SUPPLEMENT TO

Massive release of the histamine-degrading enzyme diamine oxidase during severe anaphylaxis in mastocytosis patients.

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Description of anaphylaxis events

The mastocytosis Patient A suffered from multiple (> 50) episodes of recurrent anaphylactic shock since 1998 despite the intake of 9 oral antihistamines tablets per day. The patient is allergic to several food items and wasp venom.

Index Event_A1 and Event_A2: Both severe events followed the pattern of a "typical" anaphylactic episode for this subject. It started with a flush in the face, which was treated with local ice application and further intake of ranitidine and cetirizine. When the patient subsequently developed stuffy nose and an extension of the flush to the arms and chest, the EpiPen was self-injected. Anaphylaxis was accompanied by vomiting and diarrhoea. In the case of impending anaphylactic shock the spouse was treating the patient with intravenous methylprednisolone, ranitidine and dimetindene. The typical time window from onset of symptoms before the patient went into shock and became comatose was only 15 minutes.

When the helicopter with the emergency physician arrived a blood pressure could not be recorded. The patient was transferred to the next hospital after an estimated time from onset of symptoms of about 30 minutes. The patient arrived at the hospital still with immeasurably low blood pressure in shock and still unconscious.

In Event A1 the spouse instructed the emergency physician to obtain the initial blood sample and further samples were withdrawn in the hospital. The estimate time from onset of symptoms to withdrawing the first blood sample (A1_0 min in Figure 1A and 3A) is 30 to 45 minutes. This was the sample with the highest histamine concentration and still relatively low DAO (see Figure 4A). Blood samples were not cooled after withdrawal.

Index Event_A3: Because of the recurrent anaphylaxis the treating physician recommended therapy with omalizumab. The patient experienced a mild anaphylactic reaction with nausea within an hour after the first treatment with omalizumab. The blood pressure was stable and the patient remained conscious. Since regular administration of omalizumab no severe anaphylaxis events have occurred.

Patient A was treated with cetirizine, ranitidine, levocetirizine, ketotifen, desloratidine and valium under baseline conditions before the two severe events.

The mastocytosis Patient B has also suffered from multiple episodes of recurrent anaphylaxis. Typically mast cell activation is triggered by an upper respiratory tract infection and fever. Patient B is treated only with cetirizine and ranitidine under baseline conditions.

Event_B1: Within two hours after onset of a flu-like disease the patient developed flushing and urticaria, which the subject treated with ranitidine, but decided to go to the hospital. On admission to the Emergency Department the blood pressure was 133/58 mmHg. The pulse rate was increased to 100 per minute and the temperature elevated to 38.3°C. The subject received ranitidine, diphenhydramine, methylprednisolone, odansetrone and two litres of Elo-Mel, an isotonic infusion solution. The mean arterial blood pressure decreased to 62 mmHg but fluctuated from 51 to 68 mmHg with increased pulse amplitude because of diastolic blood pressure recordings of as low as 37 mmHg. The pulse rate was constantly elevated to 110-120 per minute over the next six hours.

Event_B2: The patient called one of the authors (BJ) in the evening and reported fever and fear to develop anaphylaxis. The patient was instructed to measure blood pressure and pulse rate, which were still normal. During the night the patient started to vomit and went to the Emergency Department at 7:40 AM with intense flushing. The patient received the same intravenous treatment as described above. The body temperature was 38.5°C and the subject had to vomit a few times. The patients was hemodynamically compensated with an increased pulse amplitude (diastolic pressure ~45 mmHg) but pronounced sinus tachycardia with a heart rate of about 125 per min. The patient was transferred to an intensive care unit because of a respiratory rate of about 20 per min and remained tachycardic for about 8 hours. The pulse rate reached normal rates at about 17:00 PM. The highest DAO and lowest histamine values were measured at 16.33 PM (B2_375 min Figure 1A and 3B in the main manuscript). The blood samples in this event were immediately placed on ice after withdrawal and also withdrawn in blood collection tubes containing diminazene aceturate (DIMAZ), a potent DAO inhibitor.

