

Assessment of Potential Cross-Reactivity of Human Endogenous Matrix Metalloproteinases with Collagenase *Clostridium histolyticum* Antibodies in Human Sera Obtained from Patients with Dupuytren's Contracture

Thomas J. Edkins,^a Roland Koller-Eichhorn,^b Jack A. Alhadeff,^c Ulrich Mayer,^d Heinrich Faust,^d and Benjamin J. Del Tito^e

Edkins Pharma Services, LLC, Wayne, Pennsylvania, USA^a; Koller & Koller LLC, Zurich, Switzerland^b; Protein Therapeutics Consulting, Center Valley, Pennsylvania, USA^c; Celerion Switzerland AG, Fehraltorf, Switzerland^d; and Auxilium Pharmaceuticals, Inc., Malvern, Pennsylvania, USA^e

Collagenase *Clostridium histolyticum* (CCH) contains a fixed ratio of class I (AUX-I) and class II (AUX-II) collagenases and is used as treatment for Dupuytren's contracture. These two Zn-dependent enzymes, produced by the Gram-positive bacterium *Clostridium histolyticum*, are related functionally to matrix metalloproteinases (MMPs) which, among other functions, degrade the extracellular matrix. Since AUX-I and AUX-II exhibit sequence similarities to human MMPs, we assessed MMP-1 (interstitial collagenase), MMP-2 (gelatinase A), MMP-3 (stromelysin 1), MMP-8 (collagenase 2), and MMP-13 (collagenase 3) for cross-reactivity with anti-AUX-I and anti-AUX-II antibodies in patient serum. Serum samples from 71 subjects enrolled in a long-term clinical study (58 males and 13 females; 63 ± 10 years old [mean $\pm$ standard error]) were evaluated for cross-reactivity with the five MMPs using the two validated enzyme-linked immunosorbent assays (ELISAs). Inhibition cutoff points for anti-AUX-I and anti-AUX-II antibodies were based on assay inhibition obtained with a nonspecific protein, bovine gamma globulin, which was tested for each clinical sample. No MMP cross-reactivity was found for any of the 71 clinical antibody-positive sera evaluated. Sequence identity assessments indicated minimal, nonmeaningful alignments of the MMPs and AUX-I/AUX-II. Furthermore, clinical adverse event assessments indicated no safety signals related to MMP inhibition. The bioanalytical results, sequence identity, and clinical assessments consistently did not demonstrate cross-reactivity between CCH antidrug antibodies and endogenous human matrix metalloproteinases. The results presented here suggest that treatment of Dupuytren's contracture patients with CCH does not lead to any clinical adverse events associated with MMP inhibition.

Many protein drugs have been developed, or are being developed, to treat a large number of human diseases including lysosomal storage disorders, cystic fibrosis, AIDS, severe combined immunodeficiency disease (SCID), various anemias, and cancer. These protein drugs can be isolated/purified from bacteria, animal, or plant tissues or are recombinant DNA products. It is estimated that 90 billion dollars were spent in 2008 for protein drugs (12), including hormones, growth factors, monoclonal antibodies, and enzymes. A potential problem with the use of protein drugs is the formation of antidrug antibodies (ADAs), which could interfere with drug efficacy. In addition, these ADAs could pose safety concerns if they were to bind to endogenous proteins and interfere with their functions. One such enzyme drug is collagenase *Clostridium histolyticum* (CCH), which is comprised of two collagenases from the bacterium *Clostridium histolyticum*.

CCH is used for treatment of Dupuytren's contracture in adult patients with a palpable cord (11). Dupuytren's disease is a slowly progressive fibroproliferative disease of the palmar fascia in the hand that is believed to be one of the most common hereditary connective tissue disorders in Caucasians (2). The abnormal collagen deposition, which results in nodule and cord formation, may eventually limit hand movement by causing the affected finger(s) to bend or flex (contract) toward the palm of the hand, with loss of the ability to straighten (extend) the finger(s). When the fibrous cord retracts the finger toward the palm in the advanced stage of the disease, the resulting pathology is known as Dupuytren's contracture (20). The decreased function resulting from contracture translates into difficulties with daily activities (e.g.,

combing hair), employment, and hobbies. Historically, treatment options for Dupuytren's contracture were limited to surgical procedures, such as open fasciectomy (7), open fasciotomy (18), or needle fasciotomy (10). CCH is the first approved nonsurgical treatment option for patients with this disease (9, 23). A dose of 0.58 mg is administered from a single vial, injected intralesionally into the cord. Each cord can receive one injection at 4-week intervals up to a maximum of three injections. The collagenases lyse collagen, disrupting the contracted Dupuytren's cord (21).

CCH is a fixed combination of two enzymes from different classes that act synergistically. They are encoded by two different genes: *colG* for class I (AUX-I) and *colH* for class II (AUX-II) (15). The two enzymes have different substrate specificities. Class I hydrolyzes collagen at the carboxy and amino termini, while class II hydrolyzes the interior of collagen (17). The substrate specificities of the two classes complement each other to provide more effective degradation of collagen. Each enzyme gives rise to a distinct and high-level antibody response with a long duration, which is expected when administering this bacterial protein to humans.

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Address correspondence to Benjamin J. Del Tito, bdelcito@auxilium.com.

