Published in final edited form as:

Chembiochem. 2009 July 6; 10(10): 1625–1629. doi:10.1002/cbic.200900201.

Monoclonal Antibodies with Orthogonal Azaspiracid Epitopes

Michael O. Frederick,

Department of Chemistry and The Skaggs Institute of Chemical Biology, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037 (USA) and Department of Chemistry and Biochemistry, University of California, San Diego, 9500 Gilman Drive, La Jolla, CA 92093 (USA)

Kim D. Janda [Prof.],

Departments of Chemistry and Immunology, The Skaggs Institute of Chemical Biology and Worm Institute for Research and Medicine (WIRM), The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037 (USA)

K. C. Nicolaou [Prof.], and

Department of Chemistry and The Skaggs Institute of Chemical Biology, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037 (USA) and Department of Chemistry and Biochemistry, University of California, San Diego, 9500 Gilman Drive, La Jolla, CA 92093 (USA)

Tobin J. Dickerson [Prof.]^{*}

Department of Chemistry and Worm Institute for Research and Medicine (WIRM), The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037 (USA)

Keywords

azaspiracid; diagnostic; monoclonal antibodies; immunoassay

An incident of human illness with diarrhetic shellfish poisoning (DSP)-like symptoms reported in the Netherlands in 1995 was eventually traced back to mussels, *Mytilus edulis*, harvested from Killary Harbour, Ireland.[1] The causative toxins of this outbreak were found to be the azaspiracids (**1–11**, Figure 1), which have been proposed to originate from the dinoflagellate *Prorocentrum crassipes*. However, despite the threat this compound poses to human health and the characterization of 31 other members of this compound class since the discovery of **1**,[2] conclusive evidence of an azaspiracid producer organism has remained elusive.[3] Through heroic efforts, and with minute amounts of material, the Yasumoto and Satake group proposed a structure for azaspiracid-1;[4] however, total synthesis by the Nicolaou group found the originally proposed structure to be incorrect.[5] Degradative and synthetic efforts found the structure to be as depicted in Figure 1.[6] Containing a trioxadispiroacetal system fused onto a tetrahydrofuran ring (ABCD domain) and an azaspiro ring system fused onto a 2,9-dioxabicyclo[3.3.1]nonane system (FGHI domain), the azaspiracids represent a unique toxin both in terms of structure and toxic effects.[7]

While initially postulated to resemble diarrhetic shellfish poisoning, further study after the first report in the Netherlands demonstrated that azaspiracid poisoning (AZP) has become a widespread problem throughout Europe,[8] with the potential to become a worldwide phenomena. Indeed, various analogs of azaspiracid have since been detected in waters off of western Europe, Morocco, and eastern Canada.[2] As a result, detection methods have been sought to ensure the safety of the seafood in which the azaspiracids are often found. Currently, the EU regulatory limit for azaspiracids is 0.16 µg/g of total shellfish tissue,[9] meaning a limit

of detection for a given method should be more less than that level, yet retain the ability to specifically test for all members of the azaspiracid family. The current methods for detecting and quantifying azaspiracids are mass spectrometry and mouse toxicity assays,[10] but these methods are less desirable due to the required amounts of azaspiracid needed as a reference, or the use of animals. Additionally, studies have suggested that the azaspiracid reference test has at best a 50% chance of detecting the toxin at the EU regulatory limit.[2]

Immunodiagnosics provide an alternative diagnostic platform that can be readily and cost effectively implemented in high-throughput screening scenarios. Recently, the Forsyth and Miles groups have reported the development of polyclonal sheep antibodies from a synthetic azaspiracid hapten consisting of the FGHI domain of the molecule that could recognize the entire azaspiracid molecule.[11] However, since these antibodies are polyclonal, a detection kit using solely this polyclonal sera is not ideal (vide infra). To address the needs of an azaspiracid detection method that would be general for all members of this class of marine toxins and that would not require authentic samples of the molecule as a standard, we set out to create monoclonal antibodies to the azaspiracid molecule with distinct recognition epitopes.

