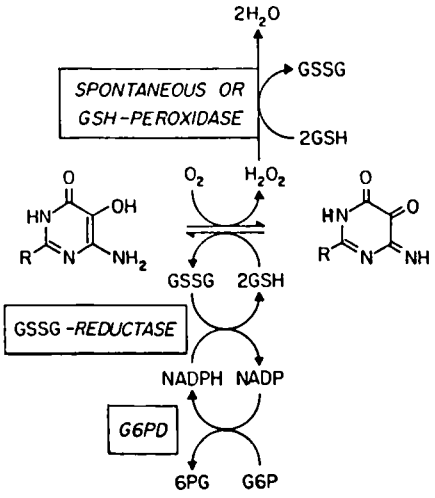


Fig. 2

Favism: The action of broad bean pyrimidines in the red blood cell. One of the 2e-redox-cycling pyrimidines is isouramil (R=OH). When isouramil reacts with O_2, H_2O_2 representing oxidant stress is formed. Both H_2O_2 and oxidized isouramil are reduced at the expense of (four) GSH molecules. The oxidized glutathione is reduced by the flavoenzyme glutathione reductase. G6PD, glucose-6-phosphate dehydrogenase; 6PG, 6-phosphogluconolactone; G6P, glucose 6-phosphate (after Chevion et al.(38)).

Table 2 Effect of BCNU and HECNU on rodent malaria

All animals were infected with $5 \cdot 10^8$ parasitized erythrocytes given intraperitoneally on day zero. From day 1 onwards the drugs were administered as two intraperitoneal injections (7.5 mg/kg body weight at 9 a.m. and 7.5 mg/kg at 1 p.m.).- Results analogous to those shown here were obtained with NMRI-mice after infection with Plasmodium vinckei, with the chloroquine-resistant strain K65 of Plasmodium berghei and with the chloroquine-sensitive strain of Plasmodium berghei, respectively.

Parasitemia on day 3 after infection with Plasmodium vinckei

Control rats (n = 5)	BCNU-treated rats (n = 5)	HECNU-treated rats (n = 5)
70%	8%	1%
60%	25%	1%
32%	20%	3%
60%	3%	6%
90%	8%	3%
all animals died before or on day 6	all animals were alive on day 6 but suffered from side effects of BCNU and the treatment was stopped. The animals died of malaria before day 11.	all animals remained apparently healthy; on day 8 their blood appeared to be free of parasites. On day 10- when the treatment was stopped-it was no longer infectious.

dent malaria being equally effective against chloroquine-resistant and chloroquine-sensitive strains of P.vinckei (Table 2). It remains to be studied to what extent the in vivo effects of HECNU are indeed caused by the inhibition of glutathione reductase, but our results are consistent with the notion that parasitized erythrocytes have a critical OSVAC balance. Increasing the oxidant stress (13) or decreasing the reducing capacity by inhibiting glutathione reductase (34) can lead to the selective destruction of parasitized cells. Being carcinogenic, BCNU and HECNU might never be considered for treating human malaria. Therefore a series of other compounds inhibiting glutathione reductase from human erythrocytes and from P. vinckei (39-41) were designed and synthesized; most of these inhibitors are carbamoylimidazolides and carbamoyloxosuccinimides. 1-Deaza-flavin is also of interest because 1-deaza-FAD glutathione reductase resembles the enzyme species found in congenital glutathione reductase deficiency of red blood cells (ref. 42 and Krauth-Siegel et al., this volume).

Some considerations on the development of flavoenzyme-directed drugs

a) Experimenta naturae including diseases (31,42-44) might be used as guidelines in drug research. For instance, the properties of the NADPH-oxidase in CGD (Table 1) or the properties of glutathione reductase in congenital glutathione reductase deficiency (42) should be studied and then be imitated by using inhibitors. Attempts in this direction have been mentioned above.

b) Specific inhibitors may be obtained by drug-tailoring in light of the three-dimensional structure of an enzyme (Fig. 3). Alternatively, one may try to link flavin with another ligand of a given flavoenzyme: If the binding sites do not overlap, the dissociation constant for the pluriligand inhibitor is expected to correspond the product of the K_i-

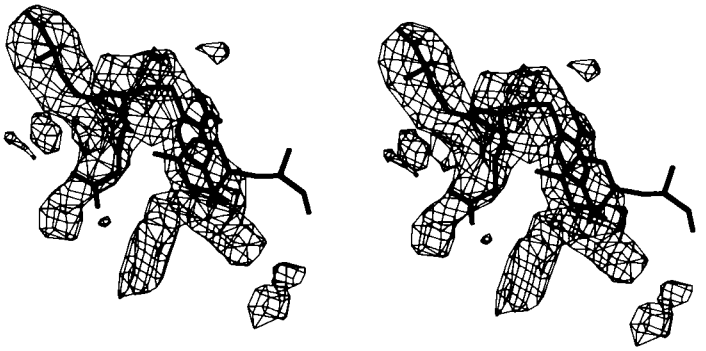


Fig. 3

Example of a drug derivative as bound to glutathione reductase at the GSSG-binding site (46). 1-Chloro-2,4-dinitrobenzene - which is used in studies on oxidant stress in liver (and in the treatment of warts) - is conjugated in vivo with GSH to give S-(2,4-dinitrophenyl)-glutathione (DNPG). As shown by X-ray crystallography, DNPG binds to glutathione reductase in an unexpected manner. This example illustrates that drugtailoring on the basis of a three-dimensional structure of a target enzyme must be accompanied by many structural analyses.
The figure shows a stereo view of DNPG as bound to glutathione reductase which is represented by Tyr-114 at the upper left hand side. The difference electron density is shown in bird cage representation. Details are given in ref. 46.

values of the individual ligands (45).

c) The effect of the drug may depend on the presence of the prosthetic group FAD or FMN. The cytostatic agent carmustine (BCNU), for instance, irreversibly modifies hologlutathione reductase in vivo but not the FAD-free apoenzyme which remains as a potential apoenzyme. This fact complicates clinical and experimental studies with inhibitors of glutathione reductase.

d) Inhibitors targeted to flavoenzymes of pathogenic bacteria or protozoa are of special interest. This is even more the case if the inhibitor can change the flavoenzyme into an

oxidase (22-24); many pathogens possess poor antioxidant capacity (12,13).

e) In general - but particularly in the case of flavin analogues - the free compound, its precursors and catabolites may have pharmacologic effects (1).

Finally one should not forget the rule of the drug industry that rational approaches to drug design are doomed to fail.

Conclusions

OSVAC processes which reflect the versatility of oxygen metabolism contribute to many pathophysiological conditions. In most OSVAC mechanism flavoenzymes play dominant roles on both sides (9-20,33,34). Consequently flavoprotein-directed compounds may become an important field of drug research. To quote again from the memoirs of Peter Hemmerich: "The chemistry of B_2 will see considerable changes and will, in the long run, be anything but a 'breadless' undertaking."

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