Antagonistic Interactions of Mefenacet with Inhibitors of Monooxygenases

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Z. Naturforsch. **46c,** 915–919 (1991); received March 26, 1991

Mefenacet, Monooxygenase, Safener, Antagonism, Induction

The monooxygenase suicide inhibitor piperonyl butoxide (PBO) antagonizes the inhibitory activity of the herbicide mefenacet in a root regeneration test system with etiolated cut oat stems. When studying the behaviour of [^{14}C]mefenacet [2-(2-benzothiazolyloxy)-N-methyl-N-phenylacetamide] in cut oat stems that had been pretreated with PBO for 24 h after cutting, an increased rate of uptake and increased concentrations of mefenacet in the tissue were found after PBO as compared to water pretreatment. Increased uptake rates and increased rates of metabolism after PBO pretreatment, supposedly by increased monooxygenation, were also observed with the substrates diuron, 2,4-D and cinnamic acid. Mefenacet induced the same responses as PBO in all systems.

The similar actions of mefenacet and PBO are interpreted to suggest that mefenacet might interfere with monooxygenase enzymes. Similar responses, *i.e.* stimulation of monooxygenation of suitable substrates, have been reported for herbicide safeners. An environmentally controlled regulatory response system is suggested to be triggered by the different classes of compounds, mefenacet and similar herbicides, monooxygenase inhibitors, and safeners, and to respond with increased enzyme activities in the monooxygenase and glutathione detoxification pathways.

Introduction

Mefenacet is an oxyacetic acid amide herbicide which has been developed for weed control in rice. Its precise mechanism of action is not known, but has been shown to be similar to the mechanism of action of the α-chloroacetanilide herbicides [1, 2]. For example, known herbicide safeners protect corn similarly from oxyacetic acid amide and from chloroacetanilide herbicide injury.

Much information has been accumulated on the interactions between safeners and chloroacetanilide herbicides. It may therefore be possible to learn more on the herbicidal mode of action of these herbicides and of mefenacet by studying their interaction with safeners. Most studies on the herbicide-safener interactions have concentrated on the stimulation of the herbicide glutathione conjugation pathway by safeners [3]. More recently, a stimulation of herbicide detoxifying monooxygenases has been reported after safener treatment [4–6]. Stimulation of monooxygenation can also be induced, among others, by the well-known monooxygenase inhibitors ABT and PBO [5, 7, 8], which both also antagonize the inhibitory activities of mefenacet and chloroacetanilides in an oat root regeneration assay [1, 9]. Of these two, PBO was less phytotoxic to oats and was also the better herbicide antagonist. We therefore decided to study in more detail the antagonism between mefenacet and PBO in regenerating oat stems as a model system to probe for new leads in the search for the mode of action of mefenacet.

Abbreviations: ABT, 1-aminobenzotriazole; BTA, 2-(2-benzothiazolyloxy)acetic acid; PBO, piperonyl butoxide.

* Based on a paper presented at the International Conference on Herbicide Safeners, August 12–15, 1990, Budapest, Hungary.

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Verlag der Zeitschrift für Naturforschung, D-7400 Tübingen
0939–5075/91/0900–0915 $ 01.30/0

Materials and Methods

Oats (*Avena sativa* L. *cv.* Flämings nova) was grown at 18 °C in the dark in vermiculite and cut 5 mm below the shoot apex after 7 days. The procedure of the rooting test has been described [1].

For the PBO pretreatment and for the incubation with radiolabeled herbicides, 5 stems were placed upright in 2 ml water containing the applied compounds ([benzothiazolylphenyl-U-^{14}C]-mefenacet, 31,350 dpm = 3.1 nmol; [phenyl-U-^{14}C]-diuron, 257,000 dpm = 2.5 nmol; [phenyl-U-^{14}C]-2,4-D, 240,000 dpm = 5.4 nmol; [side-chain-3-^{14}C]-cinnamic acid, 453,000 dpm = 3.6 nmol) and 1% methanol (MeOH). After the different incubation times the lower 15 mm of the stems (which contain

the herbicide-sensitive tissue) were extracted with MeOH. The extracts were cleared by centrifugation, dried in a nitrogen stream at 40 °C, and spotted on silica gel thin layer (TL) plates. The separation media were chloroform:acetone:MeOH = 2:2:1 for mefenacet (R_f = 0.9) and chloroform: *n*-hexane:MeOH = 8:2:1 for diuron (R_f = 0.85) and 2,4-D (R_f = 0.61). Cinnamic acid (R_f = 0.84) and its metabolites were separated in toluene: glacial acetic acid:water = 6:8.2:1. The radiolabeled compounds were located on the TL plates by scanning, and the radioactive zones were then extracted with 50% ethanol and counted in a liquid scintillation counter.

Table I. Uptake and metabolism of radiolabeled mefenacet in etiolated oat stem bases as influenced by the simultaneous addition of 150 µM piperonyl butoxide (PBO). The duration of the experiment was 24 h.

