In Vitro Selection of a Single-Stranded DNA Molecular Recognition Element for the Pesticide Malathion

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Abstract: Many large-scale applications of the organophosphate pesticide malathion have led to widespread environmental contamination. Concentrations are found in the environment well above those which are harmful to humans and environmental organisms. No current method of detection for this pesticide is rapid, cost-effective, and specific for malathion. Therefore, we utilized a stringent Systematic Evolution of Ligands by Exponential Enrichment (SELEX) process to identify a Molecular Recognition Element (MRE) for malathion. This MRE was identified from a large ssDNA library and has an equilibrium dissociation constant (K_d) in the low-nanomolar range. Additionally, it has significant selectivity for malathion in comparison to various other pesticides and metabolites of malathion, which were used as negative targets of selection. The high affinity and selectivity of the ssDNA MRE for malathion is a product of the stringent SELEX selection scheme and will be useful for rapid, inexpensive, and specific detection of malathion in the environment.

Keywords: Aptamer, *in vitro* selection, malathion, Molecular Recognition Element, MRE, pesticides, SELEX.

INTRODUCTION

Malathion is the most commonly-used organophosphate pesticide in the United States. Approximately 16.7 million pounds are used annually, with 12 million pounds being applied to crops [1]. Many other uses include in sewage systems, plant nurseries, home use, and recreational land maintenance [1]. Three major historical applications in the U.S. were Boll Weevil eradication, Medfly suppression, and mosquito suppression [2]. Its widespread usage portends its benefits.

Due to its many uses, malathion has become an environmental contaminant with multiple routes of exposure. Aerial applications often drift long distances to non-target waterways and volatilizes, leading to reduced air quality [2-5]. Runoff after application has led to levels up to 787.1 μg/L being found with ground and surface water having levels up to 6 μg/L under normal conditions [2, 6]. Furthermore, 33.7% of corn grain, 27.8% of cultivated and frozen blueberries, 24.6% of strawberries, and 19.3% of celery samples on the market tested had detectable levels of malathion [7]. Clearly, release of malathion into the environment and its interaction with humans and other organisms is occurring.

The presence of malathion in the environment is of concern to human and ecosystem health. Prior work has shown the induction of mammary epithelial cell proliferation and other carcinogenic effects by malathion through acetylcholinesterase inhibition in rats [8]. Malathion has also been found to have cytotoxic and genotoxic effects on human and other mammalian cells [9-10]. Additionally, there is a 55%-72% increase in the odds of Attention-Deficit/Hyperactivity Disorder (ADHD) being diagnosed in children that have ten-fold higher malathion exposure levels [11]. Ecologically, malathion is highly toxic to various fish species, with LC_{50} values as low as 0.1 μg/L for brown trout [12]. Many aquatic invertebrates are also highly susceptible to exposure, with LC_{50} values below environmentally relevant levels: as low as 0.5 μg/L [13]. It also has other negative effects at sub-lethal concentrations to a variety of aquatic species [14-16]. Furthermore, the pesticide has been shown to have negative survival effects at environmentally relevant concentrations on tadpoles and frogs [17], small mammals [2], reptiles [18], and non-target beneficial insects [19]. It is therefore important that individual species and entire ecosystems are protected from malathion exposure.

Currently, detection of malathion in biological and environmental samples is mostly achieved using gas chromatography (GC). This method is time- and labor-intensive, requiring expensive and expertly-used equipment [1, 20-21]. Methods of detecting an organism's exposure include monitoring blood cholinesterase activity or excretion of metabolites [1, 22]. There have also been antibody assays developed for malathion detection, however these antibodies have not been assayed for their affinity or only assayed for cross-reactivity to a few of malathion's major chemically-related metabolites [23-25]. As the antibodies were not identified for their selectivity, it is likely that they bind to major metabolites [26]. Therefore, it is necessary to identify a molecule that has been identified for its high affinity and specificity for malathion and not its metabolites.

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Identification of such a molecule, termed a Molecular Recognition Element (MRE), can be accomplished using the Selective Evolution of Ligands by EXponential Enrichment (SELEX) [27]. This is an iterative process that enriches a library with up to 10^{15} different molecules for those that bind to the target of interest under increasingly stringent conditions. A stringent SELEX process was used here which also enriches the library for the target of interest. The focus, however, was on what the MRE should not bind to utilizing many stringent negative selections. This method is ideal for identifying MREs with both high affinity and selectivity for the target of interest. In this work, the stringent SELEX method was utilized to identify a single-stranded DNA (ssDNA) MRE for the pesticide malathion. Those molecules which bound to major metabolites of malathion were removed, which included: dimethyl thiophosphate (DMTP), dimethyl dithiophosphate (DMDTP), malathion dicarboxylic acid (MDA), and malaoxon [28]. The MRE was also designed to not bind to other pesticides found in similar amounts and environments as malathion including propanil [29], 2,4-D acid [30], and atrazine [31]. Additionally, bovine serum albumin (BSA) was used as a negative target as a model for large, globular proteins. The selected MRE may be used as a sensor in a rapid, field-deployable detection device [32-34].

