Inhibition of Human Glutathione Reductase by 10-Arylisoalloxazines: Crystallographic, Kinetic, and Electrochemical Studies

Annette Schönleben-Janas,[†] Peer Kirsch,[‡] Peer R. E. Mittl,[§] R. Heiner Schirmer,[†] and R. Luise Krauth-Siegel*[,†]

Institut für Biochemie II der Universität, Im Neuenheimer Feld 328, D-69120 Heidelberg, Germany, Abteilung Organische Chemie, Max-Planck-Institut für Medizinische Forschung, Jahnstrasse 29, D-69120 Heidelberg, Germany, and Institut für Organische Chemie und Biochemie der Universität, Albertstrasse 21, D-79104 Freiburg, Germany

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A series of newly synthesized N^{10}-arylisoalloxazines—some of which are known to be antimalarial agents—were studied as inhibitors of human glutathione reductase (GR; NADPH + GSSG + $H^+ \rightleftharpoons NADP^+$ + 2GSH). The flavoenzyme was inhibited with IC_{50} values between ≤1 and 100 μM in the presence of 100 μM GSSG and 100 μM NADPH. The isoalloxazines and N^3-methylisoalloxazines with a 4′-chlorophenyl or a 3′,5′-dichlorophenyl group at N10 were found to be the most promising inhibitors of GR, although even the bulkier 10-naphthyl and -anthryl derivatives were also effective inhibitors. In contrast, at position N3 of the isoalloxazine ring, the size of the substituent was found to strongly influence the inhibitory effect. Introduction of a carboxymethyl group at N3—which markedly increased the solubility of the derivative in aqueous solutions—caused a rise in the IC_{50} values by 1 order of magnitude. 8-Fluoro- and 8-azido-10-arylisoalloxazines were potent inhibitors of GR; consequently position C8 of the benzenoid subnucleus instead of N3 should be considered for introducing substituents. No correlation was observed between the inhibitory strength of several isoalloxazines and their redox potential as measured by cyclovoltammetry. The crystallographic analysis of GR complexed with 10-(4′-chlorophenyl)-3-(carboxymethyl)isoalloxazine and 10-(3′,5′-dichlorophenyl)-3-(carboxymethyl)isoalloxazine, respectively, revealed the presence of one inhibitor molecule bound at the 2-fold axis of the homodimeric protein. This location is consistent with fluorescence titration measurements and enzyme kinetic studies in solution which gave no indication for binding at the substrate sites.

Introduction

Glutathione reductase (GR, EC 1.6.4.2) is a key enzyme in the cell's defense mechanisms against oxidative stress. The flavoprotein is responsible for the reducing intracellular milieu by catalyzing the reaction: NADPH + GSSG + $H^+ \rightleftharpoons NADP^+$ + 2GSH.[1] From a pharmacological viewpoint, human GR is an attractive target of antimalarial and antitumor drugs.[2,3] All requirements for a rational drug development are fulfilled. The three-dimensional structures of human GR[4] as well as of complexes with its substrates and several inhibitors have been solved by X-ray diffraction analysis (see ref 3 for a review). In addition, a bacterial strain that overexpresses the recombinant enzyme is available.[5]

10-(Substituted phenyl)flavins were shown to possess antimalarial activity both *in vivo* against rodent malaria and *in vitro* against the human pathogen *Plasmodium falciparum*.[6] Among the most potent compounds, the 10-(4′-chlorophenyl) and 10-[3′-(trifluoromethyl)phenyl] derivatives turned out to be effective inhibitors of GR.[7]

Here we describe a series of new 10-arylisoalloxazines studied as inhibitors of GR. The poor water solubility of the derivatives was markedly improved by introducing a carboxymethyl group at position N3.[8] Molecular interactions between the isoalloxazine derivatives and GR were studied by three different approaches: (i) inhibitor constants were determined by enzyme kinetics in solution, (ii) binding to wild-type GR and to an active site Tyr mutant[9] were followed by fluorescence measurements, and (iii) X-ray diffraction analysis of crystalline enzyme−inhibitor complexes showed the localization of the inhibitor in the protein structure.