The subject also measured blood pressure and heart rates a total of 24-times over 16 days during a period without any unusual symptoms. The mean (SD) blood pressure was 124/75 (7.7; 5.4) mmHg with a mean (SD) arterial pressure (MAP) of 91 (5.9) mmHg and a pulse rate of 68 (7.8) per minute.

The following table describes some characteristics of the DAOMAST study population.

Table S1: Mastocytosis patients included in the DAOMAST study

	Age	Diagnosis Year	Diagnosis	Tryptase (ng/ml)	KITD816V (pos/neg)
1	54	2000	ISM	13.8	pos
2	76	2011	ISM	17.4	neg
3	72	2004	ISM	53.0	neg
4	35	2010	MIS	5.7	pos
5	39	2010	ISM		pos
6	46	2002	ISM	45.1	pos
7	58	2009	ISM	21.6	neg
8	43	2012	ISM	79.4	pos
9	77	2010	ASM-CMML	12.0	pos
10	53	2008	CM	11.0	pos
11	66	2012	ISM-CEL	3.0	neg
12	57	2008	ISM	87.5	pos
13	62	1998	ISM	42.4	pos
14	61	1993	ISM	55.9	pos
15	50	1967	CM	9.1	neg
16	40	2010	CM	4.5	neg
17	37	2002	CM	15.5	pos
18	37	2006	CM	3.7	neg
19	56	2006	CM	4.0	neg
20	38	2008	ISM	95.4	pos
21	69	2010	ISM	13.9	pos
22	50	2009	ISM	19.6	pos
23	53	2003	CM	5.7	neg
24	70	1998	ISM	54.3	pos
25	34	2003	ISM	158.0	pos
26	27	2011	ISM	75.9	neg
27	67	1990	SSM	64.2	pos
28	42	2005	ISM	14.1	pos
29	45	2010	ISM	5.2	pos
30	68	2009	CM	6.5	pos
31	55	1983	ISM	51.0	neg
32	53	2009	MIS	15.2	
33	49	2011	ISM	38.3	neg
34	48	2007	ISM	13.6	pos
35	60	2005	ISM	14.2	pos
36	55	1991	ISM	51.1	pos
37	48	2008	ISM	15.0	neg
38	61	2009	ISM	63.5	pos
39	30	2011	ISM	59.0	pos
40	49	1998	SSM	109.0	
41	50	2001	ISM	30.1	neg
42	54	2002	ISM	19.1	pos
43	64	1977	ISM	73.0	pos

SM = Systemic Mastocytosis; ISM = indolent SM; SSM = Smoldering SM; ASM = aggressive SM; CMML = chronic myelomonocytic leukemia; CEL = Chronic eosinophilic leukemia; MIS = Mastocytosis in the Skin; CM = Cutaneous mastocytosis.

K_m values of human DAO for histamine and putrescine are highly variable

Table S2 summarizes published K_m values using different sources of human DAO with histamine and putrescine as substrates. Table S3 provides some descriptive statistics also after removing data from Schwelberger 2013.[1] The K_m's of histamine are very different compared to the other publications. It is possible that the used substrate concentrations were too high and substrate inhibition might have significantly interfered.