When designing an antibody-based capture assay (i.e., “sandwich” assay), two distinct antibody populations are required for detection. The first antibody is immobilized onto the solid support and serves to capture the desired analyte out of solution, while the second is conjugated to a suitable reporter enzyme (e.g., horseradish peroxidase or alkaline phosphatase) that allows for secondary signal amplification as a result of substrate turnover (Figure 2). It is desirable for the first antibody to be monoclonal as all bound antigen is uniformly displayed in a single orientation, maximizing the recognition and, consequently, the output signal that results from addition of the secondary detection antibody. The secondary antibody need not be monoclonal, but by using a monoclonal antibody can further enhance signal provided that the capture and detection antibodies have non-overlapping epitopes.

Small molecules such as the azaspiracids are inherently nonimmunogenic and therefore require covalent coupling to a carrier protein to elicit an immune response. In many cases, coupling of a small molecule to carrier proteins is achieved through the addition of a non-native functionality that can be chemically reacted with the protein side chains of the carrier (e.g., Lys residues). This requirement can be difficult to fulfill in the case of complex natural products, particularly when a semisynthetic route for the preparation of modified material is not readily apparent. Fortunately, examination of the azaspiracid structure reveals that no modification of the parent molecule is required for coupling as the terminal carboxylic acid can be exploited in standard NHS-based coupling procedures. Using this handle, we coupled synthetically prepared azaspiracid-1[6] to bovine serum albumin (BSA) and keyhole limpet hemocyanin (KLH) via lysine residues on the carrier protein surface; the former immunoconjugate was to be used in antibody screening procedures, while the latter was used to immunize mice for antibody production.[12]

Surprisingly, mice immunized with 1-KLH were found dead ~1 day after immunization. The toxic effects were reproducible, with four animals succumbing from immunization across two groups. This finding was particularly intriguing as, to our knowledge, there are no reported examples of potent toxicity resulting from administration of the immunoconjugate of a toxic molecule. Indeed, it is this exact premise that has formed the basis of many immunodiagnosics, and immunization has been previously utilized for the generation of antibodies against a wide range of potentially toxic molecules, with immunoassays for various pesticides providing an illustrative example.[13] This toxicity could not be attributed to trace amounts of free azaspiracid in the sample as all proteins were extensively dialyzed after hapten conjugation and prior to injection. Release of free hapten is unlikely as the azaspiracid molecule was coupled to the carrier protein via a stable amide bond linkage to reduce any nonspecific

hydrolysis from the carrier protein. Therefore, we questioned if the toxicity could be the result of the conjugated azaspiracid itself.

The lethal dose for azaspiracids varies based upon the specific molecule in question. A comparison study of the lethal dose of azaspiracid-1 to -5 has been performed by intraperitoneal injection[3,4] and this data indicated that azaspiracid-1, -2, and -3 are the most toxic with mouse lethal doses of 0.2 mg/kg, 0.11 mg/kg, and 0.14 mg/kg, respectively.[3b] Azaspiracid-4 and -5 have significantly less toxicity in mice (0.47 mg/kg and ~1.0 mg/kg, respectively). Interestingly, the lethal dose in mice for azaspiracid-1 is relatively independent of route of administration (intraperitoneal or oral).[14]

Given that the molecular weight of KLH ranges from 4.5×10^5 - 1.3×10^7 Da as a result of aggregation state, it is extremely difficult to determine the exact number of azaspiracid molecules present on this protein. However, the extent of immunoconjugate coupling is generally estimated by MS analysis of the corresponding BSA immunoconjugate, providing a lower limit for hapten copy number. In the case of **1**-BSA, it was determined that six azaspiracid moieties were coupled. Using this number, it was estimated that at least 1.2 µg of azaspiracid-1 was administered with each injection, albeit covalently conjugated to a carrier protein that by itself has no reported toxicity. It is likely that at least ten times this amount of azaspiracid was injected as KLH is known to display hundreds of surface lysine residues available for coupling. The lethal dose of azaspiracid-1 in mice is ~4 µg for a 20 g mouse, thus the quantity of toxin immobilized on KLH does significantly exceed the lethal dose. However, at this juncture, we cannot conclusively demonstrate the molecular basis of the toxicity of azaspiracid immunoconjugates and thus additional study will be required to explain this phenomenon.