Results and Discussion

Inhibition of Human GR by Isoalloxazines. Twenty-seven 10-arylisoalloxazines which had been synthesized and characterized as described in ref 8 were studied as inhibitors of human GR. The flavin analogues interact reversibly with the enzyme: inhibition is not time-dependent, and full activity is regained upon dilution or dialysis against an inhibitor-free solution. In order to compare the inhibitory strength among the isoalloxazines, enzyme assays were carried out at physiologic substrate concentrations (100 μM GSSG and 100 μM NADPH) while the inhibitor concentration was varied between 0.5 and 150 μM.

The compounds studied can be subdivided on the basis of their substitution pattern (Figure 1). The first group (**2a**−**e**) possesses an N3-unsubstituted isoalloxazine ring, the second group (**3a**−**e**, **18**−**20**) is N^3-methylisoalloxazines, the third one (**4a**−**d**) is 3-[(ethoxycarbonyl)methyl]isoalloxazines, and the fourth group (**5a**−**d**) comprises 3-(carboxymethyl)isoalloxazines (Table 1a). Within each group, the 4′-chloro-, 3′,5′-dichloro-, and 3′-(trifluoromethyl)phenyl derivatives were highly effective inhibitors of human GR. There was no appreciable difference between isoalloxazines **2a**−**d** and 3-methylisoalloxazines **3a**−**d**. The latter compounds are known to be potent antimalarial agents in *Plasmo-*

* Corresponding author.
[†] Institut für Biochemie II der Universität Heidelberg.
[‡] Max-Planck-Institut für Medizinische Forschung.
[§] Institut für Organische Chemie und Biochemie der Universität Freiburg.
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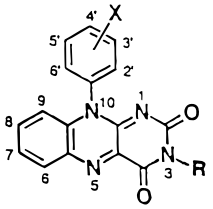

Figure 1. Structures of N^{10}-(halophenyl)-3-substituted isoalloxazines: R = H in **2a**−**e**, R = CH_3 in **3a**−**e**, R = (ethoxycarbonyl)methyl in **4a**−**d**, R = carboxymethyl in **5a**−**d**, X = 4′-Cl in **2a**, **3a**, **4a**, and **5a**, X = 3′,5′-Cl_2 in **2b**, **3b**, **4b**, and **5b**, X = 3′-CF_3 in **2c**, **3c**, **4c**, and **5c**, X = 3′,5′-$(CF_3)_2$ in **2d**, **3d**, **4d**, and **5d**, and X = 2′,3′,4′,5′,6′-F_5 in **2e** and **3e**.

Table 1. Inhibition of Human GR[a]

a. By 10-Arylisoalloxazine Derivatives

compd	X substituent	IC_{50} (μM)
	10-Phenylisoalloxazines	
2a	4′-Cl	<1
2b	3′,5′-Cl_2	<1
2c	3′-CF_3	1.0
2d	3′,5′-$(CF_3)_2$	3.5
2e	F_5	3.5
	10-Aryl-3-methylisoalloxazines	
3a	4′-Cl	<1
3b	3′,5′-Cl_2	<1
3c	3′-CF_3	1.0
3d	3′,5′-$(CF_3)_2$	5.0
3e	F_5	<1
18	H	2.0
19	10-(1′-naphthyl)	2.0
20	10-(1′-anthryl)	4.0
	10-Phenyl-3-[(ethoxycarbonyl)methyl]isoalloxazines	
4a	4′-Cl	18
4b	3′,5′-Cl_2	7
4c	3′-CF_3	8
4d	3′,5′-$(CF_3)_2$	100
	10-Phenyl-3-(carboxymethyl)isoalloxazines	
5a	4′-Cl	12
5b	3′,5′-Cl_2	14
5c	3′-CF_3	20
5d	3′,5′-$(CF_3)_2$	25

b. By 10-Pyridyl- and 10-(Methylpyridiniumyl)-3-methylisoalloxazines

compd	substituent at N10	IC_{50} (μM)
6a	4′-pyridyl	3
7a	3′-pyridyl	8
6	methylpyridinium-4′-yl	40
7	methylpyridinium-3′-yl	no inhibition up to 100 μM

c. By 8-Azido-10-(4′-chlorophenyl)-3-methylisoalloxazine (**12**) and 8-Fluoro-10-(4′-chlorophenyl)-3-methylisoalloxazine (**14**)

compd	substituent	IC_{50} (μM)
12	8-azido-10-(4′-chlorophenyl)	2.5
14	8-fluoro-10-(4′-chlorophenyl)	1.0

