MT-5950, a New Anilide Herbicide, Inhibits PSII at a Site that Slightly Overlaps the Triazine Binding Site

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The new selective acylanilide herbicide, MT-5950 [*N*-(3-chloro-4-isopropylphenyl)-2-methylpentanamide], was found to be a potent inhibitor of photosystem II (PS II) in photosynthesis. It was not as effective as atrazine in increasing variable fluorescence of wheat leaves dipped in herbicide solutions. However, in assays in which absorption across the leaf cuticle did not play a role, MT-5950 was about 10-fold more effective than atrazine in inhibiting the PSII activity of all the species tested. For example, the I_{50} values of MT-5950 and atrazine for inhibiting linear electron transport in broken chloroplasts of spinach were 0.08 and 0.8 μM , respectively. Atrazine-resistant biotypes of *Brassica napus* and *Solanum nigrum* were about 10-fold and <2-fold less susceptible, respectively, to MT-5950 than were susceptible biotypes of the same species as measured by DCPIP photoreduction and CO_2 -dependent oxygen evolution. However, MT-5950 was equally as effective as atrazine in competing for ^{14}C -labeled atrazine from thylakoid proteins. We conclude that MT-5950 inhibits PS II by binding the quinone-binding 32 kD protein at a site that overlaps the binding site of atrazine, but does not significantly overlap that portion of the atrazine binding site that, when modified, confers atrazine resistance.

A new acylanilide herbicide, MT-5950 [*N*-(3-chloro-4-isopropylphenyl)-2-methylpentanamide] (Fig. 1) has recently been patented for use in small grains. Although acylanilides are known to uncouple respiratory phosphorylation,^{1,2)} to inhibit photophosphorylation,^{3,4)} and to alter membrane permeability,^{1,2)} they generally act more effectively as inhibitors of photosynthetic electron transport at the "diuron site".^{2~4)} For example, Moreland *et al.*²⁾ found in spinach thylakoids that the ratio between the propanil I_{50} values for quenching light-dependent atebtrin fluorescence, a measure of the thylakoid pH gradient, and that of photophosphorylation was extremely high (c.600), whereas the ratio for electron transport and photophosphorylation

was nearly one. This indicates that the inhibition of photophosphorylation by propanil is almost completely due to the inhibition of electron transport. Double reciprocal plots of the binding of ^{14}C -atrazine to thylakoid membranes in the presence of propanil indicate that the binding sites overlap.⁵⁾ However, in assays of the effects on electron transport, differences in the susceptibility between atrazine-resistant and susceptible biotypes of pigweed (*Amaranthus retroflexus* L.) to the acylanilide herbicides SWEP, dicryl, karsil and propanil were 2.1,⁶⁾ 2.7,⁷⁾ 8.9,⁷⁾ and 6.0,⁷⁾ respectively. From these data, we might predict that the binding site of MT-5950 would overlap the atrazine binding site, and that atrazine-resistant biotypes would be slightly cross-

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Abbreviations: DCPIP, 2,6-dichlorophenolindophenol; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; MT-5950 = *N*-(3-chloro-4-isopropylphenyl)-2-methylpentanamide.

FIG. 1. Structure of MT-5950.

resistant to MT-5950.

The objectives of this paper are to compare the efficacy of MT-5950 and atrazine in inhibiting PSII at the whole plant level and at the molecular level; to determine the cross resistance of triazine-resistant biotypes of weeds and crops to MT-5950; and to compare competition of atrazine and MT-5950 for thylakoid binding sites with levels of cross resistance.

MATERIALS AND METHODS

Plant material. Seeds of the susceptible canola (*Brassica napus* L.) cultivar 'Tower' and the near isonuclear line with a triazine-resistant plastome (from *Brassica campestris*) were germinated in a 1:1:1 mix of peat, perlite and vermiculite, and grown in the same mixture in a greenhouse for 14 to 21 days. At this stage, secondary developmental differences due to the lower photosynthetic rates of the *R* and *S* biotypes⁸⁻¹¹) were not yet obvious. Canola seeds were the generous gift of J. Dekker at Iowa State University. Seeds of the triazine-susceptible and resistant biotypes of *Solanum nigrum* L. that were first described by Gasquez *et al.*¹² (kindly provided by David Kyle, formerly of the MSU/DOE Plant Research Lab. at Michigan State Univ.) were planted and grown as the canola. Commercial varieties of barley (*Hordeum vulgare* L. 'Morex' from the Wisconsin Crop Improvement Association) and wheat (*Triticum aestivum* L. 'Florida 302') were grown in a greenhouse as already described for canola. Wheat and barley tissues from 5- to 7-day-old seedlings were used. Common chickweed (*Stellaria media* (L.) Vill.) growing locally in a field was collected. A commercial source for spinach (*Spinacea oleracea* L.) was used.