Table S2: Collection of published DAO Michaelis Menten K_m values for histamine and putrescine

Substrate	Km µM	DAO source	Assay type	Reference
PUT	42	Purified rhDAO; CHO cells	oABA fusion	Ref. 2
	94	Placenta; purified	HRP; H_2O_2 coupling	Ref. 1
	63	Seminal plasma; purified	HRP; H_2O_2 coupling	Ref. 1
	26	Purified rhDAO; insect cells	HRP; H_2O_2 coupling	Ref. 3
	20	Purified rhDAO; insect cells	HRP; H_2O_2 coupling	Ref. 4
	19	Amniotic fluid	Radioactive PUT	Ref. 5
	83	Intestine; partially purified	Ammonia NADH test	Ref. 6
	3,4	Amniotic fluid; purified	Radioactive PUT	Ref. 7
	20	Purified placental DAO	Radioactive PUT	Ref. 8
	24	Crude placental DAO	Radioactive PUT	Ref. 8
	29	Crude DAO thyroid Ca	Radioactive PUT	Ref. 8
	33	Placenta; purified	Indigo dis; H_2O_2-based	Ref. 9
	4,3	Pregnancy plasma	Radioactive PUT	Ref. 10
	4	Serum; healthy subjects	Radioactive PUT	Ref. 11
	70	Placenta; purified	oABA fusion	Ref. 12
HIS	124	Placenta; purified	HRP; H_2O_2 coupling	Ref. 1
	108	Seminal plasma; purified	HRP; H_2O_2 coupling	Ref. 1
	2,8	Purified rhDAO; insect cells	HRP; H_2O_2 coupling	Ref. 4
	19	Intestine; partially purified	Ammonia NADH test	Ref. 6
	2,5	Amniotic fluid; purified	Radioactive HIS pH 6.7	Ref. 7
	2,8	Purified placental DAO	Radioactive HIS	Ref. 8
	3,2	Crude placental DAO	Radioactive HIS	Ref. 8
	3,2	Crude DAO thyroid Ca	Radioactive HIS	Ref. 8
	6	Placenta; purified	Indigo dis; H_2O_2-based	Ref. 9
	6	Placenta; purified	Indigo dis; H_2O_2-based	Ref. 12

PUT = putrescine; HIS = histamine; CHO = chinese hamster ovary; H_2O_2 = hydrogen peroxide
oABA = ortho-Aminobenzaldehyde; dis = disulphonate; NADH = nicotinamide adenine dinucleotide; Ca = carcinoma

Table S3: Summary statistics of published human DAO K_m values

	PUT	PUT w/o Schwelberger 2013[1]†	HIS	HIS w/o Schwelberger 2013[1]†
Mean	36	29	28	5,1
Median	26	24	4,6	3,2
SD	28,9	24,1	46,9	5,5
SEM	7,5	6,7	15	2,0
CV	81%	83%	169%	110%
Min	3,4	3,4	2,5	2,5
Max	94	83	124	19
Ratio Max/Min	28	24	50	7,6
n=	15	13	10	8

†[Substrate] 0.15 to 5 mM; possible substrate inhibition at higher concentrations; PUT = putrescine; HIS = histamine; SD = standard deviation; SEM = standard error of the mean; CV = coefficient of variation

The average histamine K_m of eight publications was 5.1 µM or converted 566 ng/ml. This is 57-times above life-threatening histamine concentrations of 10 ng/ml. Diamine oxidase should not be able to efficiently degrade histamine at these concentrations, but as shown in Figure 4A of the main manuscript human DAO in pregnancy samples is able to rapidly degrade 900 nM exogenously spiked histamine to below 1 ng/ml or 9 nM. The calculated histamine half-life was 3.4 minutes.

Calculation of pregnancy-equivalent DAO concentrations

It is very difficult to obtain plasma from anaphylaxis events once or at multiple time points and detailed activity testing is not possible because of limited material. Nevertheless, we used histamine degradation activity at 30 and 60 minutes to estimate DAO activity (Figure 4 of the main manuscript) and also calculated pregnancy-equivalent DAO concentrations (see below; Table S4, 5 and 6). As shown in Figure 4 of the main manuscript 125 ng/ml DAO in pregnancy samples can rapidly degrade 100 ng/ml histamine with a half-life of 3.4 minutes. In Figure S1A seven additional extrapolated (calculated) histamine degradation curves were added by assuming the same oxidation velocity but reduced DAO concentrations. The corresponding histamine half-lives are shown in the figure with the DAO concentrations. For example, DAO_8_54 min means that pregnancy serum/plasma at 8 ng/ml corresponds to a histamine half-life of 54 minutes. Figure S1B shows histamine degradation rates on the y-axis at corresponding histamine concentrations on the x-axis using data from Figure S1A. The fastest straight line (DAO_125) is based on real histamine degradation rates measured using

16 pregnancy sera/plasma samples in the late 2nd/3rd trimester pregnancy (Figure 4 of the main manuscript).