[a] The structural formulas of the compounds are given in Figure 1. GR activity was measured in the presence of 100 μM NADPH and 100 μM GSSG in assay buffer, pH 6.9. The residual activity (%) is given relative to controls without inhibitor in the presence of an equal volume of solvent. The IC_{50} values calculated from these data represent the inhibitor concentration which causes 50% inhibition of the enzyme at a GSSG concentration of 100 μM.

dium vinckei-infected mice and against *P. falciparum* in culture.[10]

Introduction of an (ethoxycarbonyl)methyl (**4a**−**d**) or a carboxymethyl (**5a**−**d**) group at N3 weakens the interaction with the protein as indicated by the higher IC_{50} values. Nevertheless, the hydrophilic 3-(carboxymethyl)isoalloxazines were well suited for studying crystalline enzyme−inhibitor complexes by protein crystallography (see below). The diminished inhibitory effect of these derivatives is not due to the hydrophilicity or the negative charge but rather to the size of the substituent. In a series of 10-(4′-chlorophenyl)isoalloxazines, any aliphatic or aromatic substituent at N3 larger than a methyl group caused an increase of the IC_{50} value by factors of 5−50.[11]

Table 2. Electrochemical Data[a]

a. 10-Aryl-3-methylisoalloxazines **3a**,**e**, **18**, and **19**, 10-Aryl-3-(carboxymethyl)isoalloxazines **5a**−**d**, and 10-(4′-Chlorophenyl)-8-fluoro-3-methylisoalloxazine **14**

compd	X substituent	$E_{1/2}$ (mV)	$E_{pa} - E_{pc}$ (mV)
18	H	−152	42
3a	4′-Cl	−130	46
3e	F_5	−56	42
5a	4′-Cl	−145	48
5b	3′,5′-Cl_2	−129	42
5c	3′-CF_3	−130	38
5d	3′,5′-$(CF_3)_2$	−121	46
14	4′-Cl, 8-fluoro	−143	29
19	10-(1-naphthyl)	−143	51
		−143	43[b]

b. 10-Pyridyl- and 10-(Methylpyridiniumyl)-3-methylisoalloxazines

compd	substituent at N10	$E_{1/2}$ (mV)	$E_{pa} - E_{pc}$ (mV)
6a	4′-pyridyl	−97	56[c]
7a	3′-pyridyl	−126	66
6	methylpyridinium-4′-yl	21	68
7	methylpyridinium-3′-yl	−57	62

[a] The redox potentials were measured as described under Experimental Procedures and in the legend of Figure 2. Unless otherwise stated, the cyclovoltammograms were recorded at a scan speed of 100 mV/s. [b] v = 50 mV/s. [c] v = 1000 mV/s.

Inhibition of GR by the 10-(4′-pyridyl) and 10-(3′-pyridyl) isoalloxazines **6a** and **7a** was comparable to the effect of the phenyl derivative **18**. In contrast, the methylated pyridinium salts **6** and **7** are very weak inhibitors of GR (Table 1b). The positive charge of the N10-substituent obviously interferes with binding to the protein. Both pyridinium salts **6** and **7** but not the pyridyl derivatives **6a** and **7a** cause nonenzymatic oxidation of NADPH which correlates with their high redox potentials (Table 2). $\pi-\pi$ Interactions between the N10-substituent of the isoalloxazines and aromatic side chains of the protein largely contribute to inhibition since 10-alkyl-[12] and 10-pyridinium (**6** and **7**) 3-methylisoalloxazines are much weaker inhibitors. In addition, the 10-(pentafluorophenyl)-3-methylisoalloxazine (**3e**) is an even better inhibitor than the corresponding 10-phenyl derivative **18**. This might be due to the π-acceptor properties or the higher hydrophobicity of the fluorinated compound. Even bulkier aromatic moieties are acceptable as substituents at N10 since the 10-naphthyl- and 10-anthrylisoalloxazines **19** and **20** are effective inhibitors of GR (Table 1a).