Variable fluorescence. *In vivo* chlorophyll fluorescence transients were determined with a Model SF-20 Branker fluorometer, as before.^{13,14} Leaf discs were taped to the probe, the adaxial side facing the probe, with black electrical tape. A 2-min dark adaptation period was allowed before a slow transient (50-sec duration) was measured. This was followed by a 1-min dark period and measurement of F_0 , at 1-msec into a 2-sec fast transient. Digital data were acquired, stored and plotted, using a high-speed electronic recorder.

CO_2 -dependent O_2 evolution of leaf discs. CO_2 -dependent oxygen evolution was measured polarigraphically as before¹³) by placing ten 4-mm leaf discs, that had been incubated in darkness in 1% sucrose-1 mM MES (pH 6.5) for 24 hr at 22°C with or without herbicide, into 50 mM HEPES-NaOH (pH 7.6), 0.33 sorbitol, 2 mM EDTA, 10 mM KCl, 5 mM Na_2HPO_4 and 10 mM $NaHCO_3$ at 25°C. Light intensity was $500 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ PAR.

DCPIP reduction and whole chain electron transport. Chloroplasts from canola, spinach and chickweed were isolated by the method of Brewer *et al.*¹⁵) A modification of the method of Leegood and Walker¹⁶) was used to isolate wheat chloroplast fragments. Leaves from 5 to 7 day-old plants were cooled to 4°C, cut into 2-cm sections with a razor blade and then covered with a semi-frozen solution of 0.33 M sorbitol, 10 mM $NaHCO_3$, 10 mM EDTA, 5 mM $MgCl_2$, 0.1% bovine serum albumin, 0.2% ascorbate, and 20 mM tricine brought to pH 8.4 with NaOH. The tissue was homogenized for 4 sec at maximum speed with a Sorvall blender, and then successively filtered through 2 layers of cheesecloth, 8 layers of cheesecloth and 1 layer of Miracloth. The filtrate was centrifuged at 4000 *g* for 90 sec, and the pellet was washed two times in the extraction buffer without ascorbate. The washed chloroplasts were again centrifuged at 3000 *g* for 90 sec, and the pellet resuspended in 0.33 M sorbitol, 10 mM $NaHCO_3$, 0.2 mM phosphate, 10 mM EDTA and 25 mM tricine at pH 8.4 with 1000 units of catalase per liter. This preparation was diluted to 2 mg of chlorophyll per milliliter for assays.

DCPIP reduction was monitored spectrophotometrically at 580 nm with an Aminco DW-2 spectrophotometer. A blue blocking filter (Corning 4~96) was placed over the photomultiplier tube, and actinic light from an Aminco side illumination attachment was filtered through a red filter (Corning 2~64) and 10 cm of water.

Whole chain electron transport from water to methyl viologen was measured by polarigraphically determining oxygen reduction with the method of Brewer *et al.*¹⁵)

Binding studies. Chloroplast fragments of wheat and barley were isolated from 21-day-old seedlings by a modification of the methods of Pfister *et al.*¹⁷) Minced leaves were homogenized in cold (0~3°C) 0.4 M sorbitol, 100 mM tricine, 10 mM NaCl, 50 mM ascorbate and 0.05% bovine serum albumin brought to pH 7.8 by NaOH with three 4-sec full-speed bursts of a Sorvall Omnimixer. The homogenate was filtered through 2 layers of cheesecloth, then 1 layer of Miracloth, and followed by 10 layers of cheesecloth. The filtrate was then centrifuged at 1000 *g* for 10 min. The pellet was resuspended in 10 mM tricine and 10 mM NaCl brought to pH 7.8 with NaOH, and then centrifuged for 5 min at 3000 *g*. The pellet was resuspended in 50 mM tricine, 100 mM sorbitol, 5 mM $MgCl_2$ and 10 mM NaCl at pH 7.8, and diluted with this buffer to a concentration of 50 μg chlorophyll per ml. Competitive

binding of ^{14}C -atrazine to thylakoid fragments in the presence of various amounts of non-radiolabeled PS II inhibitors was then measured.

To 100 ml of the chloroplast fragment solution, 168 nCi of ^{14}C -atrazine and enough nonradiolabeled atrazine to bring it to $0.4\ \mu\text{M}$ was stirred in darkness for 15 min at room temperature. One ml of the solution was pipetted with a glass pipette into microcentrifuge tubes, into which non-radiolabeled PS II inhibitors has been placed. The tubes were incubated for 30 min at room temperature in darkness and then centrifuged at $12,000g$ for 3 min. Samples of 0.8 ml of supernatant from each tube were mixed with 10 ml of Aquasol, and the radioactivity measured by liquid scintillation spectroscopy, the radioactivity being corrected for background and quenching. Samples were triplicated in each experiment and the experiments were repeated.