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Matrix metalloproteinases (MMPs) are Zn-dependent endoproteases that are similar in function to the two collagenases in CCH. MMPs are involved in a number of important physiological processes, including cell proliferation, migration and differentiation, and tissue remodeling (3, 4). The MMPs comprise a group of at least two dozen soluble and membrane-bound enzymes, including interstitial collagenase (MMP-1), gelatinase A (MMP-2), stromelysin (MMP-3), collagenase 2 (MMP-8), and collagenase 3 (MMP-13).

MMP-1, -2, -3, -8, and -13 are of particular interest, since they exhibit some sequence similarity over a range of 30 to 150 amino acids to AUX-I and AUX-II, respectively, the two enzymes that make up CCH. Even though sequence identity is relatively low, *in silico* analysis can only provide limited evidence for the lack of potential cross-reactivity, and additional *in vitro* safety assessments are needed to thoroughly assess potential safety effects (13). Thus, two competitive enzyme-linked immunosorbent assays (ELISAs), one for antibodies to each enzyme in CCH (anti-AUX-I and anti-AUX-II), were developed and validated. The two methods were used to measure potential cross-reactivity of the five MMP enzymes of interest with AUX-I and AUX-II human antibodies (i.e., anti-CCH antibodies). The ELISA methods were used to evaluate serum samples from 71 subjects from a long-term safety study. All results showed no cross-reactivity for any of the five MMPs evaluated.

The sequence identity does not extend to domains or subdomains of AUX-I and AUX-II compared to the MMPs. Similarly, clinical safety assessments indicate no treatment-emergent adverse events related to inhibition of human MMPs, including musculoskeletal events, such as polyarthritides, osteolysis and shoulder girdle pain, or reduction in range of motion.

The results are presented in the context of an overall risk-based approach to potential MMP cross-reactivity with antibodies to CCH (6), where a lack of any clinically significant impact is demonstrated from the substantial antibody response to administration of these bacterium-derived therapeutic enzymes. These data suggest that the risk of *in vivo* cross-reactivity is minimal or non-existent.

MATERIALS AND METHODS

MMP competitive ELISA procedure and reagents. AUX-I (class I collagenase) and AUX-II (class II collagenase) were manufactured by Auxilium Pharmaceuticals, Inc. (Horsham, PA). Both proteins were biotinylated at Celerion (Fehraltorf, Switzerland; formerly MDS Pharma Services). Rabbit polyclonal antibodies were produced and affinity purified by Squarix GmbH Biotechnology (Marl, Germany). Recombinant human MMPs (MMP-1^{F20-N469}, MMP-2^{L34-C660}, MMP-3^{Y18-C477, K45E}, MMP-8^{F21-G467}, and MMP-13^{L20-C471}) were obtained from R&D Systems (United Kingdom), and bovine gamma globulin (BGG) was purchased from Sigma-Aldrich (Switzerland). Streptavidin-poly-horseradish peroxidase conjugate was obtained from Fitzgerald Industries (Acton, MA).

In order to assess potential cross-reactivities of ADAs with endogenous MMPs, two competitive bridging ELISAs were developed and validated. Diluted antibody-positive study serum samples (anti-AUX-I or anti-AUX-II) were analyzed by bridging ELISAs using AUX-I or AUX-II as a capture molecule, a biotinylated analyte-specific detection conjugate, and a streptavidin-peroxidase conjugate as tracer, which was detected by using 3,3',5,5'-tetramethylbenzidine (TMB). For each sample, the inhibitory effects of free human MMPs (MMP-1, MMP-2, MMP-3, MMP-8, and MMP-13) on antibody determination were tested. Free antigen (AUX-I or AUX-II) was used as a positive control for a true antibody interaction, and BGG was applied as a negative control for nonspecific

interactions. Reference values for each sample were established by control experiments with buffer alone.

All samples were assayed in duplicate. Noncompetitive bridging ELISA reactions were evaluated by anti-AUX-I or anti-AUX-II positive-control (PC) samples (10 ng/ml and 25 ng/ml of affinity-purified anti-AUX-I and anti-AUX-II antibodies, respectively) and blank serum negative-control samples (NC). In addition, low-positive-control samples were applied (4 ng/ml and 10 ng/ml of affinity-purified anti-AUX-I and anti-AUX-II antibodies, respectively) to assess assay performance.

Maxisorp ELISA plates were coated with 100 μ l/well coating solution (0.25 μ g/ml AUX-I in 0.2 M carbonate buffer [pH 9.4] or 0.25 μ g/ml AUX-II in 0.2 M carbonate buffer [pH 9.4]). Plates were incubated overnight at 4°C, washed with 1 $\times$ phosphate-buffered saline (PBS; pH 7.4), 0.1% Kathon, 0.05% Tween 20 and blocked with 1 $\times$ PBS (pH 7.4), 0.1% Kathon, 1% bovine serum albumin (BSA), 0.2% Tween 20 (dilution buffer) for 1 h at 24°C.

To ensure that competition experiments were performed with comparable antibody titers, all study samples were diluted such that an optical density (OD) value of 1.0 ± 0.5 (target OD $\pm$ allowed tolerance) was obtained for noninhibited control experiments. The applied antibody titer was approximately two times the titer of the PC sample (Tables 1 and 2) and ensured that the antibody signal was effectively blocked by an antibody-binding agent.