Fraction	Treatment	
	Mefenacet	Mefenacet + PBO
	% of given	
[^{14}C]Uptake	55.7 ± 2.1	47.7 ± 2.6
[^{14}C]Extract	21.7 ± 0.6	13.8 ± 0.1
	% of extract	
Mefenacet	4.3 ± 2.9	20.7 ± 2.1
	µM in tissue	
Mefenacet	0.14 ± 0.10	0.43 ± 0.04

Results and Discussion

PBO significantly reduces the inhibitory activity of mefenacet on the regeneration of roots in etiolated cut oat stems (Fig. 1). The similar inhibition after application of α-chloroacetanilide herbicides was shown to be similarly antagonized by PBO as well as ABT [9]. Possible explanations for the observed antagonism could be a decreased uptake and/or an increased metabolism rate. Uptake and metabolism of [^{14}C]mefenacet were therefore studied in the chosen system. In the experiment recorded in Table I mefenacet and PBO were applied simultaneously for 24 h. Whereas the mefenacet uptake was nearly unaffected by PBO, the rate of metabolism was decreased, leading to increased levels of mefenacet remaining in the tissue on a percentage as well as on a molar concentration basis.

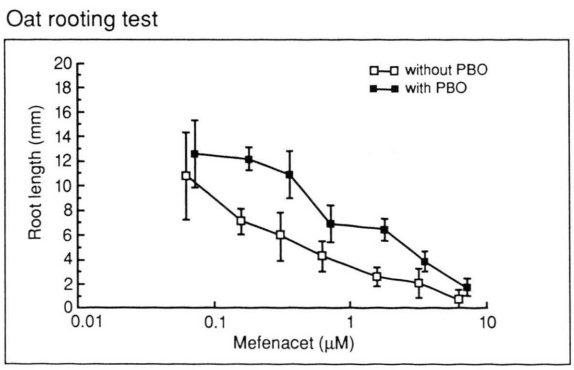

Fig. 1. Antagonism of 150 µM piperonylbutoxide (PBO) with mefenacet in an etiolated oat stem root regeneration test. □—□ without PBO, ■—■ with PBO.

Mefenacet is metabolized by amide splitting, yielding BTA and the respective amine. BTA is further metabolized by ring hydroxylation and subsequent conjugation. The amidase enzyme was measured *in vitro* [10], and PBO was found to inhibit the enzyme by *ca.* 30% at 150 µM. An amidase inhibition *in vivo* by PBO might therefore have caused the lowered mefenacet metabolism rate (Table I).

Since PBO has been shown in other systems to cause a delayed stimulation of monooxygenation after original inhibition [8], similar studies with a 24 h pretreatment were conducted in order to differentiate between immediate and delayed effects. Table II reports the results of such an experiment: after a 24 h pretreatment in water or PBO the oat stems were then incubated with radioactive mefenacet either in the presence or in the absence of new PBO for both parts of the experiment. Preincubation in PBO stimulated both the mefenacet uptake and the extractable radioactivity in the stem bases. Addition of new PBO led to additional increases. After the 2 h mefenacet incubation time only small differences were obtained in the percentage of the total mefenacet left. A small increase of the mefenacet fractions after the addition of new PBO may have been caused by the inhibition of the amidase. However, the metabolite fractions BTA and origin (conjugates) show, that in the stems that had been pretreated for 24 h with PBO more label accumulated at the origin and less occurred in BTA. This result can be taken as an indication that in the presence of PBO monooxygenase enzymes may

Table II. Uptake and metabolism of radiolabeled mefenacet in etiolated oat stem bases as influenced by a 24 h PBO pretreatment and/or by a simultaneous (2 h) PBO treatment (both 150 μM). The mefenacet incubation time was 2 h.

	Pretreatment in			
	Water		PBO	
	Incubation in		Incubation in	
Fraction	Water	PBO	Water	PBO
	% of given			
[^{14}C]Uptake	11.0 ± 3.1	15.3 ± 1.1	26.9 ± 0.5	38.8 ± 1.2
[^{14}C]Extract	2.0 ± 0.6	6.8 ± 0.8	5.4 ± 0.9	10.3 ± 0.3
	% of extract			
Mefenacet	51.7 ± 8.3	78.7 ± 5.5	59.7 ± 3.8	69.3 ± 4.0
BTA	34.7 ± 7.1	14.3 ± 2.1	12.7 ± 0.6	12.0 ± 0.0
Origin	13.1 ± 1.5	7.0 ± 6.2	27.7 ± 3.2	18.7 ± 4.0
	μM in tissue			
Mefenacet	0.38 ± 0.13	1.95 ± 0.24	1.17 ± 0.21	2.59 ± 0.16

have been induced which led to more efficient further metabolism of BTA. The combined effects of increased uptake rates and modulated detoxification after PBO pretreatment have again caused considerably higher levels of mefenacet in the tissue (compare Tables I and II).