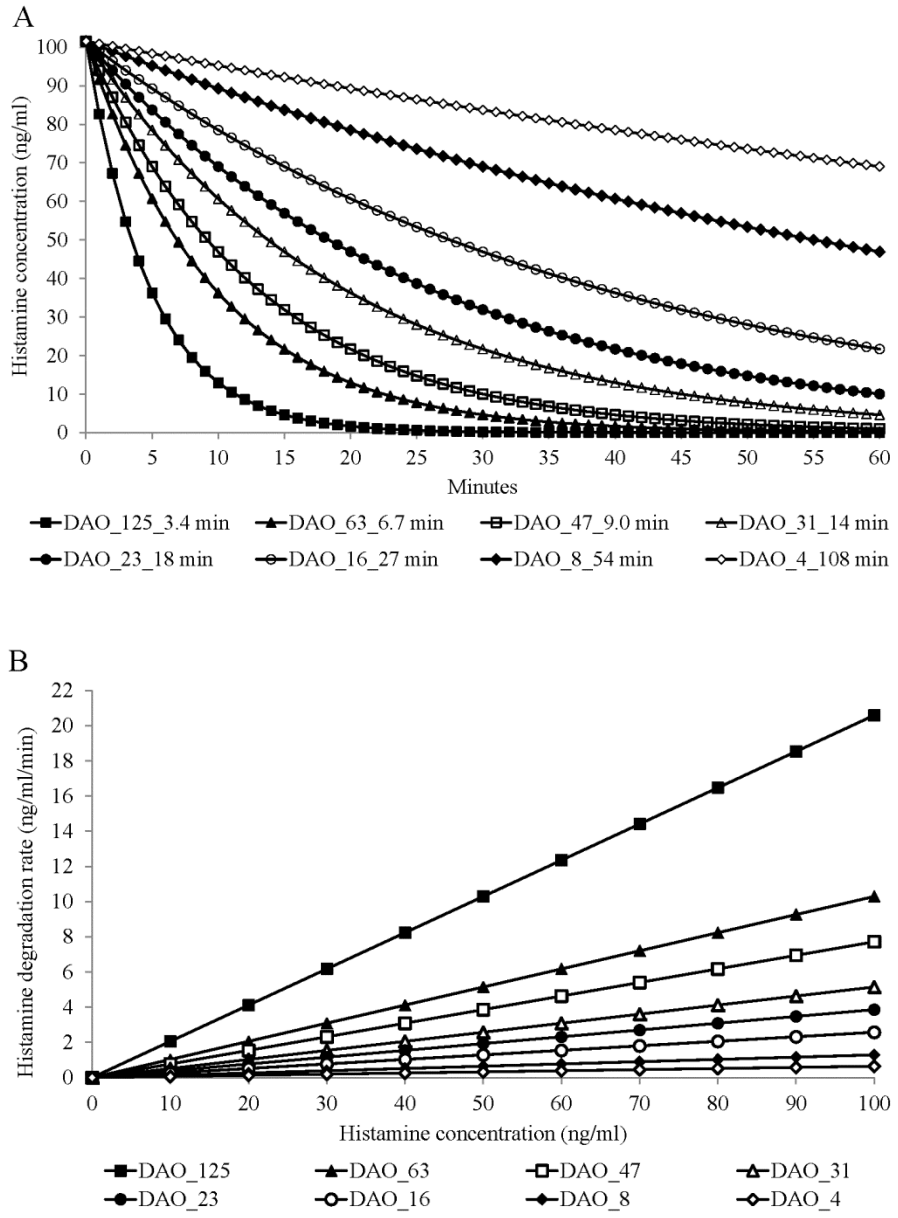

FIGURE S1: Histamine degradation capacity at different DAO concentrations

A, The fastest exponential histamine elimination curve (black squares) is the mean curve derived from 16 pregnancy sera/plasma samples. The label DAO_125_3.4 min means that the average DAO concentration in the 16 pregnancy samples was 125 ng/ml and the half-life for degrading 100 ng/ml histamine 3.4 minutes. The other curves were constructed using lower DAO concentrations. The histamine concentrations after 30 and 60 min can be used to calculate the pregnancy-equivalent DAO concentrations in unknown samples (see Figure S2 and text). They can be compared to measured DAO antigen ELISA values. **B**, The data from Fig. S1A are converted and presented with the histamine degradation rate in ng/ml/min on the y-axis at different histamine concentrations (x-axis).