MATERIALS AND METHODS

In Vitro Selection for Malathion MREs

Twelve rounds of SELEX were performed to obtain a MRE specific for the pesticide malathion (Fig. **1**). A ssDNA library with a diversity of 10^{15} molecules was enriched for those that bound to malathion and not the negative targets (Fig. **2**). The library was designed within our laboratory and designated RMW.N34. The ssDNA library was commercially synthesized and consists of two 23 base constant regions for polymerase chain reaction (PCR) amplification that flank a 34 base random region (Eurofins MWG Operon; Huntsville, AL).

The malathion derivative malathion monocarboxylic acid (Fig. **2b**) was covalently biotinylated using EDC-NHS chemistry. It was reacted with 1-ethyl-3-[3-dimethylamino-propyl]carbodiimide (EDC) (ThermoScientific; Rockford, IL) and then incubated with Amine-PEG_2-Biotin (Pierce; Rockford, IL) according to manufacturer's instructions. The biotinylated derivative was bound to streptavidin-coated magnetic beads (New England Biolabs; Ipswich, MA) and washed to provide the immobilized target (IT).

For Round 1(+) selection, the ssDNA library was incubated with 50 µL IT in a total of 500 µL selection buffer containing 100 mM sodium chloride, 20 mM Tris-HCl, and 2 mM magnesium chloride (1X selection buffer; SB). The mixture was incubated at room temperature on a rotisserie for 48 hours. The IT with bound ssDNA was removed from the solution by magnetic separation, washed three times with 1 mL SB, and resuspended in SB. This was used as a template for PCR amplification. PCR amplification was performed with the following reaction ingredients: enriched ssDNA library, 400 nM forward and biotinylated reverse RMW.N34 primers (Eurofins MWG Operon) (forward: 5'-TGTACCGTCTGAGCGATTCGTAC-3', biotinylated reverse: 5'-Biotin-GCACTCCTTAACACTGACTGGCT-3'), 250 µM deoxynucleotide triphosphates, 1X GoTaq Reaction Buffer (Promega; Madison, WI), 7 units *Taq*, and pure water. Thermal cycling conditions were: initial denaturation of 95°C for 5 minutes; cycling at 95°C for 1 minute, 63°C for 45 seconds, and 72°C for 1 minute; and final extension of 72°C for 7 minutes. Large-scale amplification was performed using either a 4 mL or a 2 mL total volume PCR, which respectively preceded positive and negative selection rounds.

The amplified DNA was then purified by agarose gel electrophoresis using the GFX PCR purification kit (GE Healthcare; Piscataway, NJ). Recovered dsDNA was then incubated with streptavidin agarose resin (Pierce) for separation of single strands. This mixture was placed in a flow-through column and washed with five column volumes

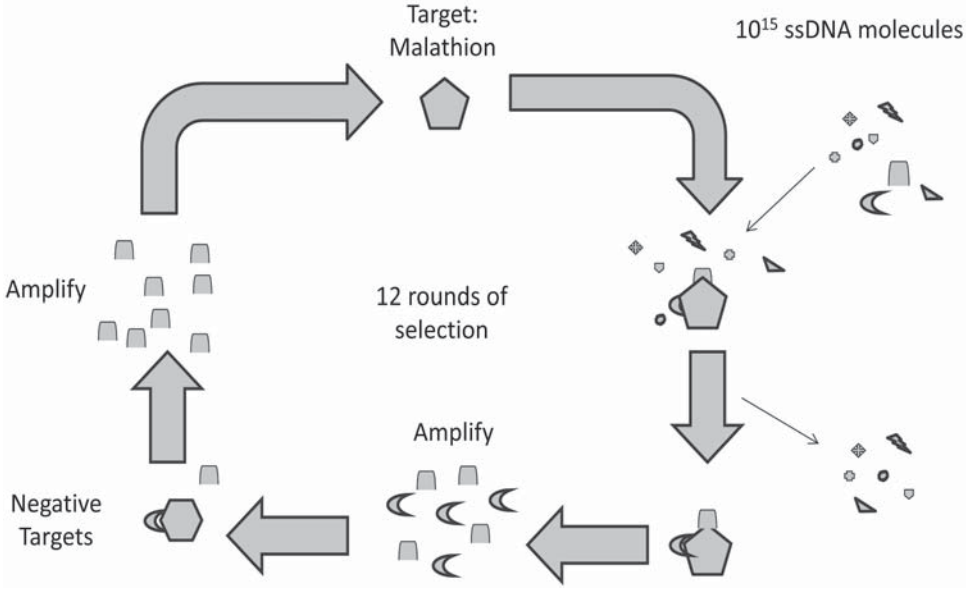


Fig. (1). SELEX scheme for selection of a malathion MRE. 10^{15} different single-stranded DNA (ssDNA) molecules were incubated with malathion. Those that bound were amplified and subjected to closely-related targets. Those do not bind those were amplified and re-subjected to malathion under more stringent conditions. This process continued for 12 rounds.