Immunoconjugate toxicity is an unprecedented phenomenon and prevents a significant roadblock to the procurement of monoclonal antibodies. Additionally, azaspiracid has been shown to result in necrosis of B- and T-lymphocytes in lymphoid organs, including the spleen, thymus, and Peyer's patches,[14b] causing concern that the generation of high antibody titers would be difficult, if not impossible. We speculated that if the dose per injection could be reduced, titers against azaspiracid-1 could still be obtained; thus, we next turned to alternative immunization protocols. Empirically, we determined that a ten-fold reduction in the amount of **1**-KLH in each injection (10 µg immunoconjugate/mouse/injection) mixed with RIBI adjuvant system (RAS) could be tolerated by mice without pronounced toxicity; however, even though the pronounced mortality was abrogated with this lower dose, the animals continued to display signs of reaction to the conjugate (e.g., fur ruffling).

Gratifyingly, at this dose, appreciable titers could be observed at 21 days (1:1,600–1:3,200/mouse) by employing a standard regimen of initial immunization, followed by a single booster injection in RAS at day 14. While the titer obtained was not suitable for monoclonal antibody production, this data did validate our hypothesis that a dose reduction could result in appreciable antibody titers against **1**-KLH. In order to increase the titer, the mice were boosted again with 10 µg of **1**-KLH in a suspension of alum adjuvant at day 35, and then bled at day 42. At this stage, antibody titers had increased slightly (1:3,200-1:6,400/mouse). After an additional month, animals were again boosted with 10 µg of **1**-KLH in alum, at which point the titers had ceased to increase. Thus, spleens were harvested and used to generate antibody-secreting hybridomas.

Surprisingly, despite the low dose immunization protocol required by the toxicity of the immunoconjugate, a relatively large panel of 67 antibodies were obtained (Table 1). Roughly half of these antibodies showed good titer against the **1**-BSA immunoconjugate ($\geq 1:6,400$), demonstrating the overall specificity for the hapten across the entire panel. To determine the relative binding affinity of each antibody for azaspiracid molecules, competition ELISA

experiments were performed using synthetically prepared azaspiracid-1 and -2 as competing antigens. In general, most antibodies within the panel bound both molecules with high affinity ($K_{d,app} < 1 \mu\text{M}$) with the tightest binding observed for mAbs 6B11 and 9E8, both of which bound azaspiracid-1 with a relative binding affinity of $0.2 \mu\text{M}$ (Table 1). These two antibodies also displayed the best binding for azaspiracid-2, demonstrating that antibodies from this panel could recognize multiple members of the compound class (Table 1).

In order to select antibodies that could be used in an ELISA-based azaspiracid diagnostic, the relative epitopes of each antibody must be characterized so as to identify a pair that can bind simultaneously to the antigen. With most small molecules, this is an extremely difficult task as the sheer size of the antibody molecule inherently prevents a second antibody from binding due to steric hindrance. In the case of azaspiracid, we felt that the molecule was of sufficient size to have one antibody bind to the ABCDE ring system, while a second antibody could recognize the FGHI ring system. To test this hypothesis, the panel of monoclonal antibodies was screened for their ability to bind the FGHI fragment **12** of azaspiracid (Figure 3). This region was selected for study as it is conserved across all known members of the azaspiracid class of molecules and has previously been shown to be immunogenic,[11] suggesting that this could be a dominant epitope within the panel, and antibodies with this epitope could serve as capture reagents in a sandwich assay. Binding of a given monoclonal antibody to this fragment implies that the epitope of the antibody is at least partially contained within the FGHI ring system, whereas weak or no binding indicates the epitope must reside within the ABCDE domain. Screening of the ten antibodies that bound **1** or **2** the tightest showed that six antibodies possessed a FGHI epitope, while four others possessed an epitope that was comprised of some portion of the ABCDE domain (Table 1). Interestingly, the ability to select antibodies against multiple regions of this small molecule from a single immunization also argues that the azaspiracid molecular structure has sufficient in vivo stability to be recognized and processed by the immune system.

