Mepanipyrim haptens and antibodies with nanomolar affinity

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Mepanipyrim is an anilinopyrimidine fungicide used worldwide for crop protection. With the aim of developing useful immunoreagents for mepanipyrim immunoanalysis, two new functionalized derivatives were prepared and antibodies were generated. Affinity and specificity were assessed by direct and indirect competitive ELISA using homologous and heterologous conjugates. Although all antibodies were selective for the target analyte, the immunizing hapten structure was revealed as determinant for high-affinity antibody production (IC50 = 3 nM).

Introduction

A number of modern fungicides with innovative modes of action have been developed during the last decade for prevention of mold resistance and reduction of economic impact caused by plant pathogens. The anilinopyrimidine group of pesticides is one of such chemical tools. Mepanipyrim (Chart 1), the first introduced fungicide of that family,1-3 was included in Annex I of EU Directive 91/414/EEC in 2004, and it was also approved by the Environmental Protection Agency on the same year.4, 5 Mepanipyrim is considered very toxic to aquatic organisms, and it may cause long-term adverse effects in the aquatic environment.6 Despite evidences of carcinogenic effects in rats and mice, mepanipyrim was deemed safe for human and animal health, provided that good agricultural practices are followed.7 Maximum residue limits in the 0.5–3.0 mg kg \(^{-1}\) range have been explicitly set up for a number of commodities, such as grapes, strawberries, tomatoes, aubergines, and courgettes.

Multiresidue analytical methods based on gas or liquid chromatography, preferentially coupled to mass spectrometry detection, are widely recognized as the most appropriate procedures for pesticide determination in enforcement monitoring programmes. Different authors have successfully developed instrumental methods for mepanipyrim analysis.8, 9 Moreover, molecularly imprinted polymers have recently been developed for selective solid-phase extraction of anilinopyrimidine fungicides.10 However, to overcome limitations usually ascribed to chromatographic methods, in particular moderate sample throughput, high analytical costs, and handling of solvent residues, immunoanalysis is definitely envisioned as an appealing technology. Among the different types of common antibody-based methodologies, the enzyme-linked immunosorbent assay (ELISA) is certainly the most extended technique because it brings together the high sensitivity and selectivity commonly exhibited by antibodies with the simplicity, portability, affordable cost, and ability to simultaneously analyse multiple samples of a microtiter plate.

Fig. 1 Molecular structures of anilinopyrimidine fungicides and the two synthesized haptens

<table>
<thead>
<tr>
<th>Compound</th>
<th>R(^1)</th>
<th>R(^2)</th>
<th>R(^3)</th>
</tr>
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<tr>
<td>Mepanipyrim</td>
<td>1-propynyl</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>Hapten MP(_m)</td>
<td>1-propynyl</td>
<td>(CH(_2))(_5)CO(_2)H</td>
<td>H</td>
</tr>
<tr>
<td>Cypromidin</td>
<td>cyclopropyl</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>Pyrimethanil</td>
<td>methyl</td>
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</table>

In the last few years, our group has contributed to expand the portfolio of reagents available for pesticide immunosensing by addressing the generation of antibodies to a number of modern fungicides, including the other two members of the anilinopyrimidine family of fungicides, i.e., cypromidin and pyrimethanil (Fig. 1).11, 12 The aim of the present study was the generation of high-affinity and selective antibodies to mepanipyrim. To that purpose, two derivatives carrying the same functionalized six-carbon aliphatic chain at two different tethering sites of the mepanipyrim framework were synthesized. Rabbit polyclonal antibodies were produced and their performance in terms of affinity and selectivity to mepanipyrim was assessed by competitive assays (cELISA). To the best of our knowledge, the immunoreagents herein described are the first ever reported for mepanipyrim immunosensing.

