Molecular Aspects of Herbicide Action on Protoporphyrinogen Oxidase

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Protoporphyrinogen oxidase, the last enzyme of the common tetrapyrrole biosynthetic pathway, is inhibited by several peroxidizing compounds resulting in accumulation of photodynamic tetrapyrroles, mainly protoporphyrin IX. The inhibition characteristics of two chemically unrelated compounds were studied using membrane bound protoporphyrinogen oxidase from corn etioplasts. As shown by Lineweaver-Burk-analysis, the inhibition of enzyme activity by the diphenyl ether oxyfluorfen and the cyclic imide MCI 15 are competitive with respect to the substrate. The competitive interaction of protoporphyrinogen and the two chemically unrelated inhibitors indicate a relative specificity of the binding site. The reversibility of oxyfluorfen inhibition was evaluated by dilution experiments and was shown to be independent of the presence of DTT. The analysis of structure-activity-relationship on protoporphyrinogen oxidase inhibition was investigated with para-substituted derivatives of phenyl-3,4,5,6-tetrahydrophthalimides. The results obtained by QSAR-calculation yielded a good correlation of the inhibitory activity determined by the lipophilicity of the para-substituent. These data point to one binding region of the inhibitors within a lipophilic environment associated with the active center of the enzyme.

Introduction

Several herbicides like diphenyl ethers, lutidine derivatives and cyclic imides have been reported to cause photodestruction in plants by an accumulation of high amounts of tetrapyrroles [1–6]. Protoporphyrin IX, the main accumulating intermediate, acts in the light as a photosensitizer and induces radical formation with subsequent degradation of cellular constituents [7–10]. It has been demonstrated that most of the peroxidizing herbicides act as inhibitor of protoporphyrinogen oxidase (EC 1.3.3.4), which catalyzes the penultimate step in both heme and chlorophyll biosynthesis, the oxidation of protoporphyrinogen to protopophyrin IX [11–15]. The inhibition of protoporphyrinogen oxidase surprisingly leads to the accumulation of the reaction product, protoporphyrin IX. Two different possibilities for the generation of protoporphyrin IX are discussed: First, because of its chemical instability an autooxidation of the accumulated substrate protoporphyrinogen to protoporphyrin IX is assumed [10, 16]. Second, an enzymatic conversion of protoporphyrinogen – delocalized from the biosynthesis pathway – by a herbicide-resistant nonplastidic isoenzyme has been proposed [17]. However, in both cases the formed protoporphyrin seems to be separated from the following biosynthesis step, namely the insertion of iron or magnesium by chelatases, by preventing the proposed substrate channeling from protoporphyrinogen oxidase to the chelatase. In addition, halt of the biosynthesis results in a deregulation of the overall heme and chlorophyll biosynthesis apparently caused by the lack of heme feedback inhibition [18, 19]. Simultaneous treatment of plants with heme or inhibitors of porphyrin synthesis like gabaculine and dioxoheptanoic acid with the peroxidizing herbicide diminished the phytotoxic effect [4, 10, 19, 20].

Little is known about the inhibition of protoporphyrinogen oxidase and the molecular mechanisms necessary for the development of photodynamic injury. It has been reported that the effect of acifluorfen-methyl on protoporphyrinogen oxidation depends on the source the enzyme derives from, the purification state of the preparation and even on assay conditions [12, 17]. Recently, it has been shown that several chemically unrelated com-

Abbreviations: oxyfluorfen, 2-chloro-1-(3-ethoxy-4-nitrophenoxy)-4-(trifluoromethyl)benzene; chlorophthalim, N-(4-chlorophenyl)-3,4,5,6-tetrahydrophthalimide; MCI 15, N-(4-(4-chlorobenzyloxy)-phenyl)tetrahydrophthalimide; MCI 2, N-cyclohexyl-3,4,5,6-tetrahydrophthalimide; DTT, dithiothreitol.