In total, the results of this study demonstrate the application of the total synthesis of complex and scarce natural products to the preparation of clinically relevant diagnostics, that would have been difficult to achieve otherwise. Despite the relatively small size of the azaspiracids, antibodies were generated that have non-overlapping epitopes, a primary requirement in the development of a sandwich ELISA-based detection system. Additionally, during the course of this study, the secondary finding that the **1**-KLH conjugate possesses potent toxicity represents an unprecedented result that is worthy of additional study, particularly as it is related to the mechanism behind this phenomenon. Nonetheless, by using the entire molecule as the hapten, antibodies could be generated from a single fusion against mutually exclusive epitopes, and thus, generate the requisite analytical reagents for the development of an optimized and efficient assay for a potent marine toxin.

Experimental Section

Immunoconjugate Preparation

Azaspiracid-1 (5 mg) was treated with 1.3 eq. 3-sulfo-*N*-hydroxysuccinimide and 1.3 eq. 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride for 24 h in DMF. This reaction was then added to solutions (5 mg/mL) of keyhole limpet hemocyanin (KLH) and bovine serum albumin (BSA) in PBS (50 mM, pH 7.4), respectively, and allowed to react 24 h at 4 °C. After reaction, unreacted azaspiracid was removed by dialysis (50,000 molecular weight cut-off filter) against PBS.

Murine Immunizations

A group of two mice (129GIX+, 8 weeks of age) were injected intraperitoneally with 10 µg of **1**-KLH in PBS which had been premixed with 100 µL of RIBI adjuvant system (RAS) (200 µL total volume). After two weeks, all mice received a booster injection identical to the initial immunization. Mice received two additional booster injections of 10 µg of **1**-KLH in PBS that had been premixed with 100 µL of alum (200 µL total volume) at days 35 and 70. One month later, a 10 µg injection of **1**-KLH was administered intravenously, and the animals were then sacrificed and the spleens removed. Hybridoma generation followed from this point as per literature procedures.[12]

Competition ELISA Experiments

An ELISA plate (CoStar; 96-well) was coated overnight at 4 °C with 125 ng **1**-BSA or **1**-KLH and then blocked with 50 µL of blocking buffer (4% skim milk powder in PBS) for 1 hour at 37 °C. Typically, 25 µL of a 1:1,000 dilution of the desired monoclonal antibody in blocking buffer containing serial dilutions of competing antigen was then added and incubated 1 hour at 37 °C. After washing, 25 µL of a 1:1,000 dilution of a goat-anti-mouse/horseradish peroxidase conjugate in blocking buffer was added and incubated for 1 hour. The plate was developed with the colorimetric reagent ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) for 20 minutes and the absorbance measured on an ELISA plate reader at 400 nm. The $K_{d,app}$ was defined as the concentration of competing antigen that resulted in one-half of the optical density measured in the absence of competing antigen.

Acknowledgments

We gratefully acknowledge D. Kubitz and the TSRI Antibody Production Core Facility for assistance in the preparation of monoclonal antibodies and Dr. S. De Lamo Marin for assistance with immunoconjugate preparation. Financial support for this work was provided by The Skaggs Institute for Chemical Biology, the National Institutes of Health (USA), and a predoctoral fellowship from the National Science Foundation (to M.O.F.).