As representatives of derivatives substituted at C8, the 8-fluoro- (**14**) and 8-azido- (**12**) 10-(4′-chlorophenyl)-3-methylisoalloxazines were studied; the photolabel **12** may be used to fix a flavin analogue covalently to a macromolecule.[13] Both compounds are effective inhibitors of GR (Table 1c). Possibly C8 is even better suited for the introduction of substituents than N3.

Fluorescence Emission Spectra of Compound 3a Bound to GR. Tyr114 represents the catalytic site of GR since it tunes the interaction between the substrate

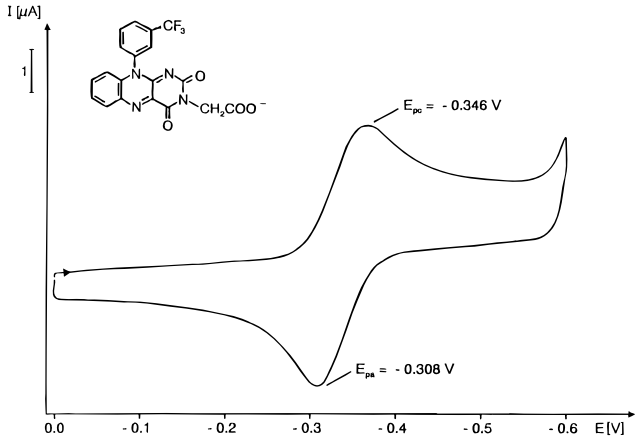

Figure 2. Cyclovoltammogram of the 10-(3′-CF_3-phenyl)-3-(carboxymethyl)isoalloxazine (**5c**). The curve shows the characteristics of a pseudoreversible transfer of two electrons. The half-wave potential, $E_{1/2} = (E_{pa} + E_{pc})/2$, is defined as the average of the anodic (E_{pa}) and cathodic (E_{pc}) peak potentials. The voltammogram was recorded under the following conditions: potential of the reference electrode (197 mV) set to zero, total current $I = 100$ μA, sweep rate $v = 100$ mV/s, recorder speed $x = y = 20$ mV/cm. The resulting electrochemical data are $E_{1/2} = (-346 - 308\ (\text{mV}))/2 + 197\ (\text{mV}) = -130$ mV and $\Delta E = -308 + 346 = 38$ mV.

glutathione disulfide and the enzyme's redox active dithiol Cys58/Cys63. In order to test if the isoalloxazine inhibitors bind close to the active site, wild-type GR and the Tyr114Leu mutant,[9] respectively, were titrated with compound **3a** and the fluorescence emission spectra were recorded. Since Tyr114 participates in substrate fixation by making direct hydrogen bonds with GSSG,[14] differences in the fluorescence intensities of compound **3a** bound to wild type and mutant GR would be an indication for inhibitor binding at the active site.

Because of the probable dimerization of isoalloxazines at concentrations above 8 μM with concomitant self-quenching of fluorescence,[15] the protein concentration was chosen between 1 and 2 μM and the inhibitor was added in steps of 0.2 μM. The titration curves for wild-type GR and the Tyr114Leu mutant were practically identical (data not shown), which indicates that the inhibitor is not bound in the vicinity of Tyr114. This finding—obtained for the enzyme in solution—is consistent with the crystallographic results (see below). The stoichiometry of inhibitor molecules bound per protein subunit could not be determined by fluorescence measurements. The titration curves did not show a clear break, probably due to unspecific adsorption of the compound to the protein surface as has been reported for other flavins.[16]