Experimental

Chemicals and intruments

Mepanipyrim (\(N\)-(4-methyl-6-prop-1-ynylpyrimidin-2-yl)aniline, CAS registry no. 110235-47-7, Mw 223.3), cypromidin, and pyrimethanil fungicide standards were purchased from Fluka/Riedel-de-Haën (Seelze, Germany). Ultraviolet–visible spectra and ELISA absorbances were read with a PowerWave HT from BioTek Instruments (Winooski, VT, USA). ELISA plates
Hapten synthesis

Hapten MP\(_n\) was synthesized directly from mepanipyrim and hapten MP\(_m\) from 1-iodo-3-nitrobenzene and 2,4-dichloro-6-methylpyrimidine as detailed in Scheme 1 and Scheme 2, respectively. Experimental details and full spectrometric characterization of the synthesized products can be found in the Supplementary Information File.

Conjugate preparation

Different conjugates were prepared, following previously optimized procedures,\(^3\) by reaction of the purified N-succinimidyl ester of each hapten (MP\(_n\) and MP\(_m\)) with the free amine groups of three different carrier proteins: bovine serum albumin (BSA) for immunizing conjugates, ovalbumin (OVA) for coating conjugates, and horsedradish peroxidase (HRP) for enzyme tracers. Conjugates were purified by Sephadex G-25 gel filtration using PB as eluent. Final hapten-to-protein molar ratios (MR) were estimated from absorbance measurements at 280 nm of the conjugate assuming that the molar absorptions of both, the hapten and the protein, do not change upon conjugation. For details see the Supplementary Information File.

Antibody production

Animal manipulation was carried out in compliance with Spanish laws and guidelines (RD1201/2005 and law 32/2007) and according to the European Directive 2010/63/EU concerning the protection of animals used for scientific purposes. Experiments were approved by the Bioethics Committee of the University of Valencia. Two female New Zealand white rabbits were subcutaneously injected with 0.3 mg of immunizing conjugate (BSA–MP\(_n\) or BSA–MP\(_m\)) in 1 mL of a 1:1 mixture of PB and complete Freund’s adjuvant. Animals were boosted at 21-day intervals with the same immunizing emulsion but using incomplete Freund’s adjuvant. Whole blood was collected by intracardiac puncture 10 days after the fourth injection. Blood samples were allowed to coagulate overnight at 4 °C. Then, sera were separated by centrifugation and the polyclonal antibodies (pAb) were precipitated twice with a saturated ammonium sulphate solution. The four antisera were stored as precipitates at 4 °C.

Antibody-coated direct competitive ELISA

Ninety-six-well polystyrene ELISA plates were coated with 100 μL of antiserum diluted in CB, and sealed plates were incubated overnight at room temperature. Coated plates were washed four times with washing solution and received, afterwards, 50 μL per well of analyte standard in PBS plus 50 μL per well of enzyme tracer solution in PBST. No blocking step was required. The immunological reaction took place during 1 h at room temperature, and then plates were washed as before. Finally, signal was generated by addition of 100 μL per well of freshly prepared 2 g L\(^{-1}\) o-phenylenediamine solution containing 0.012% (v/v) H\(_2\)O\(_2\) in enzyme substrate buffer. The enzymatic reaction was stopped after 10 min at room temperature by addition of 100 μL per well of 2.5 M sulphuric acid. The absorbance was immediately read at 492 nm with a reference wavelength at 650 nm.

Conjugate-coated indirect competitive ELISA

ELISA plates were coated with 100 μL per well of coating conjugate solution in CB by overnight incubation at room temperature. Coated plates were washed as described above, and then received 50 μL per well of analyte in PBS plus 50 μL per well of antiserum diluted in PBST. No blocking step was required. The immunological reaction was carried out during 1 h at room temperature and plates were washed again. Next, 100 μL per well of a 1/10\(^7\) dilution of peroxidase labelled goat anti-rabbit immunoglobulin pAb in PBST containing 10% foetal bovine serum was added, and plates were incubated 1 h at room temperature. After washing, signal was produced and absorbance was read as aforementioned.