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pounds cause a competitive displacement of the diphenyl ether acifluorfen and it was concluded that all these compounds share the same binding site of the enzyme [21]. Additionally, the inhibition of acifluorfen is competitive with respect to protoporphyrinogen as determined with corn and mouse organelles, although a mixed type inhibition was reported for protoporphyrinogen oxidase from yeast mitochondria [22]. The low inhibition constants indicated a high affinity of acifluorfen to the enzyme. Comparison of protoporphyrinogen with acifluorfen suggested a similarity of one half of the protoporphyrinogen molecule with the competitive inhibitor and a bicyclic nature of protoporphyrinogen oxidase inhibitors seems to be necessary for their activity [23]. Contradicting results were reported about the reversibility of binding of the photobleaching herbicides. A reversible binding of S 23121, a N-phenylimide type herbicide, on solubilized protoporphyrinogen oxidase of corn has been found [24] as well as an irreversible inhibition of acifluorfen in the presence of DTT has been reported [12].

This study should contribute to better knowledge of the action of protoporphyrinogen oxidase inhibitors by kinetic studies on the membrane bound enzyme from corn etioplasts. It could be shown that the inhibition by the diphenyl ether oxyfluorfen was reversible and that substrate and inhibitor compete for the enzyme. The analysis of structure-activity correlations from para-substituted cyclic imides showed an influence of the lipophilicity of the ligand on the inhibitory activity.

Materials and Methods

Corn etioplasts preparation

Etioplasts were prepared from greening corn seedlings (*Zea mays cv.* Anjou) germinated in vermiculite for 6 days at 30 °C in darkness [14]. After a greening period of 2 to 4 h at a light intensity of 300 μEinstein/m^2 × sec seedlings were harvested and etioplasts were isolated by differential centrifugation steps according to [25]. The homogenization medium contained 50 mM Tris/HCl (pH 7.3), 0.5 M sucrose, 1 mM EDTA, 1 mM $MgCl_2$ and 0.2% (w/v) bovine serum albumin. For kinetic studies etioplasts adjusted to a protein content of about 30 mg/ml were used immediately after preparation.

Determination of protoporphyrinogen oxidase activity

Protoporphyrinogen oxidase activity was measured in a modified direct fluorimetric assay as described in [26]. The standard reaction medium contained in 3 ml 0.1 M Tris/HCl, pH 7.45, 1 mM EDTA, 5 mM DTT, 0.03% Tween 80 (w/v), 0.3–0.6 mg of etioplast protein and approximately 5 μM protoporphyrinogen unless indicated otherwise. Under substrate-limited conditions (Lineweaver-Burk-plots) the rate of protoporphyrin formation was followed during the initial 5 min after addition of protoporphyrinogen. For each set of experiment the nonenzymatic rate measured in presence of heat inactivated enzyme was subtracted separately from the enzymatic formation of protoporphyrin IX. The final concentration of protoporphyrinogen was determined after chemical oxidation with 0.1% methylethylketoperoxide to protoporphyrin and recording the fluorescence at an excitation and emission wavelength of 405 nm and 633 nm, respectively. An authentic protoporphyrin IX standard was used to calculate the concentration from the measured relative fluorescence.

Chemicals

Oxyfluorfen was supplied by Rohm & Haas, U.S.A., the cyclic imides by Mitsubishi Kasei Corp., Japan and were dissolved in DMSO. All other compounds from highest available purity were provided from Sigma Chemicals, Munich, Germany.

Statistics

All I_{50} values are means from at least three independent experiments. The Lineweaver-Burk-plots from Fig. 1 show a typical experiment for MCI 15 and oxyfluorfen. The inhibition type was verified four times, but because of the varying protoporphyrinogen concentrations caused by autoxidation during storage over the measuring time no mean values were calculated. If not indicated otherwise all other experiments were replicated three times and variations lower than 10% were obtained. The quantitative structure-activity relationship was calculated in a multiple linear regression analysis using a F test. The contribution of the physicochemical parameters were confirmed by a t test.

Protein content

The amount of protein was determined by the method of Lowry *et al.* [27] with bovine serum albumin as a standard.