Redox Potentials of 10-Arylisoalloxazines. The redox potentials of several 10-arylisoalloxazines were studied by cyclic voltammetry[17] in order to reveal a possible correlation between the binding strength of the inhibitors and their $\pi-\pi$-acceptor properties (Table 2). A dependence of the inhibitory potency on the redox potential was suggested by the observation that the pentafluorophenyl-substituted isoalloxazine **3e** was a more effective inhibitor than the phenyl derivative **18**, both compounds having the same steric requirements. To our knowledge, this is the first application of cyclovoltammetry to flavin analogues in aqueous solution. Figure 2 shows the cyclovoltammogram of **5c** which represents a typical curve for a reversible transfer of two charges. In the case of a two-electron transfer, $E_{pa} - E_{pc}$ (the difference between the anodic and cathodic peak potentials) is usually lower than 56 mV, which is the theoretical value of a one-electron transfer. As references the redox potentials for $K_3[Fe(CN)_6]$ (451 mV), riboflavin (−215 mV), and 3-methyl-10-phenylisoalloxazine (**18**; −152 mV) were measured. These values agree well with the literature values (430,[18] −208,[19] and −2 mV at pH 4.7[19]—corresponding to −140 mV at pH 7.0—respectively). As expected, electron-withdrawing aryl substituents at N10 of the isoalloxazine shift the potentials to more positive values (Table 2). For instance, the redox potential of the pentafluorophenyl-substituted isoalloxazine **3e** is nearly 100 mV more positive than that of the corresponding phenyl derivative **18**. Also in the group of the hydrophilic carboxymethyl derivates **5a**−**d**, a positive shift of the redox potential correlates with an increasing tendency of the aryl moiety to accept electrons.

The assumption based on the pairwise comparison between **3e** and **18** could not be confirmed. In general, there was no correlation between redox potential and inhibition constant.

The derivatives with the most positive redox potentials, the methylpyridinium salts **6** and **7**, are not significant inhibitors of GR (Tables 1b and 2b). However, they oxidize the substrate NADPH in the absence of the enzyme. This phenomenon was also observed—although to a lesser extent—for the pentafluorophenyl derivatives **2e** and **3e**, again compounds with high redox potentials. Because the ΔE values of the pyridyl-3-methylisoalloxazines (**6**, **6a**, **7**, **7a**) exceed 56 mV, it cannot be excluded that with these compounds *one*-electron transfer reactions were measured.

X-ray Diffraction Analysis of GR−Inhibitor Complexes. Single crystals of human GR were soaked with 10-(4′-chlorophenyl)-3-(carboxymethyl)isoalloxazine (**5a**) and 10-(3′,5′-dichlorophenyl)-3-(carboxymethyl)isoalloxazine (**5b**), respectively, as described under Experimental Procedures. The 3-carboxymethyl derivatives had been selected because of their solubility in aqueous solution.

Binding of these compounds to the enzyme at high ionic strength was confirmed by determining IC_{50} values in the presence of 1 M ammonium sulfate in the assay mixture. Under these conditions the IC_{50} value of compound **5a** was 50 μM.

The difference Fourier maps of the crystalline enzyme−inhibitor complexes at 3 Å resolution showed electron density exclusively in a cavity at the 2-fold axis of the homodimeric protein (Figure 3). The isoalloxazine moiety of the inhibitors was found to be sandwiched between the Phe78 residues of the two subunits. The size of the cavity allows the accommodation of only 1 inhibitor molecule/GR dimer. As the inhibitor does not possess a C_2 symmetry but binds at the 2-fold axis, it breaks the symmetry of the crystal. In addition, the intercalation of the inhibitor in the crystal lattice occurred randomly. Consequently we were not able to assign a unique conformation, and the structures were not further refined. The electron density for the 3′,5′-dichlorophenyl derivative **5b** was 2 times as high as for the 4′-chlorophenyl derivative, although both compounds have similar IC_{50} values in solution. The results