In order to obtain the targeted OD response, two dilution steps were required: first, study samples were individually diluted in blank matrix to achieve a comparable antibody response for each study sample, and second, all samples were diluted 1:10 in dilution buffer in order to optimize the signal-to-noise ratio. The final sample dilution (predilution in matrix and subsequent 1:10 dilution with dilution buffer) that was required for each sample was determined in a preliminary experiment in which a dilution series was applied to each sample. The required dilution was then calculated as follows: $\text{dilution} = DF_1 + \{[(OD_1 - 1.0)/(OD_1 - OD_2)] \times (DF_2 - DF_1)\}$, where DF_1 and DF_2 define the dilution factors of the last dilution resulting in an OD value of >1.0 and the first dilution resulting in an OD value of ≤ 1.0 , respectively, and OD_1 and OD_2 represent the corresponding OD values obtained at DF_1 and DF_2 .

For subsequent cross-reactivity assessments, the final 1:10 matrix dilution step was evaluated with dilution buffer (noninhibited reference experiment) and with dilution buffer that was supplemented with the corresponding competition reagent (MMP-1, -2, -3, -8, or -13, AUX-I, AUX-II, or BGG). The concentrations of the competition reagents in the dilution buffer were 1.0 μ g/ml for the initial cross-reactivity screening and 0.01, 1.0, and 5.0 μ g/ml for the confirmatory assay. Thus, the applied concentrations within the experimental setup were 0.9 μ g/ml for cross-reactivity screening and 0.009, 0.9, and 4.5 μ g/ml for the confirmatory assay. The total dilution that was applied for cross-reactivity assessment ranged from 1:10 to 1:100,000 for anti-AUX-I-positive samples and from 1:10 to 1:20,000 for anti-AUX-II-positive samples.

After preincubation of study samples with MMP competition reagents for 90 min at room temperature, samples were transferred to preblocked microtiter plates and incubated for 1 h at 24°C. After washing, antigen-biotin conjugate was added to each well, and microtiter plates were incubated for 1 h at 24°C. Streptavidin-poly-horseradish peroxidase conjugate was used at 1:10,000 in dilution buffer. Horseradish peroxidase activity was detected by using 3,3',5,5'-tetramethylbenzidine and stopped by addition of 1 M H₂SO₄. The optical density was determined at 450 nm.

Figure 1 shows a schematic of the competitive ELISA method for anti-AUX-I serum samples.

In summary, the competition experiments were conducted between the five MMPs and AUX-I or AUX-II. When antibodies are present in clinical serum samples as a result of administering CCH (AUX-I and AUX-II), they may cross-react with the MMPs and cause inhibition of binding of the AUX-I- or AUX-II-biotin conjugate. Validation results of the two assays are presented in Table 1 and Table 2. Characteristics assessed included the minimum required sample dilution factor, interrun

TABLE 1 Validation parameters of the MMP competition assay for anti-AUX-I^a

Validation parameter ^b	Description/validation value
Analyte	Anti-AUX-I antibodies
Matrix	Human serum
Assay method	ELISA
Reference substance	Affinity-purified anti-AUX-I antibody (eluate A)
Minimum required dilution	1:10 in dilution buffer
Dilution linearity	Not applicable; semiquantitative assay
Stability ^c	
Freeze-thaw	10 cycles (−80°C/room temp)
Short term	3 h at room temp, overnight at 5°C
Long term	26 mos at −80°C
Precision of anti-AUX-I positive-control samples [*]	
Intra-assay	% CV ≤ 8.4
Interassay	% CV ≤ 15.0
Specificity [*]	No relevant cross-reactivity observed with AUX-II
Antidrug antibody detection	
Antibody detection cutoff point	Run specific; (mean OD of NC) + (0.066 × mean OD of PC)
Interrun precision and accuracy [mean OD (% CV)] (<i>n</i> = 22)	
OD of NC	0.264 (1.2)
OD of QC ^c	0.421 (7.7)
OD of PC	0.609 (7.1)
Cutoff point for anti-AUX-I-positive samples	0.304 (8.8)
Cross-reactivity assessment	
Competition specificity cutoff point	Mean inhibition with BGG + (3.09 × SD)
Competition effect of reagent at 0.9 vs 0.009 μg/ml [mean % inhibition (SD)]	
AUX-I	118.1 (7.6) vs 96.2 (8.7)
MMP-1	−3.7 (3.4) vs −1.4 (4.1)
MMP-2	1.4 (4.0) vs 2.5 (3.5)
MMP-3	2.4 (3.6) vs 2.7 (9.9)
MMP-8	0.4 (3.9) vs 0.6 (4.7)
MMP-13	2.0 (4.7) vs 4.6 (2.3)
BGG	0.9 (6.0) vs N/A ^d

^a The validation parameters for anti-AUX-I determination and binding competition analysis are provided. A run-specific antibody detection cutoff point was established to discriminate between anti-AUX-I-positive and anti-AUX-I-negative samples and to assess run validity. Overall variability of assay competition obtained with the unrelated control protein (BGG) was applied to determine a specificity cutoff point for cross-reactivity assessment.

^b Results marked with an asterisk were obtained from the validated bridging ELISA method for immunogenicity titer determination.

^c QC, quality control.

^d N/A, not applicable. Control experiments with BGG were performed only at 0.9 μg/ml.

precision and accuracy, mean cutoff value (in OD units), and the competition level of AUX-I or AUX-II with the MMPs and/or BGG as irrelevant protein (i.e., negative control).

