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Hapten design for compound-selective antibodies: ELISAS for environmentally deleterious small molecules

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Abstract

We have developed an enzyme-linked immunosorbent assay (ELISA) for the herbicide simazine with virtually no recognition of propazine and very low (8%) recognition of atrazine. In this research we have developed a generalized ''size-exclusion'' concept for designing immunogen hapten structures. The hapten should contain appendages smaller than those of the target analyte thereby generating polyclonal antibodies that exclude recognition of analytes larger than the target molecule. The use of this size-exclusion model, when extended to include coating/tracer haptens, is also able to predict suitable structures for ELISA development.

Keywords: Enzyme-linked immunosorbent assay (ELISA); Simazine; Atrazine; Propazine; *s*-Triazine herbicides; Diuron; Arylurea herbicides; Hapten design

1. Introduction

The wide use of the *s*-triazine and arylurea compounds as pre- and post-emergent herbicides for the control of broad-leaf weeds in agriculture, along highways and other rights-of-way, has distributed these substances throughout our environment. This usage [1,2] is detailed in Table 1. Note that whereas atrazine is the major triazine herbicide consumed throughout the USA, simazine has greater usage in California and thus is of special interest to our regulatory agencies for monitoring thousands of water and soil samples annually [3]. The University of California, Davis, located in the agricultural central valley, has a vested interest in clean soil and ground water. This is the additional impetus to develop an immunoassay which is selective for simazine in the presence of atrazine. Since most enzyme-linked immunosorbent assays (ELISAs) detect both atrazine and simazine, an ELISA selective for simazine then would allow quantitation of both. Cyanazine, a moderately used herbicide in California, shows very low cross-reactivity in our assays. It is currently being phased out of usage in California, and thus is of less importance at this time.

2. Immunogen hapten design

The principal objectives of this research are to develop ELISAs that selectively quantify one chemical compound in the presence of others in its class (i.e.

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Table 1
Herbicide usage in the USA and California ($kg \times 10^{-6}$)

	USA [1]	California [2]
Triazines		
Atrazine	32.9	0.02
Cyanazine	14.6	0.29
Prometryn	0.6	0.10
Propazine	NA[a]	0
Simazine	1.8	0.38
Arylureas		
Diuron	1.8	0.49
Linuron	0.9	NA[a]

[a] Not available.

analyzing for simazine in the presence of other *s*-triazines) and to meet the demands of regulatory agencies for inexpensive, reasonably accurate and precise assessment of pesticide levels in the environment. Our efforts have been directed towards developing immunoassays for the most commonly used herbicides, simazine and atrazine (Fig. 1), with a secondary concentration on the arylureas, monuron (VI) and diuron (VII). In-depth presentations of these hapten design strategies may be found in our review articles [4–6].

Since selectivity among a class is dependent on the immunogen, structural design of the hapten becomes increasingly important as the size of the target molecule becomes smaller and/or the number of determi-