To calculate pregnancy-equivalent DAO concentrations the remaining histamine concentrations after 30 and 60 minutes from Figure S1A were used to obtain the pregnancy-equivalent DAO concentration curves (Figure S2). The mean of the two calculated DAO concentrations was used as final value. For histamine concentrations <5 ng/ml, a default value of 67 ng/ml DAO was used. This was necessary only with pregnancy samples.

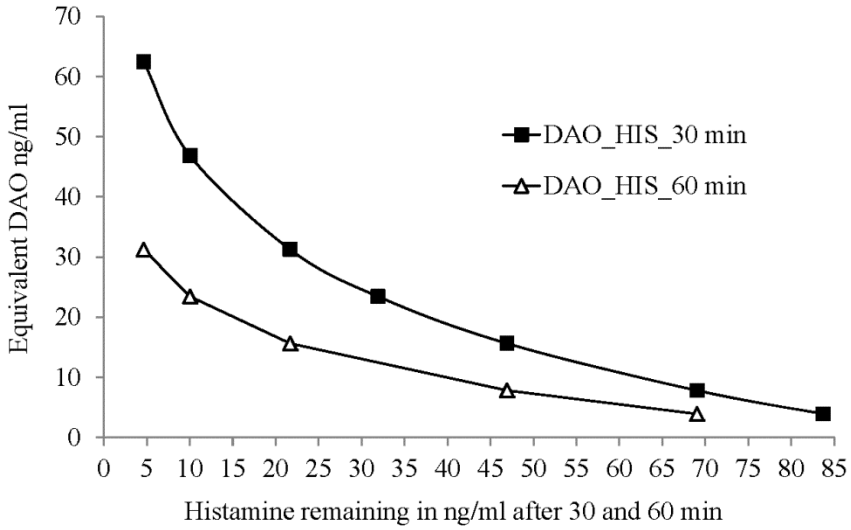

FIGURE S2: Remaining histamine after 30 and 60 min incubation dependent on DAO concentrations calculated from data in Figure S1A.

The equations for 30 and 60 min are [DAO] ng/ml = 69.2 * $\exp^{(-0.040 * [HIS] \text{ ng/ml } 30 \text{ min})}$ and [DAO] ng/ml = 34.1 * $\exp^{(-0.047 * [HIS] \text{ ng/ml } 60 \text{ min})}$ respectively. Histamine values <5 ng/ml were not included in the regression analysis. It would have been necessary to use two or three decay rate equations and the variability at this fast degradation velocities is too high.