MMP competitive ELISA method calculations. For the calculation of reagent-specific inhibition, the background signal of the blank serum (NC) was taken into account in order to adjust for interrune variability. Specific inhibition with all reagents was calculated for each study sample as follows: % inhibition = [(OD_{buffer control} − OD_{competition reagent}) / (OD_{buffer control} − OD_{blank serum})] × 100.

For cross-reactivity assessment, a specificity cutoff point was determined that reflected the assay variability of the specific sample set. Therefore, variability of the competition assay was determined with an unrelated control protein (BGG) that was applied to each study sample, and a statistical cutoff value for ADA cross-reactivity was calculated. After exclusion of outliers, the specificity cutoff value was calculated as follows: cutoff_{specific inhibition} = (mean inhibition_{BGG}) + (3.09 × SD_{inhibition BGG}). Outliers were defined as values lower than the lower quartile minus 1.5 times the interquartile range or higher than the upper quartile plus 1.5 times the interquartile range.

If an MMP-dependent inhibition level above the cutoff was obtained, antibodies were defined as potentially cross-reacting with the competition reagent.

All samples that were considered to be potentially cross-reactive were evaluated in a confirmatory assay in order to confirm specific ADA cross-

reactivity with human MMPs and to identify false-positive results. Therefore, the corresponding study samples were supplemented with three different MMP concentrations to test for dose-dependent signal inhibition. A study sample was considered confirmed as cross-reacting with a given MMP if the inhibition level was above the cutoff point as confirmed for the 1.0-μg/ml dose (percent inhibition_{1.0 μg/ml MMP} > cutoff_{specific inhibition}) and a dose-dependent inhibition was observed within the confirmation assay (percent inhibition_{5.0 μg/ml MMP} > percent inhibition_{1.0 μg/ml MMP} > percent inhibition_{0.01 μg/ml MMP}).

Sequence similarity assessments. Sequence similarities were assessed for AUX-I and AUX-II with the corresponding MMPs by using a pairwise sequence alignment algorithm. The applied program (LALIGN; www.ebi.ac.uk/Tools/psa/lalign/ [accessed 11 July 2011]) calculated the best non-intersecting alignments between two sequences. Pairwise sequence alignment was performed using the default settings of the alignment algorithm (matrix, BLOSUM50; gap open, −12; gap extend, −2; expectation value threshold, 10.0). The sequence identity/similarity data represent the values of the sequence alignment with the highest score for each pair of proteins.

Validation summary of MMP competitive ELISA methods. Table 1 and Table 2 present the validation summaries for the competitive ELISA methods for anti-AUX-I and anti-AUX-II antibodies, respectively. Interrun precision was assessed in 22 analytical runs. The coefficient of variation (CV) of the negative control (pooled human serum), positive control

TABLE 2 Validation parameters of the MMP competition assay for anti-AUX-II^a

Validation parameter ^b	Description/validation value
Analyte	Anti-AUX-II antibodies
Matrix	Human serum
Assay method	ELISA
Reference substance	Affinity-purified anti-AUX-II antibody (eluate A)
Minimum required dilution	1:10 in dilution buffer
Dilution linearity	Not applicable; semiquantitative assay
Stability ^c	
Freeze-thaw	10 cycles (−80°C/room temp)
Short term	3 h at room temp, overnight at 5°C
Long term	26 mos at −80°C
Precision of anti-AUX-II positive-control samples [*]	
Intra-assay	% CV ≤ 12.6
Interassay	% CV ≤ 21.9
Specificity [*]	No relevant cross-reactivity observed with AUX-I
Antidrug antibody detection	
Antibody detection cutoff point	Run specific; (mean OD of NC) + (0.078 × mean OD of PC)
Interrun precision and accuracy [mean OD (% CV)] (<i>n</i> = 22)	
OD of NC	0.209 (2.9)
OD of QC ^c	0.375 (8.0)
OD of PC	0.607 (12.8)
Mean cutoff point for anti-AUX-II-positive samples	0.256 (16.3)
Cross-reactivity assessment	
Competition specificity cutoff point	Mean inhibition with BGG + (3.09 × SD)
Competition effect of reagent at 0.9 vs 0.009 μg/ml [mean % inhibition (SD)]	
AUX-II	111.3 (4.2) vs 94.8 (3.1)
MMP-1	−1.1 (6.7) vs −1.8 (6.7)
MMP-2	−0.3 (4.6) vs −0.3 (5.5)
MMP-3	1.4 (5.2) vs 1.2 (5.2)
MMP-8	1.2 (5.5) vs 2.1 (6.2)
MMP-13	2.0 (5.8) vs 1.3 (6.6)
BGG	4.0 (6.8) vs N/A ^d

^a The validation parameters for anti-AUX-II determination and binding competition analysis are provided. A run-specific antibody detection cutoff point was established to discriminate between anti-AUX-II-positive and anti-AUX-II-negative samples and to assess run validity. Overall variability of assay competition obtained with the unrelated control protein (BGG) was applied to determine a specificity cutoff point for cross-reactivity assessment.

^b Results marked with an asterisk were obtained from the validated bridging ELISA method for immunogenicity titer determination.

^c QC, quality control.

^d N/A, not applicable. Control experiments with BGG were performed only at 0.9 μg/ml.

(polyclonal rabbit antibody diluted in human serum), quality control (polyclonal rabbit antibody further diluted in human serum), and OD cutoff for antibody determination ranged from 1 to 9% for anti-AUX-I and from 3 to 16% for anti-AUX-II. The competition effect for AUX-I and AUX-II was ~95% at 0.09 μg/ml and ~115% at 9 μg/ml in each assay, while the competition effect for each MMP was <5% in all cases.