Results

Investigation of reversibility of oxyfluorfen inhibition on protoporphyrinogen oxidase

The reversibility of oxyfluorfen inhibition on the membrane-bound enzyme was determined by dilution experiments. Dilution should lead to the detachment of the inhibitor if a reversible binding had occurred. Membranes of corn etioplasts were preincubated for 60 min either with dithiothreitol and oxyfluorfen or with oxyfluorfen alone (Table I). After the incubation time the assay was started under standard conditions by the addition of both the preincubation mixture and protoporphyrinogen to a 3 ml cuvette resulting in a 60-fold dilution of etioplasts and oxyfluorfen. The percentage of inhibition in relation to a simultaneously treated control without an inhibitor was compared with the inhibition we obtained at this 60-fold lower concentration (3.3 nM) without preincubation (Table I A). We found a good agreement with both samples indicating a reversible binding of the inhibitor to protoporphyrinogen oxidase. These results were also obtained with lower concentrations of oxyfluorfen – the treated sample was nearly uninhibited after dilution to a final concentration of 0.3 nM (Table I). An effect of DTT on the reversibility as reported by Jacobs *et al.* [12] was not observed. Neither preincubation with DTT nor without DTT resulted in a remaining inhibition after dilution. In order to proof a possible slow irreversible binding we prolonged the preincubation period with oxyfluorfen up to 12 h (data in brackets). During this time the enzyme activity decreased up to 50 percent as shown for control values in A. In the presence of DTT a stronger loss of activity was observed which could be explained by the inhibitory effect of reducing agents like DTT or glutathione themselves on protoporphyrinogen oxidase activity [28, 29]. Nevertheless, the binding of oxyfluorfen remained reversible even after this incubation time. This was also confirmed for a solubilized fraction of protoporphyrinogen oxidase (data not shown).

Inhibition of protoporphyrinogen oxidase activity by oxyfluorfen and MCI 15

In order to characterize the inhibition type of peroxidizing herbicides on protoporphyrinogen oxidase we used the diphenyl ether oxyfluorfen and for comparison the chemically unrelated cyclic

Table I. Effect of oxyfluorfen preincubation on protoporphyrinogen oxidase activity from corn etioplast membranes. Membranes were preincubated for 1 h on ice with the inhibitor concentrations indicated in column 1. The dilution of membranes and inhibitor was carried out by addition of the preincubated sample to the 3-ml assay mixture. The assay mixture contained all compounds described in Materials and Methods. Activity of protoporphyrinogen oxidase activity was recorded directly after addition of protoporphyrinogen. The data in brackets represent the activities obtained after a 12-h incubation time with oxyfluorfen. DTT, 5 mM.

Preincubation condition, oxyfluorfen [nM]	Oxyfluorfen concentration after dilution [nM]	Protoporphyrinogen oxidase activity [nmol/hxmg protein]
A.		
–	– (= control)	8.5 [6.0]
0.3	no dilution	8.3 [6.1]
3.3	no dilution	3.0 [2.6]
200	no dilution	0.3 [0.1]
B. Preincubation (−)DTT		
–	–	8.3 [6.5]
20	0.3	8.1 [6.5]
200	3.3	4.0 [3.7]
C. Preincubation (+)DTT		
–	–	7.8 [4.3]
20	0.3	7.8 [3.7]
200	3.3	3.3 [3.0]

imide MCI 15 in kinetic studies. Oxyfluorfen and MCI 15 are potent inhibitors of protoporphyrinogen oxidase from corn etioplasts with I_{50} values of about 1.5 nM and 5 nM, respectively (data in preparation for publication). Double reciprocal plots of the initial velocity *versus* the substrate concentration at three different inhibitor concentrations (Lineweaver-Burk plots, Fig. 1 A, B) show a linear, competitive inhibition of protoporphyrinogen oxidase in crude membrane extracts with respect to protoporphyrinogen by both classes of peroxidizers. Secondary plots of the slope of the lines from Fig. 1 *versus* the applied inhibitor concentrations correlate linearly and yield binding constants (K_i values) of 0.2 nM and 0.4 nM for oxyfluorfen and MCI 15, respectively.

Using a ^{14}C labeled inhibitor for binding studies on protoporphyrinogen oxidase the competitive interaction of protoporphyrinogen and both oxyfluorfen and the cyclic imide chlorophthalim (MCI 11, see Table II) was confirmed. Both peroxidizing compounds displaced the labeled inhibitor in dependence on their relative activity. Oxyfluorfen which was shown to be a more active inhibitor than chlorophthalim [26] caused a stronger displacement of the inhibitor at the same concentration (data not shown).