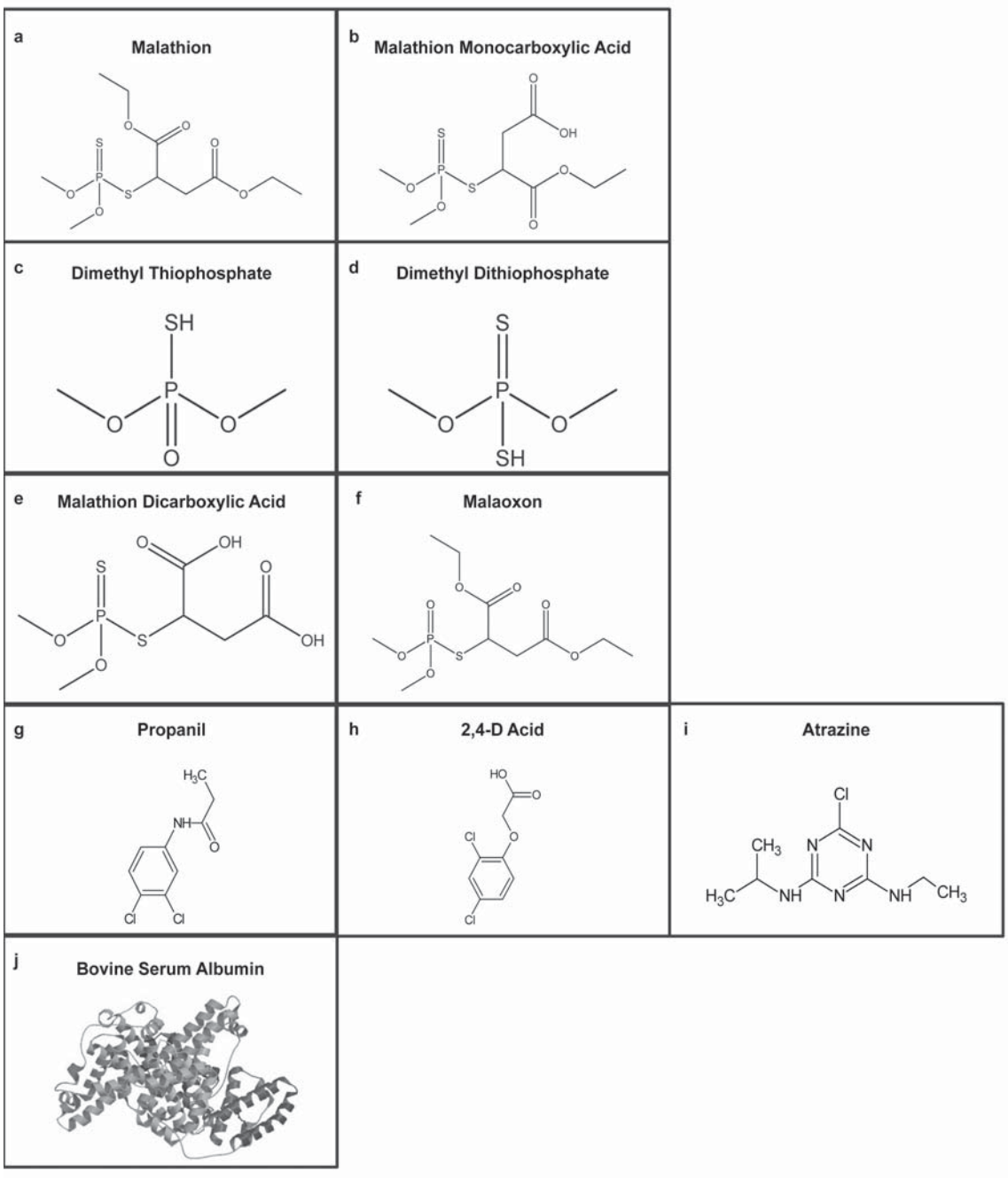


Fig. (2). Chemical structures of molecules used in selection of a malathion MRE. (**a**) Structure of the target of selection, malathion. (**b**) Structure of malathion monocarboxylic acid, a malathion derivative used in magnetic bead immobilization. (**c**) and (**d**) Structures of dimethyl thiophosphate and dimethyl dithiophosphate used as negative targets in Round 8(-). (**e**) Chemical structure of malathion dicarboxylic acid, used in Round 9(-) selection. (**f**) Structure of malaoxon, used in Round 10(-) selection. (**g**), (**h**), and (**i**) Structures of propanil, 2,4-D acid, and atrazine, used successively in Round 7(-) of selection. (**j**) Ribbon structure of bovine serum albumin (BSA) used as a negative target in Round 11(-) of selection [49].

of phosphate-buffered saline (PBS). The single forward strand of amplified DNA was eluted from the column using five column volumes of 1 M sodium hydroxide. The eluted ssDNA was prepared for ethanol precipitation by adding 0.1 volume of 3 M sodium acetate (pH 5.2), 2.5 volumes of cold 100% ethanol, and glycogen at 10 μg/mL. The solution was frozen and then centrifuged at 13,000 Xg for 1 hour, the pellet washed with 70% ethanol, and centrifuged again for 15 minutes. The pellet was dried under a vacuum and resuspended in SB. The amount of ssDNA present was verified to be at least 10^{13} sequences using a NanoDrop spectrometer (ThermoScientific).