Data treatment

Experimental values were fitted to a four-parameter logistic equation using the SigmaPlot software package from SPSS Inc. (Chicago, IL, USA). Antibody affinity was estimated as the concentration of analyte causing a 50% reduction (IC\(_{50}\)) of the maximum absorbance (\(A_{\text{max}}\)). Cross-reactivity (CR) values were calculated according to the equation:

\[
\text{CR} = \left[ \frac{\text{IC}_{50} (\text{mepanipyrim})}{\text{IC}_{50} (\text{compound})} \right] \times 100
\]

Results and discussion

Synthesis of haptenes and preparation of bioconjugates

Hapten MP\(_n\), which carries the hydrocarbon spacer arm attached to the amine nitrogen atom, thus leaving both aromatic rings unchanged, was readily prepared in two steps from mepanipyrim (Scheme 1). First, N-alkylation of the sodium amide of mepanipyrim with tert-butyl 6-iodohexanoate (1) gave the N-alkylated derivative 2. After mild acid hydrolysis of the tert-butyl ester moiety, the pursued hapten MP\(_n\) was obtained in a moderate 46% overall yield for the two-step sequence.

The synthesis of hapten MP\(_m\) was based on the palladium-catalyzed amination of 2-chloropyrimidines 4 with aniline 8, which has the C6 hydrocarbon chain that constitutes the spacer arm at the required position of the phenyl ring (Scheme 2). The former was prepared in good yield from commercially available 2,4-dichloro-6-methylpyrimidine (3) via regioselective Sonogashira palladium-catalyzed coupling reaction with propyne through the C-4 position.
relatively fast and efficiently at 100 °C using the bidentate ligand xantphos as phosphine and K₂CO₃ as base to give the anilinopyrimidine derivative 9 in 78% yield. Finally, the synthesis of hapten MPₘ was readily completed by acid hydrolysis of the tert-buty1 ester moiety of 9 using formic acid at room temperature. The overall yield for the transformation of dichloropyrimidine 3 into hapten MPₘ through this convergent sequence was ca. 49%.

Activation of the carboxyl moiety of both haptens, MPₙ and MPₘ, was easily accomplished using N,N'-disuccinimidyl carbonate (DSC), a procedure that allows obtaining the N-succinimidyl ester derivative of the hapten, i.e. compounds MPₙ-NHS (Scheme 1) and MPₘ-NHS (Scheme 2), in high yield. These activated haptens were obtained in a high degree of purity after their purification by column chromatography, thus allowing the preparation of the required biocjugates in stoichiometric controlled conditions. The calculated final MRs of the immunizing conjugates were 14 and 15 for BSA–MPₙ and BSA–MPₘ, respectively. Concerning assay conjugates, final MRs were 6 and 7 for OVA–MPₙ and OVA–MPₘ, respectively, and 2 for both enzyme tracers.

and the particular assay conditions that afforded the lowest IC₅₀ value and an Aₚₖ closest to 1.0 for each pAb/tracer combination. Antibodies obtained with hapten MPₙ showed high recognition of its homologous enzyme conjugate (the conjugate carrying the same hapten than the immunizing conjugate). In fact, saturated absorbance signals were obtained under most of the tested conditions, so immunoreagents had to be used at low concentrations in order to obtain adequate signals and satisfactory inhibition curves. However, even under these limiting-reagent conditions, MPₙ-type antibodies displayed low affinity to mepanipyrim, with IC₅₀ values about 100 nM. On the contrary, pAbs MPₙ#1 and MPₙ#2 did exhibit affinity values to mepanipyrim in the low nanomolar range using the homologous enzyme tracer at appropriate concentrations. Fig. 2A clearly shows the differential binding properties towards mepanipyrim of both groups of antibodies, with pAb MPₙ#2 displaying the
highest affinity (IC\textsubscript{50} = 3 nM). Remarkably, all assays showed very low background signals. Finally, none of the antibodies recognized the heterologous conjugate in this cELISA format; that is, MP\textsubscript{n}-type antibodies did not bind tracer HRP–MP\textsubscript{n} sufficiently, and MP\textsubscript{m}-type antibodies did not bind tracer HRP–MP\textsubscript{m}.