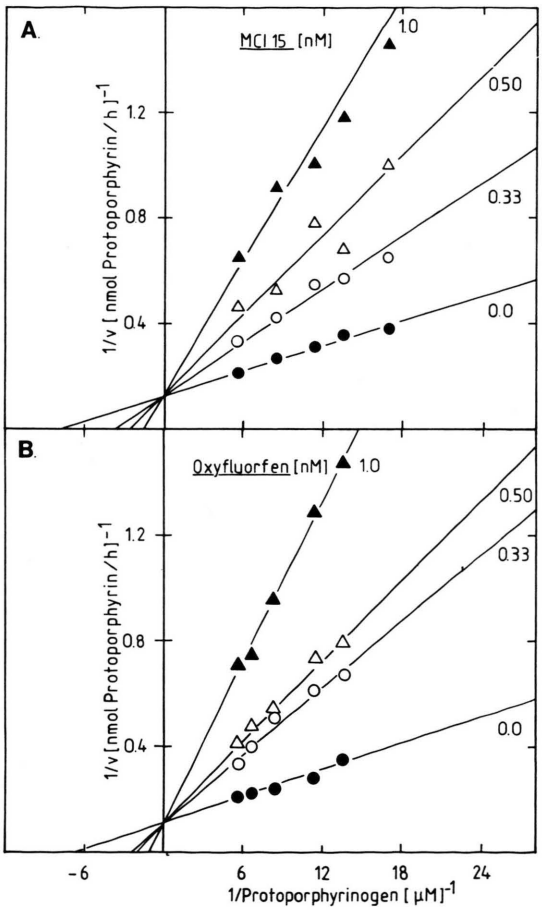
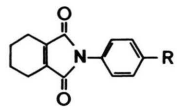


Fig. 1. Initial inhibition of membrane-bound protoporphyrinogen oxidase by MCI 15 (A) and oxyfluorfen (B) as a function of protoporphyrinogen concentration. Each rate of protoporphyrin formation was corrected by the non-enzymatic conversion of protoporphyrinogen in the presence of heat-inactivated etioplasts. The regression coefficient was better than 0.97.

Effect of physicochemical parameters of the para-substituents on inhibitory activity on protoporphyrinogen oxidase

The data presented above suggest one common binding site on protoporphyrinogen oxidase for both the active inhibitors and the substrate protoporphyrinogen because a competitive pattern of

Table II. pI_{50}-Values for inhibition of protoporphyrinogen oxidase from corn etioplasts by *para*-substituted cyclic imide derivatives and lipophilicity of the varying substituent. For the cyclic imides already introduced the published number was used [41]. The new compounds (168, 169, 171–174) were designated by the original number. The pI_{50}-values were the mean values of three independent experiments. Quantitative structure-activity analysis was performed by using the Hansch-Fujita lipophilicity constants (π, ref. [42]) and Coburn's multiple linear regression program, QSAR-PC from Biosoft, Cambridge, England.



Compound	Ligand R	Lipophilicity (π)	pI_{50}
12	$-Br$	0.86	7.8
11	$-Cl$	0.71	7.6
7	$-OCH_3$	−0.02	7.0
171	$-SCH_3$	0.61	6.7
169	$-CF_3$	0.88	6.6
174	$-F$	0.14	6.4
8	$-NO_2$	−0.28	6.4
168	$-H$	0	5.8
6	$-CH_3$	0.56	5.7
172	$-NH_2$	−1.23	4.8
173	$-CONH_2$	−1.49	4.2

inhibition and binding was obtained. This is unlikely because of the broad range of structures existing within active inhibitors of protoporphyrinogen oxidase. Competition in kinetic studies implies that either the inhibitor or the substrate (or a second competitive inhibitor) are bound to the enzyme. This is not only true for one common binding site but also for overlapping non-identical binding sites as claimed for the D-1 protein [30, 31]. It was proposed that a conformational change of the enzyme induced by binding of one inhibitor leads to the displacement of substrate or the competitive inhibitor.