Round 2(+) selection proceeded similar to Round 1(+), however the incubation period was 22 hours. Positive selections occurred with this method, with decreasing incubation periods for increased stringency, through Round 6(+). Following Round 2(+), the DNA was prepared for the

next round as specified above. However, it was then incubated with immobilization substrate (IS) for Round 2(-) selection for 16 hours in 100 µL total volume of SB. To prepare the IS, Amine-PEG_2-Biotin (Pierce) was incubated with streptavidin-coated magnetic beads (New England Biolabs) and washed to remove unbound biotin reagent. After the incubation period, the IS with bound ssDNA was separated from the solution by magnet. Washes with 25, 25, and 50 µL of SB were performed and pooled with the total 100 µL of incubation supernatant. This served as a template for PCR amplification, which was performed as previously described. Negative selections similar to Round 2(-) were performed in Rounds 3 and 5, with longer incubation periods for increased stringency.

Beginning in Round 7(+), a competitive elution was performed in order to obtain a MRE which bound to malathion in solution. For this, the enriched library was incubated with 50 µL of IT in a total volume of 500 µL SB for 3 hours. The beads were then washed three times with 1 mL SB. In this round, however, 100 µL of 10 mM malathion in SB was then added to the IT and incubated for 5 minutes. The IT was then removed from the solution by magnet, and the supernatant served as the template for PCR amplification. The enriched and amplified library was then prepared for Round 7(-) selection as noted above. This method of selection by competitive elution was performed through Round 12(+) with decreasing incubation times and malathion concentrations. This continued until Round 12(+) when the incubation with the IT was immediate, and elution with 100 µL of 100 nM malathion was immediate.

In Round 7(-), competitive elutions were performed with other pesticides found in similar environmental locations as malathion. The enriched library was incubated with the IT, the supernatant removed, and the beads washed 3 times. Then, the beads were incubated for 24 hours at room temperature on the rotisserie with 500 µL of 1 µM propanil in SB. The supernatant was removed and the beads were washed twice with 1 mL SB. The IT was then incubated with the same volume and concentration of 2,4-D Acid, washed, incubated with atrazine, and washed. The beads were then resuspended in SB and used as a template in PCR amplification. The DNA was then prepared for the next positive round of selection.

Round 8(-) selection was done similar to the previous negative round, however dimethyl thiophosphate, then dimethyl dithiophosphate were used as negative targets. Round 9(-) was performed with 500 µL of 1 mM malathion dicarboxylic acid for 15 minutes. Round 10(-) selection was performed with 500 µL of 1 mM malaoxon with an incubation period of 1 minute. The final negative selection, Round 11(-), was performed in the same manner with bovine serum albumin (BSA) as the elutant.

Sequencing of the Enriched ssDNA Library

Following every third round of selection, a random sample of at least 30 ssDNA molecules present in the enriched library was taken. The library was PCR-amplified with non-biotinylated primers as described above and ligated into the pCR2.1 vector (Invitrogen; Carlsbad, CA) according to manufacturer's instructions. This was cloned into competent bacteria, which were plated onto selective agar. Colonies with inserts were amplified, and plasmids containing individual library inserts were purified using the AxyPrep Plasmid Prep Kit (Axygen; Union City, CA). Purified plasmids were sent for sequencing (Eurofins MWG Operon) using the M13R primer complimentary to a region upstream of the pCR2.1 vector. At least 30 sequences were obtained for each enriched library. These were then analyzed for consensus sequence families to identify structures binding to malathion.

Determination of MRE Binding Affinity and Specificity

The Round 12 sequences were analyzed to produce consensus sequence families, and from those three were chosen for further characterization. These were chosen based on their inclusion in consensus families as well as their structure and stability as predicted by the Mfold DNA web server using buffer salt and 25°C as binding conditions [35-37]. The chosen sequences were designated R12.20, R12.14, and R12.29. These sequences were subjected to fluorescent saturation binding assays to determine their affinity for malathion.

Concentrations of 0, 0.25, 0.5, 0.75, 1, 5, 10, 15, 20, and 25 nM MRE were used in fluorescence saturation binding studies essentially as previously described [38-40]. In a 200 µL total volume of SB, 10 uL of IT was incubated with each concentration for five minutes. Unbound MRE was removed and each incubation was washed five times with 200 µL SB, then the immobilized target was resuspended in 200 µL SB and heated to 95°C for 10 minutes to denature and elute bound MRE. Eluted ssDNA in SB was placed in a 96-well microplate and measured in a Synergy 2 microplate reader equipped with a tungsten halogen lamp with excitation wavelength of 490 nm and an emission filter at 520 nm using Gen5 1.06 software (Biotek US; Winooski, VT). All fluorescence readings on the plate were normalized to 100 µL of a 1 nM solution in SB of the fluorescent MRE and the no ssDNA incubation. For each concentration set, the same was done with washed streptavidin-coated magnetic beads to ensure binding over background to the beads. Each set of incubations were performed in triplicate. R12.14 and R12.29 did not have greater binding to immobilized malathion than to the magnetic beads alone. Therefore, binding affinity was not determined for these sequences and further studies were performed only on R12.20. To determine the dissociation constant (K_d) of the MRE, data were analyzed with Origin 8 (OriginLab Corporation; Northampton, MA) using nonlinear regression analysis and fit with the equation, $Y=((B_{max}*X)/(K_d + X)) + NS*X$, where B_{max} is the maximum binding, K_d is the dissociation constant, and NS is nonspecific binding as previously described [41].