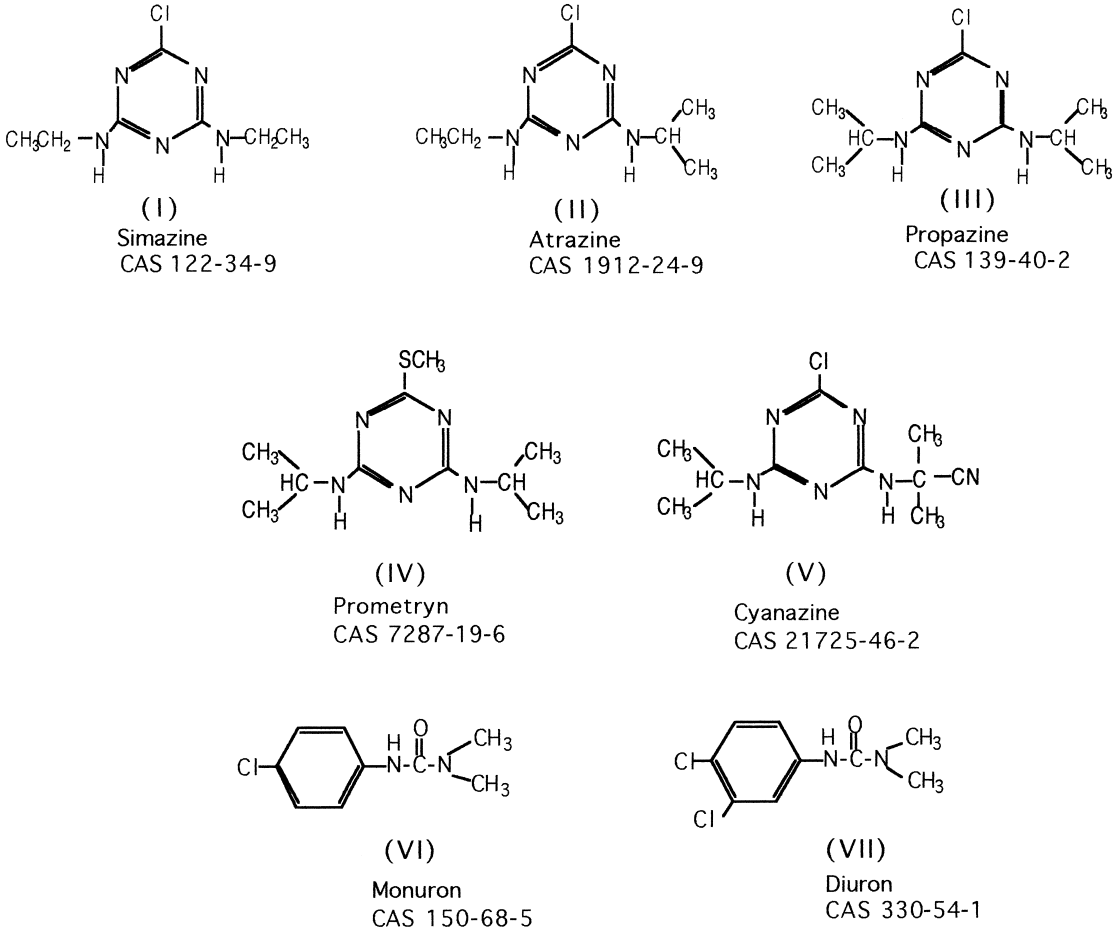


Fig. 1. Chemical structures of some *s*-triazine and arylurea herbicides. Roman numerals are used in the text to refer to the compound. Chemical abstract numbers are given below each structure.

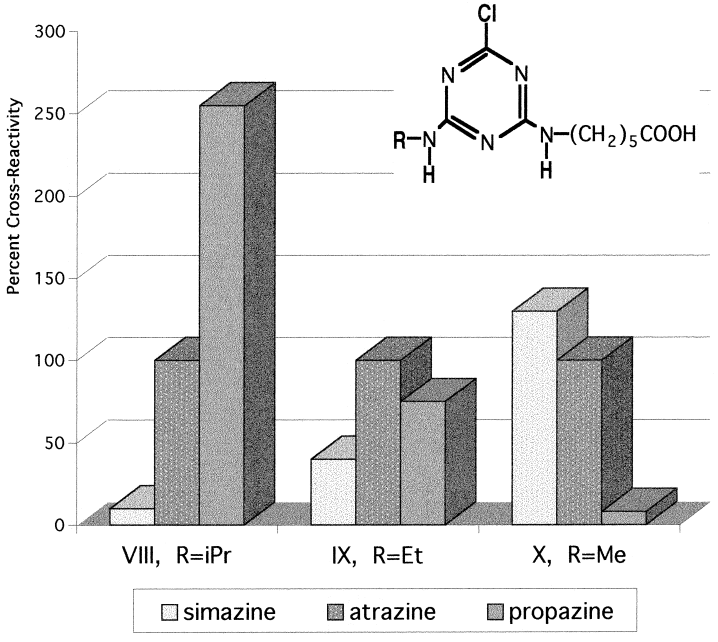


Fig. 2. Relative triazine cross-reactivities using a 6-position handle immunogen. All rabbit polyclonal antibodies were raised to the structure shown in the inset with variations on the R group at the 4-amino position (using the convention of position 2 on the triazine having the chlorine). The bars represent the cross-reactivites to simazine, atrazine and propazine assigning atrazine 100% cross-reactivity. Roman numerals indicate structure number.