To get an insight into the basic properties of a peroxidizing compound required for binding we examined a set of cyclic imide derivatives on protoporphyrinogen oxidase inhibition. The structures were identical with the exception of the substituent on *para*-position of the benzene ring (Table II). The determination of the I_{50}-values showed different effects on the inhibitory activity which is represented by a factor of about 10^4 between the most active compound MCI 12 and the inactive imide MCI 173. The quantitative relationship between the physicochemical parameters of the varying *para*-substituent and the inhibitory activity was investigated in a multiple regression analysis. The QSAR calculation showed that only the lipophilicity (listed in Table II) of the ligand influences the activity of the compound for protoporphyrinogen oxidase inhibition while steric dimensions or electronic properties have no significant effect. This is expressed by the following regression equation:

$$pI_{50} = 1.138\ (\pm 0.184)\ \pi + 6.183.$$
$$n = 11; \quad s = 0.64; \quad F = 20.4.$$

The regression coefficient r was calculated as 0.83.

As shown in Table II a more lipophilic compound like MCI 12 or 11 has a stronger inhibitory activity than a compound like MCI 168, which is characterized by a hydrogen ligand with a relative lipophilicity of zero. An exception is represented by MCI 169 which is substituted with the most lipophilic ligand $-CF_3$ but was determined with a pI_{50}-value on protoporphyrinogen oxidase inhibition of only 6.6. It has to be considered, however, that the lipophilicity data of Table II refer to the substituent not to the whole molecule.

Discussion

Since it has been reported that the phytotoxic effect of peroxidizing herbicides is based on the accumulation of high amounts of tetrapyrroles intensive studies have been performed concerning both the inhibition of porphyrin biosynthesis and the physiological processes involved in radicalic degradation of cell constituents [1–5, 9, 32]. After identification of protoporphyrinogen oxidase as the target enzyme main work was focused on the characterization of the inhibitor-enzyme interaction. This included the investigation of structure similarities of the inhibitors [21–23, 33] as well as the characterization of protoporphyrinogen oxidase especially from plants which has been purified so far only from barley [12, 17, 34–36]. Interesting and partly contradictory features of protoporphyrinogen oxidase inhibition have been obtained, including loss of inhibition after purification of the enzyme [12], different inhibition types for the plant and yeast enzyme [22], increasing inhibition by acifluorfen-methyl in the presence of DTT [12, 17, own findings] and confusing reports about the reversibility of inhibitor binding on protoporphyrinogen oxidase [12, 24]. Usually, both partners involved in inhibition *e.g.* enzyme and effector should be investigated for structural properties fitting together. Change in inhibition by purification of the enzyme exemplify that variation of one partner can result in alteration of the inhibition and binding characteristics. This study focuses on the molecular action of the inhibitor on the membrane-bound enzyme to identify common structural elements necessary for successful inhibition.

Prior to inhibition type analysis the reversibility of inhibitor binding was investigated. Since protoporphyrinogen oxidase is a membrane-bound enzyme reversibility cannot be evaluated by procedures like gel filtration or dialysis without prior solubilization of the enzyme. Because of the results of Jacobs *et al.* [12] and our own observations on the corn enzyme about change in inhibition properties by purification (data in preparation) we decided to proof the reversibility at the membrane-bound enzyme by dilution experiments.

Table I shows that the preincubation of etioplast membranes with oxyfluorfen had no effect on protoporphyrin formation after dilution, indicating a reversible binding on protoporphyrinogen

oxidase. These results are in good agreement with recent work on solubilized corn protoporphyrinogen oxidase reporting a displacement of [^{14}C]-S23121 after binding by the unlabeled identical compound [24]. In contrast, Jacobs *et al.* found a strong inhibition of barley protoporphyrinogen oxidase activity by acifluorfen-methyl in presence of DTT which could not be restored after dialysis [12]. The authors suggested an irreversible binding when both acifluorfen-methyl and DTT are present since the inhibition with the herbicide alone had no effect on protoporphyrin formation. In our experiment DTT did not influence the detachment of oxyfluorfen after dilution (Table I).

Kinetic studies were carried out under standard assay conditions including 5 mM DTT to decrease the autooxidation rate. As shown in Fig. 1 A, B, the diphenyl ether oxyfluorfen and the cyclic imide MCI 15 are competitive inhibitors with respect to the substrate protoporphyrinogen. This confirmed the previous work reporting a common binding site on protoporphyrinogen oxidase for four chemically unrelated compounds [21]. The K_i-values of the inhibitors below 1 nM indicate a high binding affinity of the inhibitor to the target enzyme. It might be possible that DTT, which was found to enhance the inhibitory activity of acifluorfen-methyl, also influences the inhibition constants and therefore the affinity of the enzyme for the inhibitor. At the moment it is not clear which biochemical processes are involved in the effect of DTT on inhibition by peroxidizing herbicides. However, the comparison of oxyfluorfen and acifluorfen-methyl, two potent diphenyl ether herbicides, showed similar I_{50}-values and inhibitor constants of protoporphyrinogen oxidase inhibition, the latter about 10-times lower than the I_{50}-value (Fig. 1 and unpubl. data, [22]).