RESULTS

Variable fluorescence

Typical traces of variable fluorescent transients of wheat leaves dipped in 50 or $500\ \mu\text{M}$ atrazine or MT-5950 after 3, 6 or 24 hr are shown in Fig. 2. A square trace, typical of PSII inhibitors, was observed at the $500\ \mu\text{M}$ application rate at all times after treatment with atrazine; however, the full effect was not observed at 3 hr with $500\ \mu\text{M}$ MT-5950. At $50\ \mu\text{M}$, atrazine began to affect the fluorescence kinetics at 24 hr; however, little or no effect was observed at this time with $50\ \mu\text{M}$ MT-5950.

These data could indicate that MT-5950 is a less effective PSII inhibitor than is atrazine, or that it is not absorbed as well as atrazine.

Atrazine-resistant biotypes of canola and black nightshade were not resistant to MT-5950 as measured by the variable fluorescence induced by $10\ \mu\text{M}$ of each herbicide in leaf discs (Fig. 3). Without further experimentation, these data would indicate that an alteration in the triazine binding site in the resistant biotypes does not influence the inhibition of photosynthesis by MT-5950.

CO_2 -dependent O_2 evolution

Carbon dioxide-dependent oxygen evolution from leaf discs of barley and wheat incubated in MT-5950 had an I_{50} value of approximately $0.5\ \mu\text{M}$ (Fig. 4). The slopes of the dose-response curves for the two species were considerably different. This could be accounted for by a more heterogeneous population of barley than of wheat leaf discs with respect to the MT-5950 sensitivity, or by a more heterogeneous herbicide binding environment in barley than in wheat.

Triazine-resistant canola had an I_{50} value of about $10\ \mu\text{M}$, whereas that of susceptible canola was about $1.5\ \mu\text{M}$ (Fig. 5A). Thus, the resistant-to-susceptible ratio was approxi-

FIG. 2. Variable Fluorescence of Chlorophyll in Intact Leaves of Wheat Seedlings Dipped into Solutions of MT-5950 or Atrazine with an Adjuvant (5 drops of Tween 20 per 100 ml).

The three sets of transients represent typical values at 3, 6 and 24 hr after treatment.

FIG. 3. Variable Fluorescence of Chlorophyll in Intact Leaf Discs of Atrazine-resistant and Susceptible Biotypes of Canola and Black Nightshade after Floating on Solutions of MT-5950 or Atrazine for 3 hr.

mately 7 with this assay. With black nightshade, the MT-5950 I_{50} values for the triazine-resistant and susceptible biotypes were about 2.5 and 1.5 μM , respectively (Fig. 5B). There was about a 10-fold difference between the sensitivity of triazine-susceptible canola to atrazine and MT-5950, whereas this difference was only about 4-fold with the black nightshade biotypes.

Whole chain electron transport

An almost precise 10-fold difference in the sensitivity of whole chain electron of spinach to atrazine and MT-5950 was measured, with I_{50} values of 0.8 and 0.08 μM , respectively (Fig. 6).

DCPIP reduction

The I_{50} values for MT-5950 inhibition of photochemical reduction of DCPIP by broken chloroplasts of wheat and chickweed were 0.03 and 0.018 μM , respectively (Fig. 7). The ratios of atrazine to MT-5950 I_{50} values with this assay were about 5 and 10 for wheat and chickweed, respectively.

Only a small (2<-fold) difference was

FIG. 4. Carbon Dioxide-dependent Oxygen Evolution of MT-5950-treated Leaf Discs of Barley and Wheat. The discs were incubated for 24 hr in the dark before assays were made with an oxygen electrode.

FIG. 5. Carbon Dioxide-dependent Oxygen Evolution of Herbicide-treated Leaf Discs of Triazine-resistant and Susceptible Biotypes of Canola (A) and Black Nightshade (B).

The leaf discs were incubated for 24 hr in the dark before assays were made.

FIG. 6. Whole Chain Electron Transport Assay (Water to Methyl Viologen) of Broken Spinach Chloroplasts as Affected by Atrazine and MT-5950.

FIG. 7. Comparison between the Inhibition of DCPIP Photoreduction by MT-5950 and Atrazine in Chloroplast Fragments of Wheat and Chickweed.