nant groups becomes fewer. Contrary to many immunogen hapten designs, we purposely avoid the use of any functional group on the target molecule for conjugation to proteins. For example, the use of succinic anhydride on an amino-substituted target molecule converts a basic moiety into a neutral amide function, introducing a strong hydrogen-bonding determinant, vastly dissimilar to the native amino group. Such hapten coupling also masks a key determinant group. Finally in small molecules, the amide would be immediately adjacent to the other determinant groups to which one wishes to make antibodies, thus misdirecting antibody production. The following criteria have governed our structure design for immunizing haptens for polyclonal (PAbs) and/or monoclonal (MAbs) antibody production:

1. The hapten should be a near perfect mimic of the target structure in size, shape (geometry) and electronic properties.
2. The chemistry of the target molecule must be understood, reviewed and evaluated for methods of insertion of the attachment handled by established chemistry.
3. The hapten handle (required for connecting the target-mimic to a carrier protein) should in itself not elicit antibody recognition. Thus, an innocuous methylene handle often is most appropriate, the length of which should be evaluated using one or more homologs.
4. An appropriate functional group for covalent attachment of the handle to the protein must be compatible with the chemistry of the functional groups on the target.
5. Several immunizing carrier proteins should be evaluated (here we have found that keyhole limpet hemocyanin (KLH), *Limulus polyphemus* hemocyanin (LPH), and thyroglobulin conjugates often produce the best PAbs). However, we sometimes also try others selected from conalbumin (CONA), ovalbumin (OVA) and bovine serum albumin (BSA); these may also serve as coating antigens in the assay development process.

To date, most ELISAs for atrazine (II) are actually propazine (III) assays in that the antibodies (Abs) recognize propazine better than atrazine. This was indeed the case in our early assays (Fig. 2) where we compared the cross-reactivities of rabbit polyclonal antibodies (PAbs) produced from our immunogen haptens (VIII and IX), having the 6-position handle [7,8]. When the immunogen appendage was isopropylamino (VIII), propazine was recognized 2.5 times better than atrazine and there was a negligible (0.1 times) Ab recognition of the smaller simazine molecule. When the immunogen appendage was the smaller ethylamino group (IX), the PAbs now provided what might be called an atrazine (100% cross-reactivity (CR)) assay, but with increased recognition of simazine (40% CR) and accompanying diminished recognition of propazine (72% CR). Our subsequent study using an even smaller appendage, a methylamino group (Fig. 3, X), generated a true simazine (100% CR) immunoassay with lesser but still significant recognition for atrazine (75% CR) and low (13% CR) recognition for propazine [9].

We hypothesized that even more selective Abs would be produced if we presented the unique and

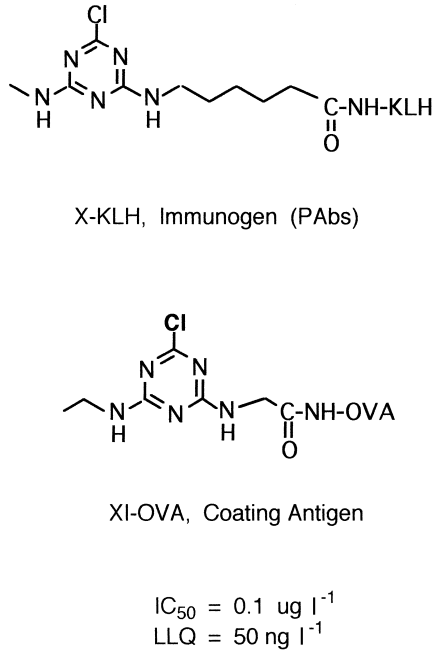


Fig. 3. The structures of haptens used as immunizing and coating antigens in a simazine immunoassay [9].