Included in the tables for reference are intra- and interassay precision results for the positive control during validation of the bridging ELISAs used to determine anti-AUX-I and -II titers: 8 and 15% CV, respectively, for anti-AUX-I, and 13 and 22% CV, respectively, for anti-AUX-II. Cutoff points for the two bridging ELISAs were determined per current standard practices (8, 16, 24). Short-term, long-term, and freeze-thaw stability results and cross-reactivity results for each antibody are also shown.

Clinical study summary and sample selection process. Once the two competitive ELISA methods were validated, as summarized in Tables 1 and 2, clinical serum samples from 71 subjects enrolled in a 5-year follow-up study were analyzed to assess potential cross-reactivities. Serum samples were selected that were positive for either or both antibodies in the anti-AUX-I and anti-AUX-II immunogenicity bridging assays, covering a wide range of titers from approximately 100 to 500,000. All samples selected were from only the second-year draw (of a 5-year study).

The following criteria were applied for sample selection: (i) subjects had a positive neutralizing antibody result (anti-AUX-I and/or anti-AUX-II) from an earlier time point and received between 1 and 8 injections of

CCH; (ii) subjects were enrolled in the long-term follow-up study, and a year 2 serum sample was available; (iii) the sample vials remained frozen until analyzed and were not subjected to any freeze-thaw cycles; (iv) the serum samples had antibody titers sufficient to give an OD reading of >0.5 in the competitive ELISA.

RESULTS

A total of 71 serum samples from year 2 of a 5-year clinical study were evaluated for cross-reactivity of either anti-AUX-I or anti-AUX-II antibodies with the five MMPs of interest. Thirty samples were tested initially, while the remaining 41 were tested on a later occasion. Discussion and analysis of each set of results are provided separately below.

Initial MMP competitive ELISA results for year 2 clinical study samples (long-term safety follow-up). Initially, 30 clinical samples were tested for anti-AUX-I and anti-AUX-II (30 samples in total) antibody cross-reactivity with MMP-1, -2, -3, -8, and -13. The specificity cutoff points for the competition assay were determined to be 24.1% and 24.4% for anti-AUX-I and anti-AUX-II, respectively (Table 3). No MMP-dependent inhibition was seen at or above the cutoff point: the maximum percent inhibition detected for any MMP applied at 0.9 μg/ml was 7% for both anti-

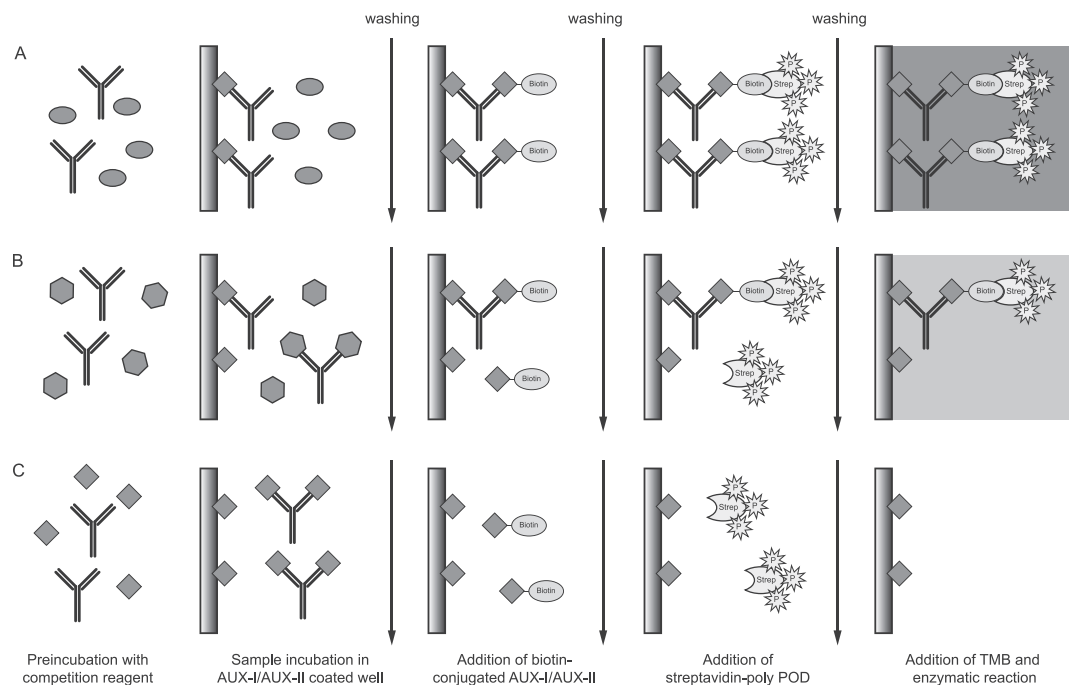


FIG 1 A competitive bridging ELISA format was applied to test cross-reactivity of anti-AUX-I- and anti-AUX-II-positive human serum samples with human MMPs. (A) Schematic illustration of lack of cross-reactivity; (B) partial cross-reactivity; (C) strong cross-reactivity. Samples were preincubated with competition reagent before they were applied to AUX-I- or AUX-II-coated wells. Biotinylated AUX-I/AUX-II was applied as a detection reagent, which was visualized with streptavidin–poly-horseradish peroxidase (P) and then addition of TMB. The washing steps are indicated by arrows.