References

1. McMahon T, Silke J. Harmful Algae News 1996;14:2.
2. a) Ofuji K, Satake M, McMahon T, James KJ, Naoki H, Oshima Y, Yasumoto T. Biosci Biotechnol Biochem 2001;65:740. [PubMed: 11330704] b) Ofuji K, Satake M, McMahon T, Silke J, James KJ, Naoki H, Oshima Y, Yasumoto T. Nat Toxins 1999;7:99. [PubMed: 10647511] c) Rehmann N, Hess P, Quilliam M. Rapid Comm Mass Spectrom 2008;22:549.
3. Twiner MJ, Rehmann N, Hess P, Doucette GJ. Mar Drugs 2008;6:39. [PubMed: 18728760]
4. Satake M, Ofuji K, Naoki H, James KJ, Furey A, McMahon T, Silke J, Yasumoto T. J Am Chem Soc 1998;120:9967.
5. a) Nicolaou KC, Pihko PM, Diedrichs N, Zou N, Bernal F. Angew Chem 2001;113:1302. Angew Chem Int Ed 2001;40:1262. b) Nicolaou KC, Qian W, Bernal F, Uesaka N, Pihko PM, Hinrichs J. Angew Chem 2001;113:4192. Angew Chem Int Ed 2001;40:4068. c) Nicolaou KC, Li Y, Uesaka N, Koftis TV, Vyskocil S, Ling T, Govindasamy M, Qian W, Bernal F, Chen DYK. Angew Chem 2003;115:3771. Angew Chem Int Ed 2003;42:3643. d) Nicolaou KC, Chen DYK, Li Y, Qian W, Ling T, Vyskocil S, Koftis TV, Govindasamy M, Uesaka N. Angew Chem 2003;115:3777. Angew Chem Int Ed 2003;42:3649.
6. a) Nicolaou KC, Vyskocil S, Koftis TV, Yamada YMA, Ling T, Chen DYK, Tang W, Petrovic G, Frederick MO, Li Y, Satake M. Angew Chem 2004;116:4412. Angew Chem Int Ed 2004;43:4312. b) Nicolaou KC, Koftis TV, Vyskocil S, Petrovic G, Ling T, Yamada YMA, Tang W, Frederick MO. Angew Chem 2004;116:4418. Angew Chem Int Ed 2004;43:4318. c) Nicolaou KC, Pihko PM, Bernal F, Frederick MO, Qian W, Uesaka N, Diedrichs N, Hinrichs J, Koftis TV, Loizidou E, Petrovic G, Rodriguez M, Sarlah D, Zou N. J Am Chem Soc 2006;128:2244. [PubMed: 16478178] d) Nicolaou KC, Chen DYK, Li Y, Uesaka N, Petrovic G, Koftis TV, Bernal F, Frederick MO, Govindasamy M, Ling T, Pihko PM, Tang W, Vyskocil S. J Am Chem Soc 2006;128:2258. [PubMed: 16478179] e)

- Nicolaou KC, Koftis TV, Vyskocil S, Petrovic G, Tang W, Frederick MO, Chen DYK, Li Y, Ling T, Yamada YMA. *J Am Chem Soc* 2006;128:2859. [PubMed: 16506764] f) Nicolaou KC, Frederick MO, Petrovic G, Cole KP, Loizidou E. *Angew Chem* 2006;118:2671. *Angew Chem Int Ed* 2006;45:2609. Nicolaou KC, Frederick MO, Loizidou EZ, Petrovic G, Cole KP, Koftis TV, Yamada YMA. *Chem Asian J* 2006;1:245. [PubMed: 17441061]
7. Vilariño, N. *Phycotoxins: Chemistry and Biochemistry*. Botana, LM., editor. Blackwell Publishing; Ames, Iowa: 2007. p. 311-318. b) James KJ, Saez MJF, Furey A, Lehane M. *Food Addit Contam* 2004;21:879. [PubMed: 15666982] c) McMahon T, Silke J. *Harmful Algae News* 1996;14:2.
8. James KJ, Furey A, Lehane M, Moroney C, Fernandez-Puente P, Satake M, Yasumoto T. *Food Addit Contaminants* 2002;19:555.
9. Lehane M, Saez MJF, Magdalena AB, Canas IR, Sierra MD, Hamilton B, Furey A, James KJ. *J Chromatogr A* 2004;1024:63. [PubMed: 14753707]
10. a) Hamilton B, Sierra MD, Lehane M, Furey A, James KJ. *Spectroscopy* (Amsterdam, Netherlands) 2004;18:355. b) James KJ, Sierra MD, Lehane M, Magdalena AB, Furey A. *Toxicon* 2003;41:277. [PubMed: 12565749] c) Moroney C, Lehane M, Magdalena AB, Furey A, James KJ. *J Chromatogr A* 2002;963:353. [PubMed: 12187990] d) Lehane M, Magdalena AB, Moroney C, Furey A, James KJ. *J Chromatogr A* 2002;950:139. [PubMed: 11990987] e) Furey A, Magdalena AB, Lehane M, Moroney C, James KJ, Satake M, Yasumoto T. *Rapid Commun Mass Spectrom* 2002;16:238. [PubMed: 11803546] f) Flanagan AF, Callanan KR, Donlon J, Palmer R, Forde A, Kane M. *Toxicon* 2001;39:1021. [PubMed: 11223091] g) Draisci R, Palleschi L, Ferretti E, Furey A, James KJ, Satake M, Yasumoto T. *J Chromatogr A* 2000;871:13. [PubMed: 10735281]
11. Forsyth CJ, Xu J, Nguyen ST, Samdal IA, Briggs LR, Rundberget T, Sandvik M, Miles CO. *J Am Chem Soc* 2006;128:15114. [PubMed: 17117862]
12. Kubitz, D.; Keinan, E. *Catalytic Antibodies*. Keinan, E., editor. Wiley-VCH; Weinheim: 2005. p. 491-504.
13. Kaufman BM, Clower M Jr. *J AOAC Int* 1995;78:1079. [PubMed: 7580321]
14. a) Ito E, Satake M, Ofuji K, Higashi M, Harigaya K, McMahon T, Yasumoto T. *Toxicon* 2002;40:193. [PubMed: 11689241] b) Ito E, Satake M, Ofuji K, Kurita N, McMahon T, James K, Yasumoto T. *Toxicon* 2000;38:917. [PubMed: 10728830]