A number of similarities and relationships among safeners, monooxygenase inhibitors, and affected herbicides have led us earlier to suggest that all these compounds primarily act by interfering with monooxygenases [9]. The basis for this concept is supported by the frequent induction of monooxygenase type enzymes by inhibitors of monooxygenases, although other influences may lead to similar effects. It was therefore of interest to compare the effectiveness of PBO and mefenacet in stimulating the uptake and detoxification of representative herbicides which are well known to be metabolized by monooxygenation in cereals [7]. Diuron and 2,4-D were selected for this purpose (Tables III and IV). PBO as well as mefenacet strongly increased the uptake rate of diuron, but not of 2,4-D. However, both significantly stimulated the detoxification rates of diuron and 2,4-D. These results support the above concept that mefenacet, as well as α-chloroacetanilide herbicides, are inhibitors of monooxygenases in susceptible plants.

A well-known inducible monooxygenase enzyme is cinnamic acid 4-hydroxylase. For a study of the respective metabolic route the metabolism of [^{14}C]cinnamic acid was studied in the oat system after a pretreatment for 24 h with either water or mefenacet (Table V). The mefenacet pretreatment again caused an increased uptake of the radiotracer. It also strongly increased the hydroxylation to *p*-coumaric acid and to further (additionally hydroxylated?) metabolites with lower chromatographic mobility. Therefore, also in this respect

Table III. Uptake and metabolism of radiolabeled diuron and 2,4-D in etiolated oat stem bases as influenced by a 24 h pretreatment with PBO (150 μM) or mefenacet (13 μM). The incubation time was 7 h for diuron and 2,4-D.

	Pretreatment in					
	Water	PBO	Mef.	Water	PBO	Mef.
Fraction	Incubation in Diuron			Incubation in 2,4-D		
	% of given					
[^{14}C]Uptake	4.3	8.4	6.4	2.3	2.1	2.5
Uptake ratio	1.00	1.95	1.49	1.00	0.91	1.09
	% of extract					
Herbicide remaining	85.9	73.2	79.4	87.8	58.5	66.8

Table IV. Uptake and metabolism of radiolabeled diuron in etiolated oat stem bases as influenced by a 24 h pretreatment with mefenacet at 3.4, 13 or 67 μM. The diuron incubation time was 5 h.

Fraction	Pretreatment in Water –	Mefenacet [μM] 3.4	13	67
		% of given		
[^{14}C]Uptake	3.8 ± 0.3	6.1 ± 0.3	6.8 ± 0.9	8.1 ± 1.2
Uptake ratio	1.00	1.61	1.79	2.13
		% of extract		
Diuron remaining	80.6 ± 0.6	81.4 ± 0.1	77.8 ± 0.8	65.6 ± 3.7

Table V. Uptake and metabolism of radiolabeled cinnamic acid in etiolated oat stem bases as influenced by a 24 h pretreatment with mefenacet at 6.7 or 67 μM.

Fraction	Pretreatment in Water –	Mefenacet [μM] 6.7	67	Water –	Mefenacet [μM] 6.7	67
	Cinnamic acid incubation time [min] 5			15		
			% of given			
[^{14}C]Uptake	4.5 ±0.2	11.0 ±0.4	8.3 ±0.2	12.9 ±0.1	16.6 ±0.2	15.0 ±0.1
[^{14}C]Extract	2.2 ±0.1	3.8 ±0.4	3.6 ±0.5	4.7 ±0.2	5.8 ±0.1	5.8 ±0.8
			% of extract			
Cinnamic acid	25.6 ±3.0	12.0 ±2.9	12.7 ±1.3	17.1 ±1.5	9.0 ±0.8	11.1 ±1.6
p-Coumaric acid	15.6 ±1.7	19.6 ±5.9	22.5 ±0.1	11.9 ±0.4	9.5 ±0.9	12.7 ±1.4
R_f 0.3–0.5	20.1 ±2.2	32.0 ±7.1	30.7 ±1.3	27.9 ±2.3	37.0 ±2.0	34.9 ±2.3

mefenacet behaves similar to the well known monooxygenase inhibitor PBO (data not shown).

Conclusions

The stimulation of uptake and of metabolic degradation, presumably by monooxygenation, occurring similarly after PBO or mefenacet pretreatment, suggest that both compounds might act similarly in the oat stem tissue. Similar responses have been reported after safener treatments [4–6]. The triggered response system could provide a regulatory adaptation of the metabolism, including monooxygenase enzyme induction, to an inhibition of monooxygenases in stress situations or after chemical interaction. It is suggested that safeners originally inhibit monooxygenases, thereby triggering adaptive responses in, *inter alia,* the monooxygenase and the glutathione-S-transferase systems. Increases of monooxygenases might also include enzymes that are involved in the herbicidal action of mefenacet. An increase of herbicidal target enzymes might explain the antagonism between PBO and mefenacet that was observed under the conditions of increased mefenacet concentrations in the tissue.

Acknowledgements

I wish to thank my colleagues in the Chemical Research and in the Metabolism Research Department who have supplied me with possible metabolites of the studied herbicides. The skillful technical assistance of Mrs. Hiester and Mr. Jantzen is gratefully acknowledged.

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