AUX-I and anti-AUX-II. Assay results are summarized in Fig. 2A and B, for anti-AUX-I and anti-AUX-II, respectively. For comparison, the maximum inhibition seen for the nonspecific negative-control reagent BGG was 8.6% and 11.0% (for anti-AUX-I and anti-AUX-II, respectively), while the mean inhibition obtained by addition of AUX-I and AUX-II during analysis was positive, ranging from 94.8 to 118%. Furthermore, there was a direct concentration response when the antigen was applied as a competition reagent, but no concentration response was observed with MMPs. Finally, the PC sample (polyclonal rabbit anti-AUX-I and anti-AUX-II) used for anti-AUX-I and anti-AUX-II immunogenicity

testing showed no, or virtually no, inhibition when used in testing with the competitive ELISA methods here. Since no cross-reactivity was observed for any of the 5 MMPs evaluated with the initial 30 samples assayed, no further confirmatory analysis was required.

Final MMP competitive ELISA results for additional 2-year follow-up clinical samples. Subsequently, 41 additional serum samples from subjects enrolled in the long-term clinical study were selected for either an AUX-I or AUX-II competition experiment with the validated ELISAs. For this additional analysis, the competition cutoff point was determined to be 22.1% and 20.0% inhibition for anti-AUX-I and anti-AUX-II, respectively (Table 3).

For MMP competition experiments, no inhibition at or above the cutoff point was observed for anti-AUX-I analysis. MMP inhibition ranged from –23.1% to 19.4% (Fig. 2C), while strong inhibition with a direct but nonlinear dose dependency was observed for all 21 samples when AUX-I was assayed at the two concentrations of 0.9 and 0.009 $\mu\text{g/ml}$, respectively. Since no result for any MMP in any serum sample was above the cutoff point of 22.1%, the confirmation step was not run, as per the requirements described above for determining the cutoff percent specific inhibition based on the inhibition results ($+3.09 \times \text{SD}$) for the BGG control.

For anti-AUX-II positive clinical samples, the competition cutoff point was 20.0% inhibition (Table 3). In contrast to results from the anti-AUX-I analysis, 4 serum samples collectively showed 7 potential positive MMP inhibition results for MMP-2, -3, -8, and/or -13. All of these results were slightly above the cutoff point of 20.0%, ranging from 20.5 to 21.9% inhibition (Fig. 2D).

In order to discriminate between false-positive competition

TABLE 3 Specificity cutoff point determination for competition and confirmation analyses			
Antibody and step	<i>n</i>	Mean % inhibition (SD)	Cutoff point (%)
Anti-AUX-I			
Competition step, 1st analysis	15 ^a	1.5 (7.3)	24.1
Confirmation step, 1st analysis	NA ^b		
Competition step, 2nd analysis	21	2.7 (6.3)	22.1
Confirmation step, 2nd analysis	NA		
Anti-AUX-II			
Competition step, 1st analysis	15 ^a	6.0 (5.9)	24.4
Confirmation step, 1st analysis	NA		
Competition step, 2nd analysis	20	1.2 (6.1)	20.0
Confirmation step, 2nd analysis	41	1.9 (6.7)	22.7

^a Three BGG replicates (negative control) were performed for each study sample.
^b NA, not applicable; no confirmation testing was performed because all samples were below the cutoff point.

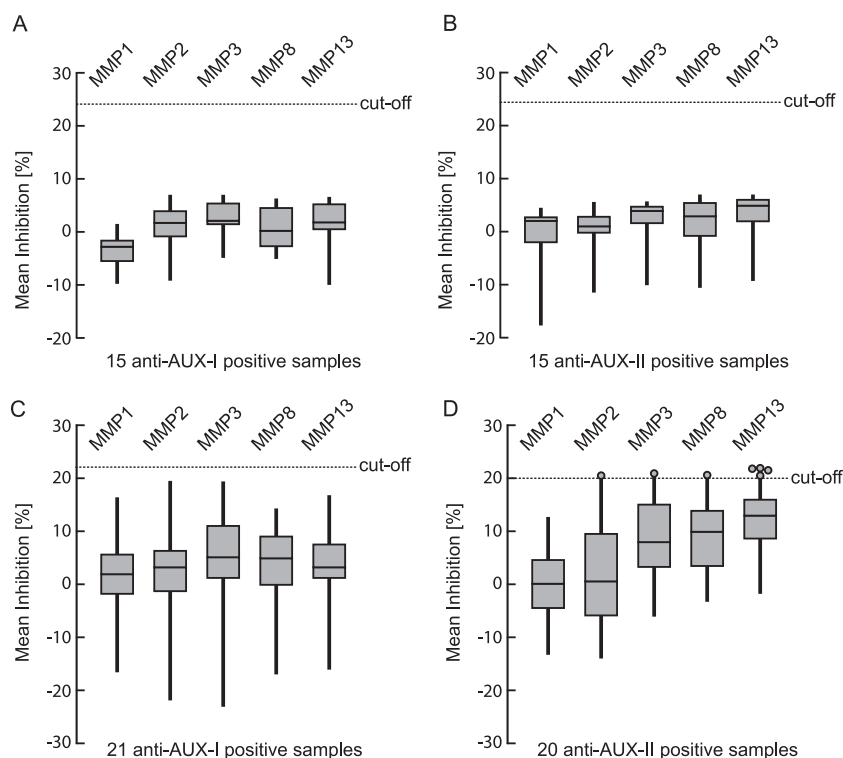


FIG 2 MMP competition assays performed for anti-AUX-I and anti-AUX-II. MMPs were applied at a concentration of 0.9 $\mu\text{g/ml}$ to ADA-positive clinical samples. Competition assays were performed on two occasions. Initially, 15 samples were analyzed both for anti-AUX-I (A) and anti-AUX-II (B). Additionally, 21 and 20 clinical samples were tested for anti-AUX-I (C) and anti-AUX-II (D), respectively. Dotted lines represent the specificity cutoff point for each analysis. Samples with an inhibition level above the cutoff point (gray circles) were further analyzed in a confirmatory assay.