To determine binding of the selected MRE, R12.20, to compounds used in the selection, 50 nM fluorescent ssDNA was used. For each elutant used, in a 200 µL total volume of SB, 10 µL IT was incubated with R12.20 for five minutes. The magnetic beads were washed five times with 200 µL SB. Then, each incubation was resuspended in 200 µL of 1 µM in SB of the following in SB: malathion, malathion monocarboxylic acid, dimethyl thiophosphate, dimethyl dithiophosphate, malathion dicarboxylic acid, malaoxon, propanil, 2-4, D acid, atrazine, and BSA. It should be noted

that this assay was used to determine binding of the MRE to malathion in solution relative to other molecules in solution. Each elutant was incubated with the immobilized target for ten minutes, and the solution was removed from the beads. This solution was placed in a 96-well plate and measured in a fluorescence plate reader. Data were normalized to an internal fluorescent standard of 1 nM MRE in 100 µL SB and background fluorescence of each elution buffer. Each set of binding studies were performed in triplicate. Data were averaged and standard deviations calculated. For each elutant, a one-tailed t-test was performed to determine statistical differences in the means.

RESULTS AND DISCUSSION

Identification of a Malathion-Specific MRE

In order to identify a MRE with high affinity for malathion that did not bind to other pesticides or closely-related structures, twelve rounds of SELEX were completed (Table **1**). The selection used negative targets including the immobilization substrate, propanil, 2,4-D acid, atrazine, dimethyl thiophosphate, dimethyl dithiophosphate, malathion dicarboxylic acid, malaoxon, or bovine serum albumin. Following Rounds 3, 6, 9, and 12, at least 30 random sequences were chosen from the enriched library for consensus sequence analysis. Sequences were analyzed and tracked throughout the *in vitro* selection to monitor sequence diversity (representative families shown in Table **2**). One sequence motif, GGTATC was found in 0, 0, 2, and 4 sequences from Rounds 3, 6, 9, and 12 respectively. This is comparison to a smaller section of this motif, GGTA, which was found in 9, 3, 11, and 9 sequences in those rounds. Thus, while the shorter motif was present throughout the selection rounds at high levels, the longer motif took 12 rounds of selection to comprise a large enough part of the library to be identifiable in sequence analysis. Another, GAGAG was found in 2, 2, 2, and 5 sequences from those rounds with other sequence families of similar size being found to increase throughout selection rounds. It is clear that there was a decrease in sequence diversity and an increase in the size of larger sequence motifs, showing that maturation of binding affinity and specificity continued to occur through 12 rounds of selection.

Three sequences were chosen, R12.14, R12.20, and R12.29 based on their presence in consensus families, structure, and stability as predicted by the Mfold DNA web server (representative families shown in Table **2**). Not all of the sequences in the greater than 30 analyzed were found to contain consensus sequence families; however these were sufficient to identify MREs with high inclusion into families and very stable structures as predicted by Mfold. The consensus sequence motifs found in R12.20 discussed above (Table **2**) comprise the entire region of the most stable stem-loop structure in its secondary structure (Fig. **3**). The GGTATC motif which increases throughout the selection rounds comprised the base of this structure, while GAGAG spanned the stem-loop. It is likely that these motifs contribute to malathion binding either through direct interaction or by stabilizing the directly-interacting bases. While these motifs are relatively short for *in vitro* selection experiments, the sequence does have high stability and affinity. These sequences have a predicted Gibb's free energy value of -8.84 kcal/mol, -5.84 kcal/mol, and -7.89 kcal/mol, respectively, for R12.14, R12.20, and R12.29.

Affinity and Specificity of Malathion-Specific MRE

Fluorescence saturation binding assays identified R12.20 as having the highest affinity and specificity (Fig. **3**). R12.14 and R12.29 were also assayed; however their binding to immobilized malathion was not above background binding to magnetic beads alone. Therefore, binding affinity and

Table 1. SELEX scheme for malathion MRE selection.