The competition of both protoporphyrinogen and oxyfluorfen with respect to binding of a strong inhibitor on protoporphyrinogen oxidase confirms the results obtained by kinetic analysis and led to the question about the nature of the binding site fitting for so many different types of compounds.

Attempts have been made to determine structural similarities between the inhibitors and the substrate protoporphyrinogen [23]. For acifluorfen the analysis of steric complementation indicated an imitation of one half of the protoporphyrinogen molecule by the bicyclic structure. This holds also true for the cyclic imides used in this study and for oxyfluorfen. On the contrary, structural similarities are very low between the known inhibitors of protoporphyrinogen oxidase which include diphenyl ether, phenylpyrrolidine carboxylates and lutidine derivatives. We assume that comparable to the D-1 protein of the photosynthetic electron transport more than one binding site is available for inhibitor binding and that these binding sites are partly overlapping [30, 31]. This includes an allosteric interaction between the active center where protoporphyrinogen is bound and the inhibitor binding region resulting in a blockage of other binding sites if protoporphyrinogen or an effector has bound before. The prevention of simultaneous attachment might be achieved by a conformational change of the protein after binding of the effector molecule. Additionally, this theory could explain the findings of Jacobs *et al.* [12]. They reported that during purification of protoporphyrinogen oxidase inhibition by acifluorfen-methyl is strongly decreased while enzymatic activity which represent protoporphyrinogen binding is further maintained. Therefore, two different binding sites must be proposed otherwise a simultaneous loss of inhibition and activity would be observed.

Interestingly, as seen in Table II, compounds which are modified in only one substituent differ remarkably in their inhibitory activity. For the related cyclic imides a quantitative structure-activity relationship was investigated revealing a requirement of lipophilicity of the ligand for strong protoporphyrinogen oxidase inhibition. In contrast, the herbicidal activity in a root growth assay of these and other related compounds was dependent on the steric dimensions of the substituents [37]. Probably, between the targetting of the enzyme in plants *in vivo* and the inhibition of protoporphyrinogen oxidase in prepared etioplast membrane *in vitro* some other mechanism are involved. For a row of diphenyl ether analogues the same discrepancy between *in vivo* and *in vitro* results was found [23]. Influence of the lipophilicity in addition to an involvement of electronic properties for inhibitory activity was observed in the enzyme assay while the lipophilic parameter did not influence the *in vivo* activity of the compound.

The influence of lipophilicity on the activity of inhibition has been reported for other herbicides

several times [31, 38, 39]. Common for all these herbicides, namely the photosynthetic electron transport inhibitors and the herbicides of carotenoid biosynthesis, is a membrane-bound target protein. Therefore, a lipophilic environment is present and generally the substrates turned over are lipophilic, too. For protoporphyrinogen oxidase the protoporphyrinogen binding site was proposed to be located within the lipophilic membrane because the affinity of protoporphyrinogen oxidase for protoporphyrinogen was dramatically enhanced by insertion of the protein into phospholipid vesicle [40]. Moreover, protoporphyrinogen is known to be a highly hydrophobic molecule. Therefore, with respect to a competition of protoporphyrinogen and the inhibitor at the enzyme molecule a requirement for lipophilicity of the inhibitor for targetting the protein within the membrane is conceivable. For acifluorfen-methyl and acifluorfen a difference in the I_{50}-values of protoporphyrinogen oxidase inhibition has been reported ([22, 23], own data). Acifluorfen-methyl which is a stronger inhibitor of protoporphyrinogen formation is more lipophilic than acifluorfen which supports our findings on a better inhibition with increasing lipophilicity. The informations obtained by this study supplement the data reported about structural properties of protoporphyrinogen oxidase inhibition. Further studies are underway to investigate the receptor structures involved in inhibitor binding and to get a clearer view of the enzyme-inhibitor interaction.

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