FIG. 8. Comparison between the Inhibition of DCPIP Photoreduction in Chloroplast Fragments of Triazine-resistant Canola (A) and Black Nightshade (B) by MT-5950 and Atrazine.

measured between the sensitivity of atrazine-resistant and susceptible canola to MT-5950 using the DCPIP photoreduction assay (Fig. 8A). Essentially, no difference was found in the I_{50} values between the sensitivities of triazine-resistant and susceptible biotypes of black nightshade to MT-5950, although a slight difference was observed in the slopes of the dose-response curves (Fig. 8B). The ratios of atrazine to MT-5950 I_{50} values for atrazine-susceptible biotypes of both canola and black nightshade were approximately 10.

Competition for the atrazine binding site

MT-5950 was as effective as atrazine and DCMU (diuron) in competing for ^{14}C -atrazine from wheat, barley and triazine-susceptible canola thylakoid membranes (Fig. 9). Bentazon was much less effective in competing

for ^{14}C -atrazine from the thylakoid membranes in all three species.

DISCUSSION

These data indicate that MT-5950 was a potent inhibitor of photosynthetic electron transport. It was approximately 10-fold more effective than atrazine at the molecular site of action, with an I_{50} value of nearly $0.01\ \mu\text{M}$ in some systems. This is similar to the results of Fuerst *et al.*,⁷⁾ who found karsil, a close analogue of MT-5950, to be 10-fold more active than atrazine at the chloroplast level. Propanil, an acylanilide that has less structural similarity to MT-5950, had similar activity to atrazine. These *in vivo* reactions do not necessarily reflect differences in the efficacy of these two herbicides, however. The fluorescence data

FIG. 9. Comparison between the Competition of Cold (Non-radiolabeled) Herbicides for the Binding Sites of Radiolabeled Atrazine on Thylakoid Fragments of Wheat, Barley and Triazine-sensitive Canola.

with intact tissues (Fig. 2) indicate that MT-5950 was not absorbed as well across the leaf tissue as is atrazine, or that it was more readily metabolized by wheat than was atrazine. Aryl acylamidase is the only enzyme known to be involved in the metabolism of acyl anilide herbicides.¹⁸⁾ The relative insensitivity of rice to propanil was due to the presence of an aryl acylamidase that hydrolyzed the herbicide.¹⁸⁾ This enzyme could not be found in crude extracts of barley or wheat.¹⁹⁾ Thus, the discrepancy between the efficacies of MT-5950 and atrazine at the molecular level, when compared to the whole-plant level, can probably be explained by differential absorption.

There was only marginal cross resistance with the atrazine-resistant biotypes of canola and black nightshade, as has been found for other acylanilide herbicides such as SWEP,⁶⁾ propanil, karsil and dicryl.⁷⁾ Recently, Haworth and Steinback²⁰⁾ found the same level of resistance (about 10-fold) of DCMU-resistant *Chlamydomonas reinhardtii* to both DCMU and propanil. In their studies, there was no cross resistance to triazines, and there was no evidence in altered binding of diuron to the 32 kD Q_B -protein of the mutant. Thus, this

alteration only affected the efficacy of the bound herbicide in influencing electron transport. This suggests a similarity in the binding properties between urea- and acylanilide-type herbicides.

Binding studies demonstrated that MT-5950 acted as efficiently as nonradiolabeled atrazine in competing for the binding site of ^{14}C -atrazine. Our findings that DCMU efficiently competed for the atrazine binding site, whereas bentazon was a very inefficient competitor, are in line with the findings of Fuerst *et al.*⁷⁾ They found very little cross resistance (*c.* 2-fold) to DCMU in atrazine-resistant mutants, despite efficient competition for the atrazine binding site. Again, these data indicate a similarity between urea and acylanilide herbicides in binding sites, as has been previously proposed.⁶⁾ Studies of the double reciprocal plots of atrazine and propanil binding by van Assche⁵⁾ have demonstrated that acylanilide herbicides bind at a site that overlaps that of atrazine. The low level of cross resistance, despite highly efficient competitive binding, indicates that, although there is significant overlap in the binding sites of triazines and acylanilides, the genetic alteration that greatly

reduces triazine binding has little effect on acylanilide binding.

At least part of the difference in sensitivity to MT-5950 of the two biotypes may have been due to the PS II density and lipid differences. Atrazine-resistant biotypes have relatively more PS II per unit of chlorophyll than do the susceptible biotypes,^{11,14} and thus, more PS II-inhibiting herbicide might be required to saturate inhibition on a chlorophyll or unit area basis. The lipid changes associated with triazine resistance²¹ might also cause membrane property alterations which could slightly change the efficacy of other PS II herbicides in other ways than in an alteration of binding properties.

In summary, MT-5950 was a very efficient inhibitor of photosynthetic electron transport, and could be a useful herbicide on weeds that have developed resistance to triazine herbicides. Our results also suggest that differences in absorption account for the selectivity of MT-5950. An improvement of the herbicide penetration into the leaf cuticle could greatly increase its efficacy.

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