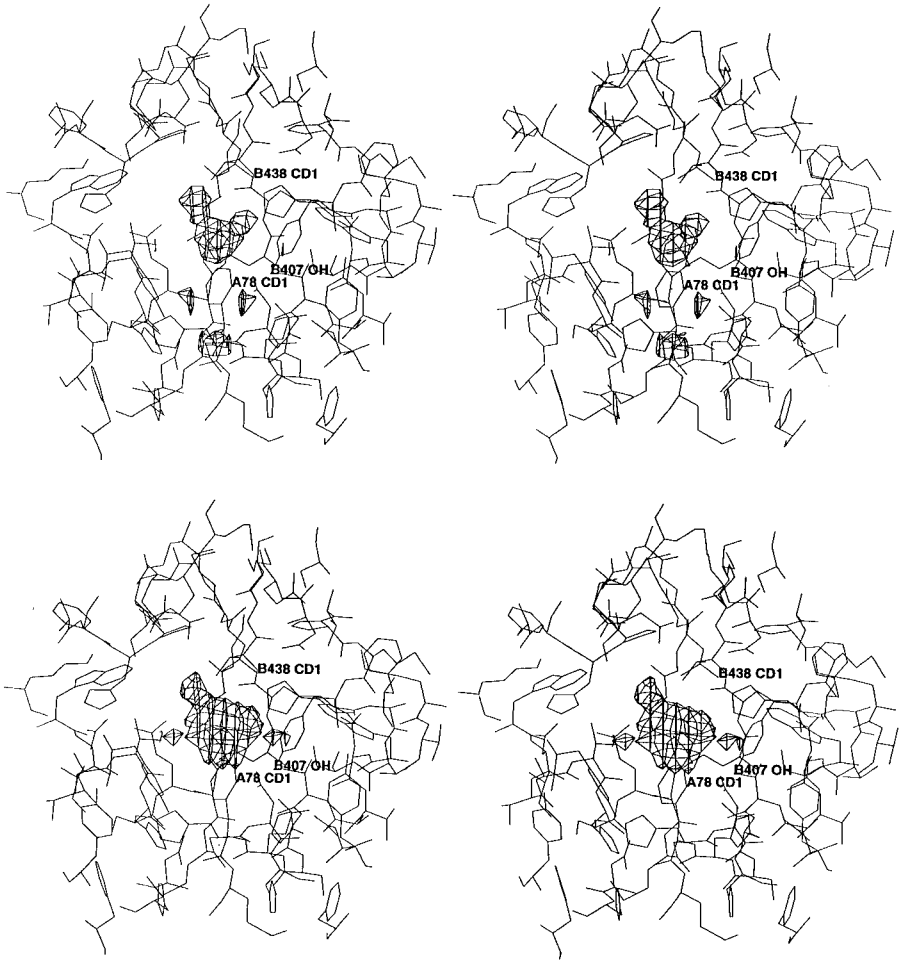

Figure 3. Human GR complexed with (a) 10-(4′-chlorophenyl)-3-(carboxymethyl)isoalloxazine (**5a**) and (b) 10-(3′,5′-dichlorophenyl)-3-(carboxymethyl)isoalloxazine (**5b**). Stereoviews of protein sections with 11 Å radius around the 2-fold axis are shown. The inhibitors are given in bird-cage presentation at cut-off levels of (a) 3.0σ and (b) 3.5σ. They bind at the 2-fold axis of the homodimeric protein in a cavity which is lined *inter alia* by the aromatic residues His75, Phe78, and Tyr407 and their symmetric counterparts in the other subunit. A 78 CD1 is a ring atom of Phe78 of one subunit; B 407 OH and B 438 CD1, respectively, are side chain atoms of Tyr407 and Leu438 of the other subunit.

of the X-ray diffraction analysis gave no indication for binding of the isoalloxazines at either substrate site. The binding mode of the isoalloxazine derivatives to GR is thus strikingly similar to that of menadione and safranin which are also located close to Phe78 and Phe78′ at the 2-fold axis.[20]

In contrast to GR, the structurally closely related parasite enzyme trypanothione reductase of *Trypanosoma cruzi* is not inhibited by the isoalloxazines **5a**,**b**. This finding agrees well with the fact that in this enzyme the cavity at the 2-fold axis is much smaller than in GR (Ch. Lantwin and R. L. Krauth-Siegel, unpublished data).