An example might clarify this calculation. An anaphylaxis plasma sample was spiked with 100 ng/ml histamine and incubated at 37°C. The histamine concentration after 30 and 60 min were 40 and 23 ng/ml respectively. These values correspond to 18 and 16 ng/ml pregnancy-equivalent DAO concentration based on Figure S2 and the corresponding equations. Therefore, the final pregnancy-equivalent DAO concentration is 17 ng/ml. Endogenous histamine concentrations have been included in the calculations. For example, an anaphylaxis plasma sample was spiked with 100 ng/ml histamine and incubated at 37°C. The endogenous histamine concentration was 30 ng/ml. The histamine concentration after 30 and 60 minutes were 70 and 45 ng/ml respectively, but this does not include the endogenous histamine. The starting histamine concentration was actually 130 ng/ml and therefore 60

ng/ml after 30 min and 85 ng/ml histamine after 60 min have been degraded. The corrected remaining histamine values after 30 and 60 min are 40 and 15 ng/ml. These values correspond to 18 and 21 ng/ml DAO based on Figure S2 and the corresponding equations. The pregnancy-equivalent DAO concentration is the mean 19.5 ng/ml. Consequently, the activity-based pregnancy-equivalent DAO concentrations are divided by the DAO ELISA measured DAO concentrations. This ratio calculates the percent activity of the DAO antigen concentrations. If the DAO concentration is > 125 ng/ml, this value was used instead of the measured DAO concentration. The DAO activity/degradation relationship was constructed based on an average concentration of 125 ng/ml DAO. Therefore, this procedure is conservative and generates higher relative activities for DAO concentrations > 125 ng/ml. For example, if the pregnancy-equivalent DAO concentration is 4 ng/ml and the DAO ELISA antigen concentration 85 ng/ml, the DAO activity is only 4.7% or DAO is 20-fold less active as expected based on the measured DAO antigen concentrations. This is the case in the anaphylaxis samples in this study. DAO activity levels during anaphylaxis were below 10% except for two cases. The exceptions were A30_30 min (196 ng/ml DAO) and B2_375 (392 ng/ml DAO). In both cases low histamine concentrations have been measured in plasma samples. It is tempting to argue that the low histamine concentrations measured in vivo are caused by the high DAO concentrations. Control pregnancy samples showed the expected activities.

Pregnancy-equivalent DAO concentrations and calculated relative activities are shown in the following Tables S4 to S6. These tables also list the histamine concentrations presented in Figures 4B, C and D of the main manuscript. For pregnancy-equivalent DAO concentration calculations the endogenous histamine concentrations have been subtracted (see above). Nevertheless, subtraction did not have any relevant effects on the DAO equivalent concentrations or activities.

Table S4: DAO activity is severely compromised in anaphylaxis events of Subject A

	HIS 30 min	HIS 60 min	DAO measured	DAO equivalent	DAO activity
A1_30 min	104	107	85	<4	4,7%
A1_60 min	92	77	138	<4	3,2%
A2_0 min	66	58	104	<4	3,8%
A2_30 min	28	14	196	20	16%
A3_0 min	115	123	2,4	<4	167%

HIS = Histamine in ng/ml after adding 100 ng/ml; DAO measured and DAO equivalent in ng/ml

Table S5: DAO activity is severely compromised in the second anaphylaxis events of Subject B

	HIS 30 min	HIS 60 min	DAO measured	DAO equivalent	DAO activity
B2_-30 min	106	110	8,1	<4	49%
B2_0 min	104	113	11	<4	36%
B2_D_0 min	112	111	11	<4	36%
B2_30 min	108	117	15	<4	27%
B2_D_30 min	108	111	15	<4	27%
B2_135 min	130	128	74	<4	5%
B2_D_135 min	121	123	74	<4	5%
B2_190 min	119	112	95	<4	4%
B2_D_190 min	120	125	95	<4	4%
B2_375 min	28	13	392	21	17%
B2_D_375 min	89	87	392	<4	3%

HIS = Histamine in ng/ml after adding 100 ng/ml; DAO measured and DAO equivalent in ng/ml; D = Diminazene aceturate added to plasma samples immediately after withdrawal

Table S6: Pregnancy serum or plasma show high pregnancy-equivalent DAO concentrations and activities in the same experiment