Table 1

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<tr>
<th>pAb</th>
<th>HRP–hapten</th>
<th>[Conjugate] (µg L\textsuperscript{-1})</th>
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<th>Slope</th>
<th>IC\textsubscript{50} (nM)</th>
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Table 2

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Fig. 2 Inhibition curves obtained with antibodies MP\textsubscript{m}#1, MP\textsubscript{m}#2, MP\textsubscript{n}#1, and MP\textsubscript{n}#2 using direct (A) and indirect (B) homologous cELISA. Results are the mean of three independent experiments.

Specificity

Hapten MPM\textsubscript{n} and MP\textsubscript{m} were designed to elicit antibodies of the outmost specificity to mepanipyrim. Accordingly, the spacer arm was introduced at sites that are shared by the other anilinopyrimidine fungicides (cyprodinil and pyrimethanil), thus leaving the distinctive pyrimidine ring unaltered (see Chart 1). Assay selectivity towards those compounds was evaluated by cross-reactivity (CR) studies. Accordingly, calibration curves were run in parallel, and recognition by the four described pAbs was evaluated in both cELISA formats.

For indirect assays, plates were coated with OVA–hapten solutions at 10, 100, and 1000 µg L\textsuperscript{-1}, and next, the competitive step was carried out using 6 antiserum dilutions (from 1/3×10\textsuperscript{3} to 1/10\textsuperscript{6}). Thus, a collection of 18 inhibition curves was obtained for each conjugate/antibody pair. Particular assay conditions affording the best inhibition curves (lowest IC\textsubscript{50} and A\textsubscript{max} closest to 1) and its parameters are listed in Table 2. Like in the direct format assays, antibodies from hapten MP\textsubscript{n} showed strong recognition of the homologous conjugate, so plates had to be coated at a low concentration (10 µg L\textsuperscript{-1}) in order to obtain satisfactory inhibition curves – higher coating concentrations resulted in poor or no inhibition. Even so, high IC\textsubscript{50} values were again obtained with MP\textsubscript{n}-type pAbs. As before, antibodies from hapten MP\textsubscript{m} exhibited high affinity to mepanipyrim when the homologous coating conjugate was used at 100 µg L\textsuperscript{-1}. Contrarily to the direct assay format, antibodies did recognize heterologous protein conjugates in the indirect format, helping to improve the apparent affinity to mepanipyrim of MP\textsubscript{n}-type antibodies, most likely because of the poorer recognition of the heterologous coating antigen. However, MP\textsubscript{n}-type antibodies did not show lower IC\textsubscript{50} values when the heterologous conjugate (OVA–MP\textsubscript{n}) was used. Despite this fact, the affinity displayed by MP\textsubscript{n}-type antibodies in homologous assays was even better than the affinity of MP\textsubscript{n}-type antibodies using heterologous conjugates, which strengthens the conclusion concerning the superior performance of hapten MP\textsubscript{n} as immunogen, as it can be clearly appreciated in Fig. 2B.

No binding was observed (CR lower than 0.1%) to any of the assayed fungicides using antibodies MP\textsubscript{m}#1, MP\textsubscript{m}#2, and MP\textsubscript{n}#2, and just a slight recognition of cyprodinil and pyrimethanil by the MP\textsubscript{m}#1 antibody was found (0.3% and 0.4%, respectively), which confirms the suitability of the proposed hapten structures for specific antibody generation.

Conclusions

To the best of our knowledge, the antibodies herein described are the first reported immunoreagents able to selectively bind mepanipyrim with nanomolar affinity. This goal was
accomplished through a newly synthesized hapten (MPm) with a carboxylate linker tethered at the phenyl ring of the mepanipyrim structure. This derivative seems to adequately mimic the three-dimensional spatial conformation and electronic properties of the target molecule. On the contrary, hapten MPn, with the spacer arm at the bridge nitrogen atom, did not seem to adequately expose the molecule to the immune system, thus resulting in antibodies of poorer affinity for the free analyte. The lower performance of this central derivatization approach was also previously observed for cyprodinil, so it seems to be a general behaviour rather than an isolated finding. Following optimization and validation, these novel immunoreagents may become important analytical tools for the determination of mepanipyrim residues in food and environmental samples when implemented either in competitive immunoassays, biosensors, or immunochromatographic strips.

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Notes and references