

# The effects of chicken egg white cystatin and proteinase inhibitor on cysteine peptidase-like activity in the sera of patients with breast cancer

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## Abstract

**Background.** The activity of autogenic proteolytic enzymes is regulated in vivo by autogenic inhibitors. They play important roles in maintaining a balance in many processes in the human body. In pathological conditions, enzymes are overexpressed and the balance is disturbed. Such uncontrolled changes may lead to the development of local or systemic cancer.

**Objectives.** To evaluate the effects of specific inhibitors, i.e., chicken egg white cystatin (CEWC) and proteinase inhibitor (E-64) on autogenic cysteine peptidases (CPs) in the sera of patients reporting for subsequent stages of treatment after being diagnosed with breast cancer. Cysteine peptidases play a vital role in the basic processes that are associated with cancer progression.

**Materials and methods.** We selected serum samples from 108 patients with a diagnosis of breast cancer (stages IIA–IIIA) who had received no previous treatment. The blood samples were centrifuged, and the resulting serum was placed in liquid nitrogen and stored at –80°C. The biochemical tests were performed at the laboratory of the Department of Physical Chemistry and Microbiology.

**Results.** For CEWC, we found an inhibitory effect in 37 out of 108 samples; for E-64, 14 out of 22 samples displayed an inhibitory effect. In the remaining blood samples, these inhibitors caused an increase in fluorescence. In a parallel test, we added pure cathepsin B to 9 serum samples, and then used CEWC to inhibit the activity of autogenic CPs. Chicken egg white cystatin completely inhibited the cathepsin B that was added to the serum without changing its effect on the autogenic CPs.

**Conclusions.** The results suggest that there may be a potential difference between the commercially available cathepsin B and its autogenic analogues found in the serum of cancer patients. The increase in fluorescence induced in the reaction between the inhibitors and autogenic CPs is still unexplained. There was no relationship between the observed inhibition/activation of CPs and any of the available indicators of the health of the patients examined.

**Key words:** breast cancer, serum cysteine peptidase-like activity, chicken egg white cystatin, E-64 proteinase inhibitor

## Background

Autogenic proteolytic enzymes have various functions throughout the body and their activity is regulated *in vivo* by autogenic inhibitors. They play important roles in maintaining the balance in many processes in the human body. Under pathological conditions, enzymes are overexpressed, the balance is disturbed, and there can be either an excess of active enzymes or a lack of active inhibitors *in vivo*. Such uncontrolled changes in the body may lead to the development of local or systemic cancer or other diseases.

It was therefore proposed to inhibit the activity of these enzymes under pathological conditions. The inhibitory effect is achieved by supplying specific exogenous inhibitors. This represents a chance to develop a new type of treatment using specific non-toxic inhibitors to alter the *in vivo* activity of enzymes that catalyze pathogenic processes. Studies on experimental animal models have demonstrated that many autogenic inhibitors obtained through chemical synthesis or from biological sources prevent the neoplastic process both *in vitro* and *in vivo*, but their toxicity in humans is too high for them to be used as new drug components.<sup>1–4</sup> It has been suggested that cathepsin B plays a particularly significant role in certain individual biological processes associated with cancer progression. For this reason, cathepsin B has become potentially the most efficient new target for anticancer therapy employing specific inhibitors. So far, many molecules regulating the *in vitro* activity of cathepsin B have been identified, and studies are continually finding inhibitors able to produce inhibitory effects *in vivo*.<sup>5,6</sup> It was also found that cathepsin B attenuates the efficiency of conventional chemotherapy.<sup>7</sup> Research has confirmed that an overexpression of cysteine peptidases – with a simultaneous reduction in their activity achieved by reducing the number of their active inhibitors – decreases immune system function and facilitates the invasion of cancer cells and metastasis. Autogenic cystatins are specific inhibitors which are able to suppress this process.