	HIS 30 min	HIS 60 min	DAO measured	DAO equivalent	DAO activity
Preg_P1	1,7	2,8	184	>67	54%
Preg_P1_D	109	116	184	<4	3,2%
Preg_P2	1,5	1,8	290	>67	54%
Preg_P3	4,4	3,4	113	>67	59%
Preg_P4	2,0	1,1	121	>67	55%
Preg_S1	1,0	1,4	197	>67	54%
Preg_S2	12	6,6	116	34	29%
Preg_S3	1,6	2,1	115	>67	58%
Preg_S4	1,0	1,0	208	>67	54%
Preg_S4_D	79	77	208	<4	3,2%

HIS = Histamine in ng/ml after adding 100 ng/ml; D = Diminazene aceturate added at 10 µM

Medications used during anaphylaxis treatment do not inhibit DAO activity

A possible explanation, why DAO activity is low in anaphylaxis compared to pregnancy samples, might be that medications used to treat these events inhibit DAO activity. We therefore tested seven compounds used for the treatment of the anaphylaxis events at high concentrations in a DAO activity assay using recombinant human DAO. Diminazene aceturate was used as positive control. None of the compounds at 5 or even 50 µM significantly reduced DAO activity (Figure S3).

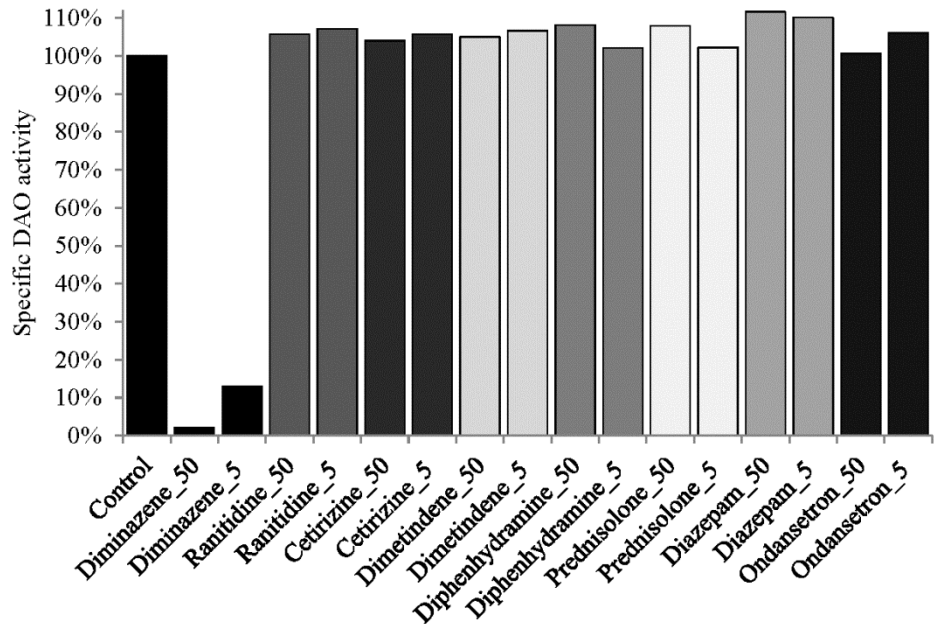

FIGURE S3: Medications used during anaphylaxis treatment do not inhibit DAO activity

Purified recombinant human DAO at 3 µg/ml[2] was used in a DAO activity assay at pH 8.0 using 400 µM putrescine as substrate. This assay is based on horseradish peroxidase hydrogen peroxide coupling and luminol oxidation described originally in Schwelberger and Feurle[15] with minor modifications. We described our assay recently.[13,14] These seven compounds were used for severe anaphylaxis treatment. Specific DAO activity is based on the mean of duplicates of 30 measurements at 5 min intervals for 180 min and subtracting wells without putrescine as negative control.

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