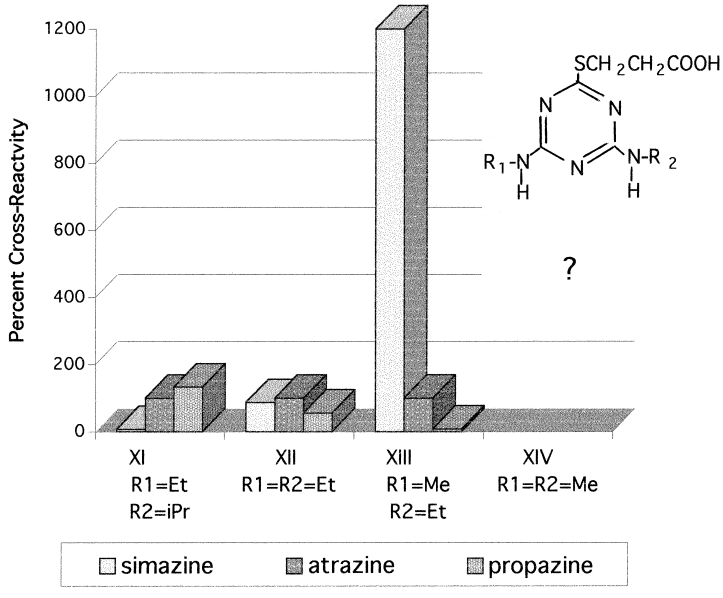
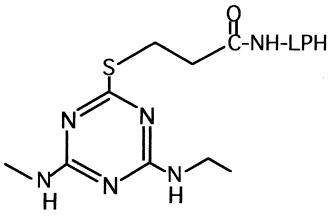


Fig. 4. Relative triazine cross-reactivities using a 2-position handle immunogen. All rabbit polyclonal antibodies were raised to the structure shown as the inset with variations on the R group at the 4- and 6-amino positions (using the convention of position 2 on the triazing having the chlorine). The bars represent the cross-reactivites to simazine, atrazine and propazine assigning atrazine 100% cross-reactivity. Roman numerals indicate compound number.

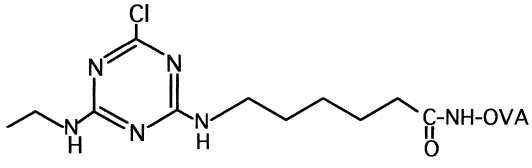
dissimilar features of simazine and atrazine structures distal to the connecting handle. Thus we were faced with the necessity of selecting a chlorine mimic capable of accommodating two appendages – the target structure and the handle. Our selection of sulfur as this connecting link is reviewed in an earlier publication [7]. It should also be noted that many 2-methylthiotriazines are commercial herbicides with physical and biological properties very similar to the 2-chlorotriazine herbicides. Thus, in our initial studies, we concentrated on immunizing hapten structures which presented these differences, ethylamino–isopropylamino (Fig. 4, XI) and the ethylamino–ethylamino (XII) moieties, distal to the common 2-mercaptopropanoic acid handle for polyclonal antibody production in rabbits. The ELISA developed from replacing the isopropylamino group with a second ethylamino group, resulted in a substantial increase in simazine recognition (about 15-fold) and a marked decrease (by one-half) in the recognition of the larger propazine molecule. A similar trend was also observed by Lawruk [10] using comparable haptens. In our most recent studies [11,12] we found that reducing the size of one appendage even more, from bis(ethylamino) (Fig. 4, XII) to methylamino–ethylamino appendages (XIII), produced antibodies with an astoundingly greater recognition for the smaller simazine molecule (12 times that of atrazine, and 1200 times that of propazine, Fig. 5). This ''size-exclusion'' effect was even more dramatic than what was observed on reducing the size of the alkylamino in the series of structures with the handle in the 6-position. Apparently antibody pockets made to the small methylamino substituent precluded any accommodation of the two isopropylamino groups of the propazine molecules.

Such an astounding effect, of course, necessitates the obvious: preparation and evaluation of the bis(methylamino)–mecaptopropanoic acid triazine XIV as the immunogen hapten. This study is currently in progress.

Concurrent with our studies, however, Lawruk et al. [10] at the former Ohmicron laboratory, have also developed a simazine-selective immunoassay. Using PAbs derived from an immunogen with an aminobutanoic acid handle (shorter than ours, Fig. 2, IX) and with a 6-[4,6-bis(ethylamino)–1,3,5-triazine-2-yl]mercaptohexanoic acid hapten for the enzyme conjugate, they developed an excellent ELISA for simazine. The assay showed an IC_{50} of 15 $\mu g\ l^{-1}$, a limit of detection of 0.07 $\mu g\ l^{-1}$, and recognition of atrazine and propazine of <1%.