results and true antibody cross-reactivity, all 7 presumptive cross-reactive samples were analyzed in a confirmatory assay in which the inhibitory effect was tested for dose dependency. Three additional replicates of BGG were assayed per competition experiment to increase the statistical power of the inhibition specificity cutoff point for the confirmatory assay. The cutoff point was calculated to be 22.7% for the confirmatory analysis (Table 3).

Figure 3 graphically displays the results of the confirmatory assay that was performed for the 7 potential cross-reactive anti-AUX-II samples. All 7 potential positive samples assayed in the

competition step were determined to be negative, with no sample showing greater than 18.5% inhibition (Fig. 3, subject FF, MMP-13, 4.5 $\mu\text{g/ml}$).

For one cross-reactivity confirmation experiment, a high negative inhibition was obtained (Fig. 3, subject HH, MMP-13, 4.5 $\mu\text{g/ml}$). This result was caused by a slightly higher signal in the presence of the competition reagent than that seen in the noncompetitive reference experiment. However, this difference only displays assay variability for ADA detection and is otherwise meaningless. Signal inhibition values varied in a similar way in the initial screening, where comparable inhibition values were occasionally observed.

Note that there was no dose dependency for subject FF. The increases in concentrations of MMP-13 from left to right in the bar graphs (0.009, 0.9, and 4.5 $\mu\text{g/ml}$) did not result in a corresponding increase in the percent inhibition. In fact, no dose dependency was found for the MMP confirmation step for any samples evaluated.

In contrast, strong dose-dependent inhibition was observed for the positive-control AUX-II for all 7 presumptive positive MMP results (Fig. 4). All results approached 100% inhibition for the higher AUX-II concentration.

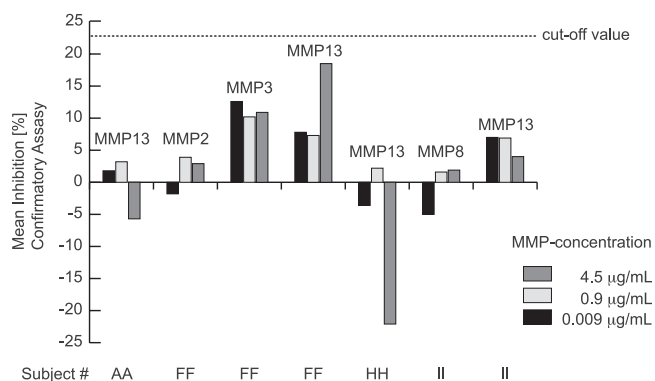


FIG 3 Summary of the competition assay for anti-AUX-II: effects of MMPs (confirmation step). Samples from subjects were assessed at MMP concentrations of 0.009, 0.9, and 4.5 $\mu\text{g/ml}$. The dotted line represents the cutoff point of 22.7%. No inhibition above the cutoff point was observed.

DISCUSSION

MMP competitive ELISA cutoff point determination and assay results. To our knowledge, there are currently no recommendations for the implementation of a specificity cutoff point for a competition bridging ELISA for determination of ADA cross-re-

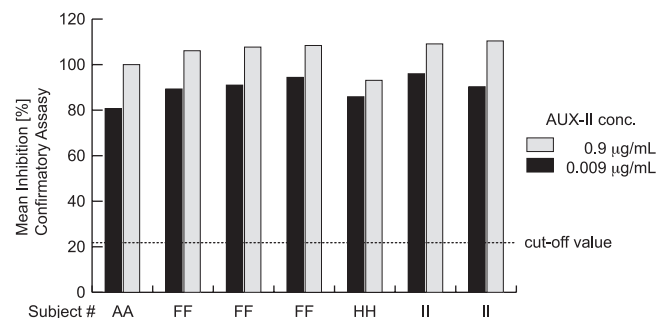


FIG 4 Summary of the competition assay for anti-AUX-II: effects of AUX-II (confirmation step). AUX-II was added at concentrations of 0.009 and 0.9 µg/ml per subject sample. The dotted line represents the cutoff point of 22.7%. Strong inhibition was observed with addition of AUX-II.

activity. Since ADA-positive samples are utilized for cross-reactivity assessments, the evaluation of drug-naïve samples for the determination of a specificity cutoff point, as suggested by Shankar et al. (19), could not be performed. Also, there was no clear evidence that assay variability was independent of the instrumental response and the presence of ADAs. In order to avoid an arbitrary fixed inhibition criterion, we decided to evaluate assay variability over a predefined OD range by using BGG as a nonspecific inhibition reagent. A study-specific statistically determined inhibition cutoff point (based on results with BGG, specifically the mean inhibition with the BGG control plus 3.09 times the SD) was then calculated and applied to determine antibody cross-reactivity. The required dilution was determined in preliminary experiments for each study sample.