Round	Positive Selection	Negative Selection
1	Immobilized Target (IT) 48 hrs	Immobilization Substrate (IS) 16 hrs
2	IT 22 hrs	-
3	IT 16 hrs	IS 24 hrs
4	IT 10 hrs	-
5	IT 6.5 hrs	IS 23 hrs
6	IT 3 hrs	-
7	IT 3 hrs, 5 min Competitive Elution (CE) w/ 10 mM Malathion (100 uL)	IT 3 hrs, 24 hr CE w/ 1 µM Propanil, 2,4-D Acid, Atrazine in succession (500 uL)
8	IT 1 hr, 5 min CE w/ 1 mM Mal (100 uL)	IT 1 hr, 24 hr CE w/ 1 µM dimethyl thiophosphate, dimethyl dithiophosphate in succession (500 uL)
9	IT 15 min, 5 min CE w/ 100 uM Malathion (100 uL)	IT 15 min, 15 min CE w/ 1 mM Malathion Dicarboxylic Acid (500 uL)
10	IT 1 min, 1 min CE w/ 10 uM Malathion (100 uL)	IT 1 min, 1 min CE w/ 1 mM Malaoxon (500 uL)
11	IT 1 min, immediate CE w/ 1 uM Malathion (100 uL)	IT 1 min, 15 min CE w/ 1 mM BSA (500 uL)
12	IT immediate, immediate CE w/ 100 nM Malathion (100 uL)	-

In vitro selection process for obtaining malathion-specific MRE. Immobilized target (IT) is malathion monocarboxylic acid bound to magnet beads. Immobilization substrate (IS) is streptavidin-coated magnetic beads plus blocked biotin reagent. Competetive elution (CE) is removal of bound ssDNA from target-coated magnetic beads by given concentration of free pesticide in solution. Times listed are incubation times in hours (hrs) or minutes (min).

Table 2. Post-round 12 sequence families.

Name	Sequence
R12.20	TGTACCGTCTGAGCGATTCGTACTATGGTATCCGAGAGGCCTACGGAATTGTTGTACAGCCAGTCAGTGTTAAGGAGTGC
R12.14	TGTACCGTCTGAGCGATTCGTACTTATTCGTCTTTGTCTTGGCTCAAAGGCGGTCAGAGCCAGTCAGTGTTAAGGAGTGC
R12.25	TGTACCGTCTGAGCGATTCGTACATCCGGTCAAGGAAAGGTATCGCGACAGTCAGAGAGCCAGTCAGTGTTAAGGAGTGC
R12.26	TGTACCGTCTGAGCGATTCGTACCTCCGGAAGTTATGTATCTTCTTTAGCTGATTCGAGCCAGTCAGTGTTAAGGAGTGC
R12.20	TGTACCGTCTGAGCGATTCGTACTATGGTATCCGAGAGGCCTACGGAATTGTTGTACAGCCAGTCAGTGTTAAGGAGTGC
R12.28	TGTACCGTCTGAGCGATTCGTACCGATTGAATTGGAGAGAGCTACGTCCTGTCTATTAGCCAGTCAGTGTTAAGGAGTGC
R12.46	TGTACCGTCTGAGCGATTCGTACTTAGGGAATTTCGCGATCTTGTCCCCTCCAAAGAAGCCAGTCAGTGTTAAGGAGTGC
R12.20	TGTACCGTCTGAGCGATTCGTACTATGGTATCCGAGAGGCCTACGGAATTGTTGTACAGCCAGTCAGTGTTAAGGAGTGC
R12.29	TGTACCGTCTGAGCGATTCGTACTGTGCAGTGGCCTCGGACTAGAGTCTCTTCGTCCAGCCAGTCAGTGTTAAGGAGTGC
R12.18	TACCGTCTGAGCGATTCGTACTATATAGCCAGGTAGTATAGTCGGCCTAAAGAGAGCCAGTCAGTGTTAAGGAGTGC
R12.20	TGTACCGTCTGAGCGATTCGTACTATGGTATCCGAGAGGCCTACGGAATTGTTGTACAGCCAGTCAGTGTTAAGGAGTGC
R12.18	TACCGTCTGAGCGATTCGTACTATATAGCCAGGTAGTATAGTCGGCCTAAAGAGAGCCAGTCAGTGTTAAGGAGTGC
R12.25	TGTACCGTCTGAGCGATTCGTACATCCGGTCAAGGAAAGGTATCGCGACAGTCAGAGAGCCAGTCAGTGTTAAGGAGTGC
R12.28	TGTACCGTCTGAGCGATTCGTACCGATTGAATTGGAGAGAGCTACGTCCTGTCTATTAGCCAGTCAGTGTTAAGGAGTGC
R12.47	TGTACCGTCTGAGCGATTCGTACGGTGAGTCCGGAGTTACTCGGTAGGGTGGGGGAGAGCCAGTCAGTGTTAAGGAGTGC

Representative sequence families following Round 12 of SELEX. Families are separated by a space with common sequences underlined or double-underlined.

specificity was not determined for these sequences. After assaying concentrations in the pM to low nM range, the dissociation constant (K_d) of R12.20 was determined to be 2.43 nM ± 0.68 nM (Fig. **4**). This is in the lower range of previously-selected ssDNA MREs for small-molecule targets [42-43]. Furthermore, in all three affinity binding assays, the lowest concentration tested, 250 pM, was consistently above background and therefore detectable. This is an extremely low limit of detection which is below the LC_{50} of 0.1 and 0.5 µg/L for brown trout and other aquatic species as noted above. This high affinity is likely to be a product of the increasingly stringent incubation conditions used in the SELEX process and will translate well to environmental sensing applications.