XIII-LPH, Immunogen



IX-OVA, Coating Antigen

Rabbit PAbs:	IC_{50} = 84 $\mu g l^{-1}$
	LLQ = 0.9 $\mu g l^{-1}$
	CR(%)
Atrazine	8
Propazine	<0.8

Fig. 5. The structures of haptens used in immunizing and coating antigens in an immunoassay of increased selectivity for simazine [12].

3. Coating/tracer hapten design

In addition to our goal of designing haptens with unique compound-selectivity among closely related structures, we have synthesized numerous analogs and homologs of the target structures for evaluation as coating/tracer haptens. Our objective was to screen multiple haptens searching for those providing ELISAs with the lowest levels of quantification (LLQ), often referred to erroneously as ''sensitivity''. (Since the analytical chemist defines ''sensitivity'' as ''the slope of the titration curve at the IC_{50} point'', we shall avoid this term and focus on the amount of analyte

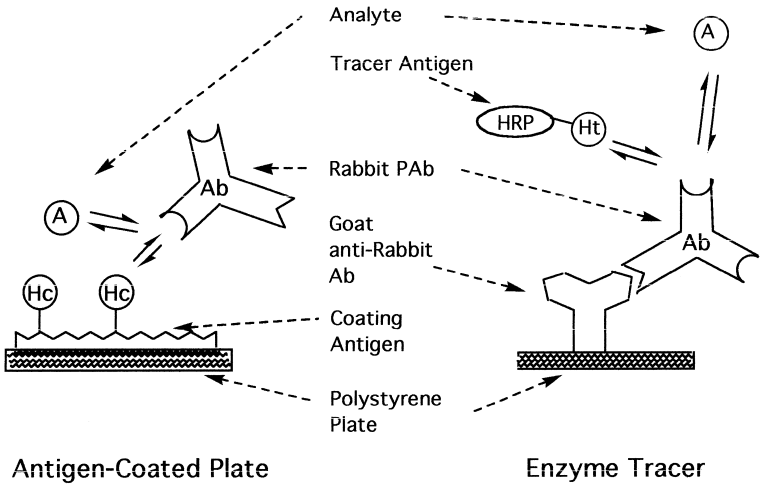


Fig. 6. Two ELISA formats commonly used in pesticide analysis. HRP – horse radish peroxidase, Ht – hapten tracer, Hc – coating hapten, A – analyte, and Ab – antibody.

present at the LLQ.) The advantages of using heterogolous haptens for immunogens and coating/tracer haptens have been reported earlier [13] and the concept is employed extensively in our laboratory for the triazines [7,8] and the arylureas [14,15]. The use of heterologous assay systems has produced lower IC_{50}s than homologous assays [8]. Variables such as the position of the handle, length of handles, position of target appendages, and size of appendages offer a variety of modifications for heterologous systems.

The conceptual steps with the two equilibria involved in two common ELISA formats (antigen-coated plate and enzyme tracer) are represented in Fig. 6. A schematic representation of the quasi-equilibria using the antigen-coated plate format is illustrated in Fig. 7. Assuming no analyte (A) is present, only the coating hapten equilibrium, K_{CH}, (variable by changing hapten structure) is in operation between antibody (Y) and coating hapten (H) and a maximum signal from a tracer antigen when attached to the Y–H is observed. On the addition of a minute quantity of analyte (A), this equilibrium is displaced towards the formation of antibody–analyte (Y–A). This dramatically reduces the Y–H amount and hence the tracer signal. Thus, for a fixed quantity of antibody, the lowest LLQ (and lowest IC_{50}) is observed when the affinity of the antibody for the analyte is greater than the affinity of the antibody for the plate-coating hapten ($K_A \gg K_{CH}$). Hence, with a fixed K_A for Y–A, one can