At 3 Å resolution no major structural changes of the protein could be observed upon binding of the isoalloxazines. Since His75 and Phe78 as well as the redox-active Cys58/Cys63 couple are constituents of the same long helix (residues 56−80), one may speculate that binding of the aromatic compounds at Phe78 can influence catalysis by altering the electronic distribution at the active site.[1] A well-known example of an asymmetric effector molecule located between subunits of a symmetric oligomeric protein is 2′,3′-bisphosphoglycerate as a ligand of hemoglobin.[21,22]

Conclusions

Human GR is an attractive target structure for antitumor and antimalarial agents. 10-Substituted (halophenyl)isoalloxazines have been shown to be active against different *Plasmodia* species on the one hand and to be inhibitors of GR on the other.[6,7]

The binding locus of the isoalloxazines in GR was evaluated. X-ray diffraction analysis of two crystalline enzyme−inhibitor complexes revealed that the isoalloxazine molecule was bound in a cavity at the 2-fold axis of the dimeric protein, which implies a stoichiometry of 0.5 inhibitor molecule/protein subunit. Kinetic studies in solution as well as fluorescence titrations and the difference Fourier analysis gave no indication for any direct interaction of the isoalloxazines with the substrate binding sites. The knowledge of the inhibitor binding site will now allow the tailoring of derivatives with even better binding properties, for instance, by introducing a reactive group that mediates covalent modification of GR.

Comparison of all 27 isoalloxazines with respect to their inhibitory action on GR clearly demonstrates the tolerance of the N10-position toward large substituents. Even 10-naphthyl or 10-anthryl derivatives are good inhibitors. In contrast, a positively charged methylpy-

ridinium group practically abolishes the effect on GR. Isoalloxazines with aliphatic N10-substituents are very poor inhibitors.[12]

Position N3 of the isoalloxazine ring is quite sensitive to the size of the respective substituents. Any substituent larger than a methyl group—for instance, a carboxymethyl or an ethoxycarbonyl moiety—increases the IC_{50} value by 1 order of magnitude. Probably the C8-position is also suited for future derivatizations since the 8-fluoro- and 8-azido-10-aryl-3-methylisoalloxazines turned out to be effective inhibitors of GR.

Experimental Procedures

Chemicals. The isoalloxazine derivatives were synthesized and characterized as described.[8]

GR Assay. All kinetic studies were carried out at physiological substrate concentrations in assay buffer (20.5 mM KH_2PO_4, 26.5 mM K_2HPO_4, 200 mM KCl, 1 mM EDTA, pH 6.9 at 25 °C). The assay mixture (1.0 mL) contained 100 nmol of NADPH and 5−10 mU of human GR.[23] In order to exclude nonspecific NADPH oxidation, the absorbance at 340 nm was monitored for 2 min. Then 100 nmol of GSSG was added, and the absorption decrease due to NADPH consumption was followed (spectrophotometers U-1100 and 150-20, Hitachi/Colora, Lorch). Inhibition of GR was studied in the presence of varying inhibitor concentrations; 1 mM stock solutions of the 10-arylisoalloxazines **2a**−**e**, the 10-aryl-3-methylisoalloxazines **3a**−**e**, **12**, **14**, and **18**−**20**, the 10-aryl-3-[(ethoxycarbonyl)methyl]isoalloxazines **4a**−**d**, and the pyridyl derivatives **6a** and **7a** were prepared in dimethyl sulfoxide (DMSO). All other compounds were dissolved in assay buffer. The residual enzyme activity in the presence of inhibitor was determined relative to a control containing solvent but no inhibitor. From these data, IC_{50} values were calculated (Table 1).