The cross-reactivity of R12.20 for negative targets used in the selection was also determined. This assay identified the binding of the MRE to malathion in solution relative to the other molecules tested. The ssDNA MRE binds to malathion significantly greater than it does to DMTP, DMDTP, malaoxon, propanil, and 2,4-D acid ($p<0.05$) (Fig.

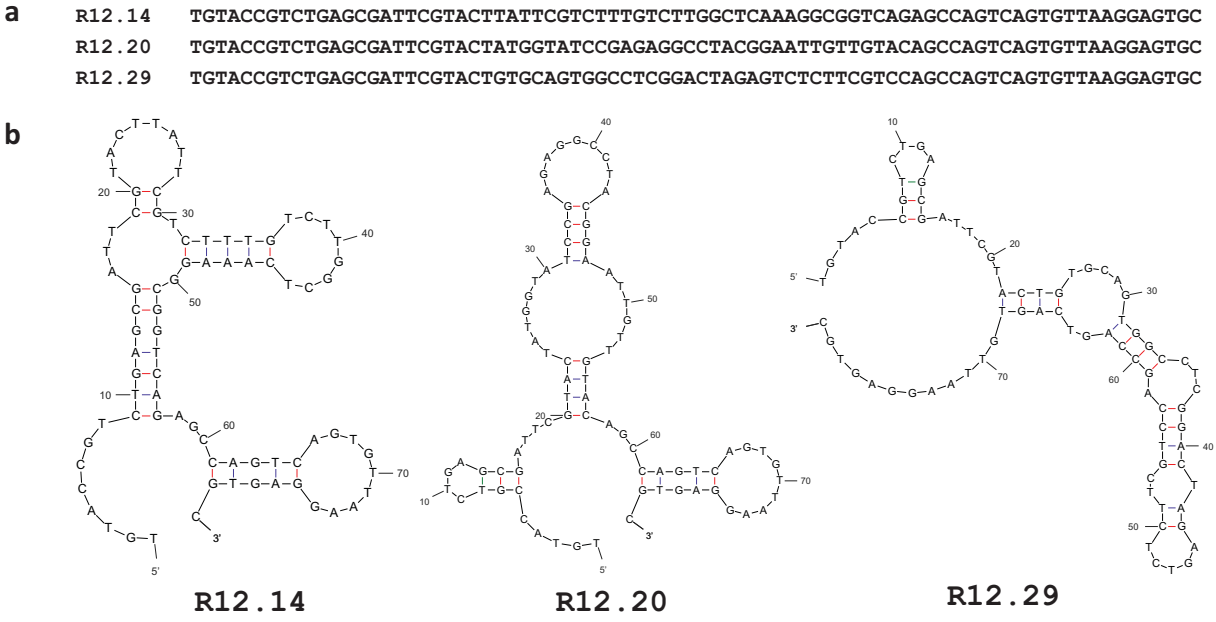
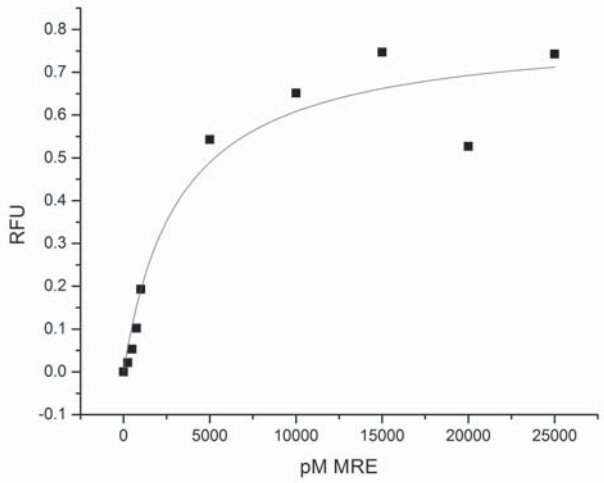


Fig. (3). Sequence and structure of malathion ssDNA MRE. (**a**) ssDNA sequence of malathion MRE sequences R12.14, R12.20, and R12.29. (**b**) Secondary structures of R12.14, R12.20, and R12.29 as predicted by Mfold [35].

5). The binding of R12.20 to malathion is 204% greater than to atrazine. Large variation, however, renders this result statistically insignificant ($p = 0.06$). Binding to malathion is also 142% greater than to malathion monocarboxylic acid (MMA), however there is no statistically significant difference in these samples ($p = 0.06$). This is expected as MMA was the molecule used to create the immobilized target used in all rounds of selection. Binding of the MRE to malathion is also 179% greater than to malathion dicarboxylic acid (MDA), however this is not significantly different ($p = 0.11$). The similarity of MMA and MDA explain the binding of R12.20 to MDA (Fig. **2b**, **e**). Binding of the MRE to BSA, however, is as strong to malathion. This may be due to the large, globular nature of the protein and its nonspecific interaction with ssDNA. This therefore suggests that R12.20 would be most successful as a sensing element in the presence of mostly small molecules, which will be achieved through use of a pre-processing size-exclusion chromatography step in future work to design an environmental sensor. The cross-binding results are particularly encouraging as the MRE differentiates malathion from three closely-related structures, including malaoxon, that some antibodies cannot discern between [26]. Future work will determine the performance of this MRE compared to current chromatographic techniques and malathion-specific antibodies in environmental sensing applications.