Figure 1.
Structure of azaspiracids-1 through -11 (**1–11**).

Figure 2. Schematic depiction of antibody-based azaspiracid detection systems. A) Assay in which the capture antibody (blue) is monoclonal and, thus, all azaspiracid molecules are uniformly oriented so as to maximize the analytical signal. B) System in which the capture antibody (blue, green and purple) is polyclonal. In this system, multiple conformations of the molecule can be displayed to the detection antibody, compromising the output signal, particularly in cases where the capture and detection antibodies share a common epitope (purple antibody).

Figure 3.
A) Molecular structure of FGHI fragment **12** used in competition ELISA screening. B) Representative competition ELISA data using the FGHI fragment and mAb 8F4.

Table 1Ten tightest binding monoclonal antibodies procured from immunization of mice with **1**-KLH.

mAb	Isotype	$k_{d,app} 1[^a]$	$K_{d,app} 2[^a]$	$K_{d,app} \text{ FGH1 fragment 12 } [^a]$
2G2	$\kappa\gamma 2\alpha$	0.6 μM	0.6 μM	9.4 μM
2H3	$\kappa\gamma 2\alpha$	0.6 μM	0.6 μM	2.4 μM
2H6	$\kappa\gamma 2\alpha$	0.3 μM	0.8 μM	>50 μM
6B11	$\kappa\gamma 2\alpha$	0.2 μM	0.3 μM	18.8 μM
8F4	$\kappa\gamma 2\alpha$	0.6 μM	0.6 μM	2.4 μM
9A12	$\kappa\gamma 2\alpha$	0.6 μM	0.8 μM	>50 μM
9E8	$\kappa\gamma 2\alpha$	0.2 μM	0.6 μM	>50 μM
10G2	$\kappa\gamma 2\alpha$	0.3 μM	0.4 μM	50 μM
14H7	$\kappa\gamma 1$	0.6 μM	0.8 μM	4.7 μM
15D7	$\kappa\gamma 2\alpha$	0.6 μM	0.8 μM	>50 μM

^a $K_{d,app}$ determined by competition ELISA. In all cases, the ELISA plate was coated with **1**-BSA and the desired small molecule competing antigen was added at a range of concentrations from 50 nM to 50 μM . $K_{d,app}$ values are defined as the concentration of competing antigen that results in half-maximal OD₄₀₀.