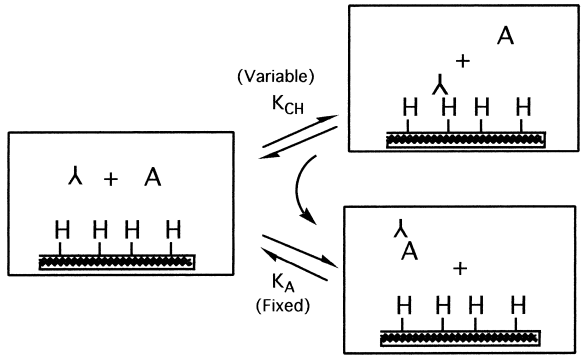


Fig. 7. Schematic representation of the quasi-equilibria using heterologous haptens in a competitive ELISA format occurring on a hapten (H)–protein coated plate. In the absence of analyte (A) the antibody will bind to the hapten-coated plate with an equilibrium constant of K_{CH}. If the antibody affinity for the hapten is the same as for the analyte, there will be a 1:1 displacement of the antibody from the plate by the hapten as indicated by the constant K_A, However, if the hapten binds to the antibody significantly less well than the analyte (in practice often about 3%), then a small amount of analyte will displace a large amount of antibody from the plate giving a large reduction in signal and a lower IC_{50}.

shift the overall equilibria by selecting a different coating hapten with a decreased affinity for the antibody (thus varying K_{CH}). Assuming no change in the slope of the curve, this produces a lower IC_{50} (as well as decreased LLQ) assay for the target analyte. Table 2 lists the coating/tracer hapten design criteria for planning heterologous hapten syntheses which are

Table 2
Coating/tracer hapten design criteria

1. Heterology of hapten structure
(a) Position of handle
(b) Composition of handle
(c) Length of handle
(d) Conjugation chemistry
2. Partial structure of target molecule
3. Consideration of cross-reactivity of hapten molecules (or derivatives)
4. Alteration of immunizing hapten's chemical composition

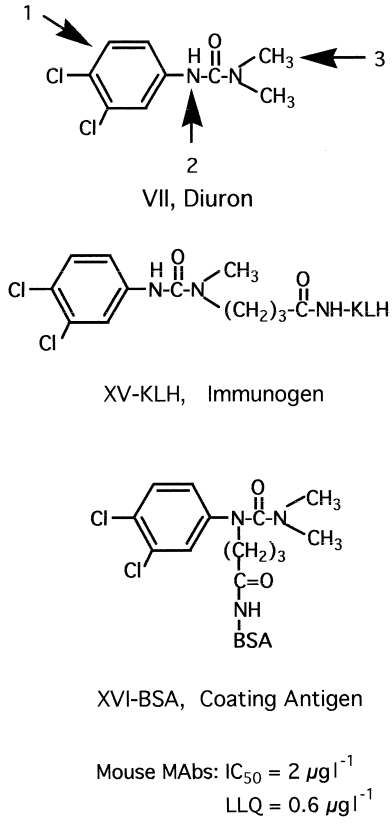


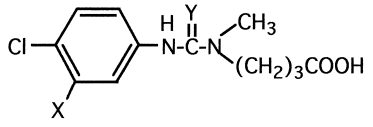
Fig. 8. The structures of haptens used for immunizing and coating antigens in a monoclonal antibody based immunoassay for diuron [15].

detailed in former reviews [7,8]. Thus, synthesizing many haptens and screening for an optimum coating antigen has led to a monoclonal antibody (MAb)-based ELISA for triazines at 40 ng l^{-1}, but it was not totally selective for any one species among the class [16].

The substantial use of diuron (VII) in California (Table 1), and its prominence in Germany for replacing the banned triazine herbicides [17] has renewed our interest in improving existing ELISAs and developing new immunoassay techniques for this herbicide. The former assay development by Karu et al. [15] was derived from an ideal hapten through an extension of an existing carbon chain (Fig. 8, XV) by methylene groups. The best coating antigen of the three evaluated consisted of an isomer (Fig. 8, XVI) with the methylene handle attached at the internal nitrogen. The IC_{50} was satisfactory at 2 μg l^{-1} with a LLQ of 0.6 μg l^{-1}.

Using our rationale for a lower antibody affinity for the coating hapten, we had found that by replacing the oxygen of the monuron immunizing hapten structure with a sulfur to make a thiourea coating hapten, we obtained a usable assay for monuron with a very low IC_{50} of 0.5 μg l^{-1} [14]. The sulfur, requiring more space than oxygen, probably could not fit well in the urea-established antibody pockets (based on a size-exclusion model). Additionally there would be a substantially lower affinity for this coating hapten structure due to the loss of hydrogen-bonding between the thiocarbonyl and the antibody.