The selected malathion ssDNA MRE will be useful for environmental sensing. Previously-developed nucleic acid



(**b**)

MRE	K_d	Std Error
R12.20	2.43 nM	0.68

Fig. (4). Fluorescence equilibrium binding assays of R12.20 for malathion. (**a**) Representative saturation binding curve of R12.20 with nonlinear regression best fit. (**b**) Average equilibrium dissociation constant (K_d) with standard error of R12.20 binding assays.

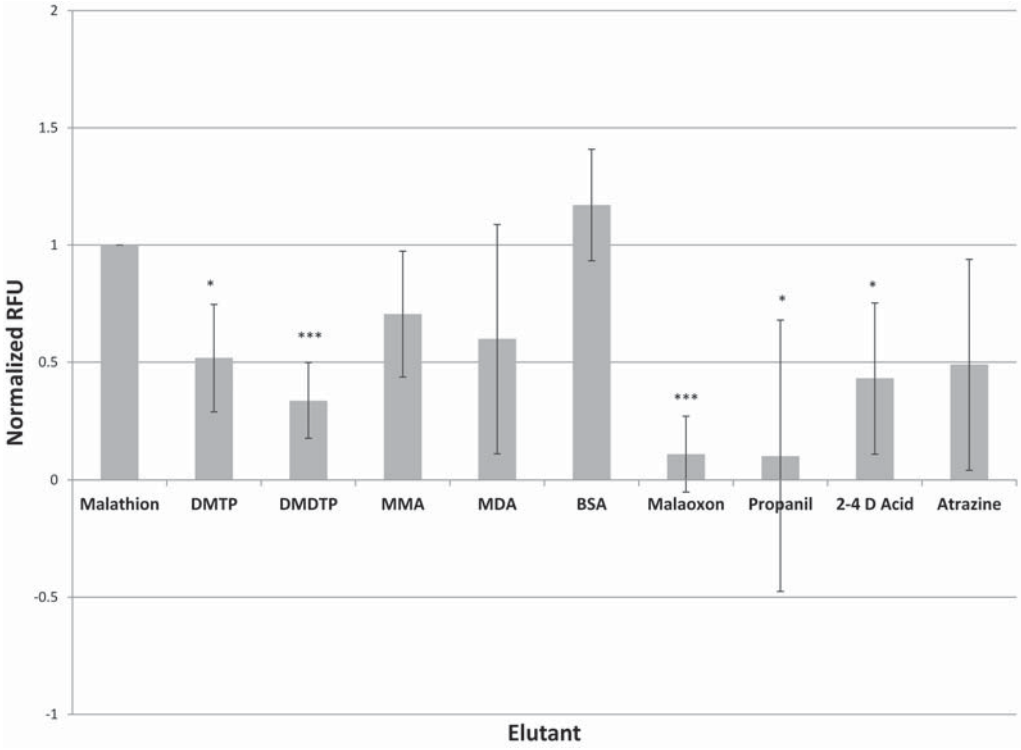


Fig. (5). Cross-binding studies of malathion MRE to other molecules used in the selection. Data were normalized to atrazine binding which was set to 100%, with error bars representing standard deviations. Statistical significance levels of $p<0.05$ are designated by '*', and levels of $p<0.001$ are designated by '***'. Malathion is the target of the selection and MMA was used to create immobilized target. DMTP, DMDTP, MDA, and malaoxon are molecules similar to malathion used as negative targets. BSA was also used as a negative target as model of a large, globular protein. Propanil, 2,4-D acid, and atrazine are other pesticides which were used as negative targets. MMA = malathion monocarboxylic acid; DMTP = dimethyl thiophosphate; DMDTP = dimethyl dithiophosphate; MDA = malathion dicarboxylic acid; BSA = bovine serum albumin.

MREs have been used as the sensing element in this capacity [44, 45]. It is possible that design of the sensor will allow for a field-usable malathion detection device that will be more rapid, inexpensive, and easier to operate than current methods. With easier detection of malathion, a greater sampling of contaminated environments will be possible. It is then possible to actively remediate these contaminated environments by biological, chemical, or physical methods [46-48].

CONCLUSION

A stringent variation of SELEX has been utilized to obtain a MRE for the pesticide malathion. Twelve rounds of selection were completed, with consensus sequence families identified and potential MREs obtained. The selected ssDNA MRE, R12.20, has a very high affinity for malathion and selectivity compared to various other pesticides and metabolites of malathion. This MRE will allow for rapid, inexpensive, and specific detection of malathion, which will lead to potential remediation of contaminated environments.

ABBREVIATIONS

SELEX = Systematic Evolution of Ligands by Exponential Enrichment

MRE = Molecular Recognition Element;

PCR = Polymerase chain reaction;

ssDNA = Single stranded deoxyribonucleic acid

CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

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