Determination of the Redox Potentials of the Isoalloxazines. The equilibrium potentials of the isoalloxazine/dihydroisoalloxazine redox pairs were studied by cyclic voltammetry[17] in aqueous solution. $K_3[Fe(CN)_6]$,[18] riboflavin, and the 3-methyl-10-phenylisoalloxazine (**18**)[19] served as reference compounds. The measurements were carried out at 20 °C using a potentiostat (EG & G Princeton Applied Research, Model PAR 362), a platinum electrode, and an Ag/AgCl reference electrode (3.0 M KCl/H_2O) in a total volume of ca. 15 mL of 20.5 mM KH_2PO_4 and 26.5 mM K_2HPO_4, pH 6.9, containing 300 mM KCl. The potentials were recorded by an *x*/*y* writer (PM 8033, Philips) and an oscilloscope (T912, Tektronics). The concentration of the compounds was 0.1−1 mM; the sweep rate was usually 100 mV s^{-1}.

The results are given with reference to the Ag/AgCl potential (E = 197 mV). The mean error of the measured potentials was about 10 mV. In aqueous solutions, O_2—which can be reduced between −0.2 and −0.8 V vs a standard calomel electrode—must be removed by flushing the sample with argon. The 3-(carboxymethyl)isoalloxazines were directly dissolved in buffer; the 3-methylisoalloxazines **3a**,**e**, **14**, **18**, and **19** were taken up in 1 mL of DMSO and then diluted with buffer to the desired concentration. The organic solvent had no influence on the redox potential.

Measurement of Fluorescence Emission Spectra. Enzyme (2.0 mL, 2 μM) in assay buffer *without* EDTA was titrated with inhibitor **3a** (10-(4′-chlorophenyl)-3-methylisoalloxazine; 200 μM in DMSO) in steps of 0.2 μM. The excitation wavelength was 450 nm. Because of the light sensitivity of the inhibitor,[24] fluorescence emission was measured for 1 s at the maximum wavelength of 522 nm (Fluorolog F 112 XE fluorescence spectrophotometer).

Preparation of Crystalline Enzyme−Inhibitor Complexes. Human GR was purified from recombinant *Escherichia coli* SG5 cells as described.[25] The enzyme was crystallized by the "hanging drop" procedure. For this purpose, 10 μL droplets of the protein solution (10−20 mg/mL) in assay buffer containing 5% $(NH_4)_2SO_4$ were allowed to equilibrate by vapor diffusion at 25 °C with 1 mL of reservoir solution consisting of 15−18% $(NH_4)_2SO_4$ in assay buffer at 25 °C. Crystals of $200 \times 200 \times 200\ \mu m^3$ which diffracted to 2.2 Å resolution were obtained within 2 weeks.

The crystals were incubated in 1 mL of soak solution containing 10 μM compound **5a** or 1 mM compound **5b**, respectively, for 35 days at room temperature in the dark. The solubility of the inhibitors in the soak solution (20.5 mM KH_2PO_4, 26.5 M K_2HPO_4, 200 mM KCl, 1 mM EDTA, 1 M $(NH_4)_2SO_4$, pH 6.9) was 12.6 mM (ϵ_{436nm} = 10.1 mM^{-1} cm^{-1}) for **5a** and 1.4 mM (ϵ_{436nm} = 11.0 mM^{-1} cm^{-1}) in the case of **5b**. The soaked crystals were mounted and subjected to X-ray diffraction analysis. Because of the light sensitivity of the inhibitors, any handling was done in the dark under a red light source; the photodegradation products of the isoalloxazines were found to be no inhibitors of GR.

X-ray Diffraction Analysis of GR−Inhibitor Complexes. The diffraction data of the crystals were collected on a three-circle area detector (Model X1000, Nicolet/Siemens) using Cu Kα radiation generated from a rotating anode with graphite monochromator (Model RU200B, Rigaku). Each data frame covered an angular range of 0.25° and was recorded for 2 min. Data were processed with the program XDS.[26] The $(2F_o - F_c)\exp(ia_{calc})$ and $(F_o - F_c)\exp(ia_{calc})$ electron density maps were calculated with reference to the unliganded human GR structure[4] and displayed using the program O.[27]

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