For cross-reactivity assessment, MMPs were applied at concentrations that were 1 to 2 orders of magnitude higher than the endogenous MMP serum concentrations (22). For the applied sample dilution, the molecular ratio of MMP to IgG was estimated to be 2,500:1 and 1,000:1 for anti-AUX-I and anti-AUX-II samples, respectively. This high excess of competition reagent ensured that even cross-reactive antibodies that showed only low affinity for an MMP could be effectively identified. Full-length proforms of the five recombinant human MMPs were used, since enzymatic activity is not required for the ELISA competition studies. To our knowledge, full-length, catalytically active human MMPs are not commercially available. The full-length proforms are preferable to the much smaller catalytically active domains (which are available), as they contain all epitopes, including those in the propeptides, which potentially could cross-react with the anti-AUX-I and anti-AUX-II antibodies.

Five MMP enzyme activity inhibition methods were concurrently validated to further assess cross-reactivity in functional assays, as recommended by regulatory guidance documents (8, 24). Since all results for all MMPs in the AUX-I and AUX-II competition ELISAs were determined to be negative, none of the 71 clinical serum samples was evaluated using any of these enzyme activity inhibition assays.

Sequence similarity assessments. Similarity analysis using the LALIGN sequence alignment program available on the website www.ebi.ac.uk (accessed 11 July 2011) revealed only limited identity/similarity between the two clostridial collagenases ColG (AUX-I) and ColH (AUX-II) and human MMPs MMP-1, -2, -3, -8, and -13. Similarity was restricted to just two to five contiguous

amino acids separated by gaps of various lengths within sequences of approximately 30 to 150 amino acids. The longest stretch of consecutive identical amino acids found in the alignment analysis was only 4 amino acids long. These short sequences of identity would probably result if AUX-I and AUX-II, which are large proteins of 113 and 112 kDa, respectively, were compared to any random protein, since the secondary structure of most proteins contains α -helices, β -pleated sheets, and β -turns that are enriched in specific amino acids and specific short sequences. Therefore, short stretches (1 to 5 amino acids) of identity/similarity are usually not meaningful. However, cross-reactivity studies of the five MMPs were carried out as an FDA postmarketing requirement (PMR) associated with the U.S. approval of CCH.

In contrast to the limited identity/similarity between clostridial and human (MMP) collagenases, much higher sequence similarity/identity was found when the protein sequences of AUX-I and AUX-II were compared to each other. The two proteins showed 42% sequence identity, which was not limited to short amino acid sequences but found for the entire sequence of 1,006 amino acids.

Even though AUX-I and AUX-II share high sequence similarity, the corresponding antidrug antibodies were very specific for one antigen, and no cross-reactivity was observed for anti-AUX-I antibodies with AUX-II protein or anti-AUX-II antibodies with AUX-I (5). Thus, the complete lack of cross-reactivity between ADAs and human MMPs, which share only very limited sequence similarities with the two antigenic collagenases, is not surprising.

Adverse events evaluation. Clinical adverse events were reviewed as an additional supporting approach to evaluate cross-reactivity, by comparison of the adverse events noted in seropositive subjects with those described in the published literature following the use of broad-spectrum MMP inhibitors in clinical trials (1, 14). These products have been studied in clinical trial settings for potential therapeutic indications, including oncology uses and osteoarthritis. In these clinical programs, dosing was limited or discontinued due to the appearance of a characteristic set of side effects mediated by broad-spectrum MMP inhibition. In one such program, a definition of MMP inhibitor-associated musculoskeletal syndrome (MSS) was used to describe these effects and included painless loss of range of motion (ROM) in large joints (particularly in the shoulders), joint stiffness and joint swelling, soft tissue pain, and fibrosis of palmar tendons (Dupuytren's contracture).

In the clinical program, medical review of all treatment-emergent adverse events across the clinical studies revealed no safety signals related to the inhibition of endogenous collagenases (MMPs). Specifically, there were no reports of musculoskeletal events, such as polyarthritis, osteolysis, or shoulder girdle pain or reduction of motion, that would be indicative of cross-reactivity resulting in inhibition of endogenous collagenases among subjects who received up to eight injections of CCH. Joint stiffness and swelling was found in the treated hand following the first dose (in the absence of antidrug antibodies) as well as following subsequent doses. There was no evidence of worsening of Dupuytren's disease as a result of treatment with CCH. The lack of individual symptoms offers clinical support that anti-AUX-I and anti-AUX-II do not cross-react with MMPs.

Conclusions. As the evidence from the *in vitro* bioanalytical studies indicates and the *in vivo* clinical studies discussed here suggest, there is no cross-reactivity of collagenolytic MMPs with anti-AUX-I and anti-AUX-II antibodies, and they are therefore of

no pharmacologic or physiologic relevance. Sequence identity evaluations support these experimental results, indicating insignificant alignments between MMPs and AUX-I and AUX-II, which were determined to be of low risk to patient safety.

These results and analyses support the lack of cross-reactivity of the two CCH antibodies, anti-AUX-I and anti-AUX-II, with the five human MMPs.

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The clinical studies discussed in this paper were conducted in accordance with Good Clinical Practice (GCP) guidelines and the World Medical Association Declaration of Helsinki and complied with all relevant federal guidelines and institutional policies. Each subject voluntarily signed and dated a consent form before participating in any study.

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