Using polyclonal antibodies made to a 'monuron' hapten (Fig. 9, XVII) and a structurally similar diuron-type thiourea-HRP tracer hapten (XVIII), Kramer et al. [17] developed a diuron ELISA with an IC_{50} of 0.1 μg l^{-1} and a linear range 0.02–1 μg l^{-1}. Employing a newly developed flow injection immunoaffinity analysis (FIIAA), and the same reagents, Kramer et al. found an IC_{50} of 0.1 μg l^{-1} and a linear range 0.02–0.5 μg l^{-1}. Using the mouse MAbs that Karu [15] produced from the diuron hapten (XV), Kramer improved on the IC_{50} in the ELISA using the thio-diuron hapten (XIX), (IC_{50} of 4 μg l^{-1} and LLQ of 0.6 μg l^{-1}) [18]. This was a substantial improvement on values obtained by Karu using the same MAb 481 and the oxygen analog of XVIII as a coating antigen (IC_{50} ca. 230 μg l^{-1}). These three successful examples in which a slight modification of immunizing hapten structures produced low μg l^{-1} assays, suggests the size-exclusion phenomenon might be applied to other similar classes of pesticides such as the organophosphates, carbamates, or the thiocarbamates. This principle might also be extended to replacing the 4-chlorophenyl moiety contained in several



	X	Y
XVII-BSA, Immunogen (a monuron hapten)	H	O
XVIII-HRP, Tracer	Cl	S

ELISA: IC_{50} = 0.1 $\mu g l^{-1}$ FIIAA: IC_{50} = 0.06 $\mu g l^{-1}$
LLQ = 0.03 $\mu g l^{-1}$ LLQ = 0.02 $\mu g l^{-1}$

	X	Y
XV-KLH, Immunogen	Cl	O
XIX-HRP, Tracer	Cl	S

ELISA: IC_{50} = 4 $\mu g l^{-1}$ FIIAA: in progess
LLQ = 0.6 $\mu g l^{-1}$

Fig. 9. The structures of haptens used for immunizing and coating antigens in an ELISA and FIIAA for Diuron [17,18].

pesticide immunogen haptens with a 4-iodophenyl for use as a coating/tracer hapten.

Another broad generalization uncovered in our years of ELISA developments was the observation from screening multiple haptens, that when the best immunogen hapten contained a long methylene chain handle, the best coating/tracer handle would be very short. This was especially true for the simazine ELISA studied by Wortberg [9] where the immunogen handle on the triazine (Fig. 2, X) was a 6-aminohexanoic acid. Coating haptens bearing aminoacetic to 6-aminohexanoic acid handles in the same position were evaluated. The IC_{50} for the shortest handle was 0.1 $\mu g l^{-1}$, whereas the IC_{50}s of the others were in the 100–1000 $\mu g l^{-1}$ range. Additionally, the other alkylamino substituents, being either ethylamino or isopropylamino, showed no marked difference in their IC_{50}s. Apparently this latter appendage only had to be larger than methylamino group of the immunogen hapten to be appropriately less recognized by the antibodies; the handle had to be short in both series.

4. Conclusions

Using a "size-exclusion" model for hapten design and synthesis, we have developed polyclonal antibodies that selectively recognize the smaller molecules of a class. Use of this same paradigm to design heterologous coating/tracer haptens has provided immunoassays with lower IC_{50}s and lower levels of quantification.

5. Nomenclature

ELISA	enzyme-linked immunosorbent assay
cross-reactivity	the ratio of the IC_{50} of the target analyte to the IC_{50} of the test analyte, expressed as a percent
IC_{50}	the equivalence point, or the concentration of analyte that inhibits maximum binding of the antibody to the antigen by 50%
lowest level of quantification (LLQ)	the lowest concentration of analyte which can be quantitatively distinguished from the background singal
selectivity	the ability of antibodies to bind preferentially to one chemical structure in the presence of other substances
sensitivity	ratio of the rate of change of signal to a corresponding change of analyte concentration

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