Cystatins are involved in many physiological and pathological processes, and a deficiency of them can disturb key immunomodulatory functions. The overexpression of cysteine peptidases suppresses immunity, decreasing the control of the autogenic inhibition of neoplastic processes. This finding leads to the hypothesis that reduced activity of pathogenic cysteine cathepsins *in vivo* caused by autogenic or exogenous inhibitors can enhance the immune system, which can then be used as a defense against the progression of cancer.<sup>8</sup> Studies on cell lines of human breast cancer have confirmed the expression of cathepsin B on the surface of mutant cells in the form of an inactive precursor that was activated by enzymes in the next stage. It was found that the active enzyme is inhibited by specific inhibitors. Findings from *in vitro* studies were confirmed *in vivo* on transgenic mice who received a graft of breast

cancer cells. In this experiment, the inhibitors of cysteine peptidases reduced the progression of breast cancer by inhibiting CPs, strongly limiting the progression of the disease.<sup>9</sup> Moreover, it was found that cathepsin D attenuates the anticancer immune response by degrading chemokines and limiting the activity of dendritic cells. On the other hand, cathepsins B, K and L play key roles in the degradation of the extracellular matrix during cancer invasion, acting directly or through the activation of precursors of proteolytic enzymes, such as metalloproteinases, collagenases, plasminogen activator, and others involved in this mechanism. It has been confirmed that specific inhibitors of cysteine peptidases successfully inhibit the cancer invasion and metastasis, and that cathepsin B and other cysteine cathepsins are important targets for anticancer therapy.<sup>2,10</sup>

The activity of CPs is regulated *in vivo* by their autogenic inhibitors – mainly from the cystatin family, including cystatins, stefins and kininogens – and a low level/activity of them in bodily fluids may be associated with a depleted ability of the body to regulate the overexpression of CPs. Therefore, some researchers have suggested that a deficiency of these autogenic inhibitors may be supplemented by their exogenous analogues, including chemically synthesized ones. A synthetic cathepsin B inhibitor, CA-074, as well as antibodies directed against cathepsin B, were able to exert a powerful anticancer activity without any further additional drugs, limiting metastasis and cancer invasion.

This information has inspired researchers to analyze a number of synthetic cysteine peptidase inhibitors as potential drugs for anticancer therapy.<sup>11,12</sup> *In vitro* studies have confirmed the ability of specific inhibitors to block active cysteine peptidases, but the toxicity of inhibitors was found to be too high to use them *in vivo* as potential drugs for anticancer therapy.<sup>13</sup> The proteolytic activity of CPs was also inhibited using the most efficient currently known inhibitors of these enzymes. Chicken egg white cystatin was found to be the most effective inhibitor of processes involved in the overexpression of cathepsins B and L (resulting in over 80% inhibition of cancer cell invasion and metastasis), and was more effective than the synthetic inhibitor, E-64. Other inhibitors derived from biological materials or through chemical synthesis also inhibited these processes, but not as effectively as CEWC. It was also confirmed that the efficient inhibition of these enzymes was associated with limited tumor aggressiveness in breast cancer.<sup>14</sup>

This paper presents the effects of CEWC on the activity of autogenic CPs in the sera of patients with breast cancer. To complement the results, the activity of CPs in selected serum samples was also inhibited using a specific inhibitor (E-64). We found that CEWC inhibited the autogenic CP-like activity in 37 out of 108 serum samples; in the remaining samples, fluorescence increased after this inhibitor was added, which may suggest the increased activity of these

enzymes in serum. To confirm this finding, the activity of CPs in selected blood samples was also inhibited using a synthetic inhibitor (E-64). We found that E-64 inhibited the activity of the same enzymes in 14 out of 22 serum samples, while in the remaining samples fluorescence increased, similar to the samples to which CEWC was added. We also found that the activity of pure cathepsin B added to the serum samples was completely inhibited by CEWC, without changing the previously observed effects of this inhibitor on the autogenic cysteine peptidases in the serum samples.

## Objectives

The aim of our study was to determine changes in groups of patients at different stages of cancer or different phases of treatment. We present the effects of specific inhibitors on the serum of patients with breast cancer. Similar results were also found after the analysis of serum from patients with prostate cancer (unpublished data).

## Materials and methods

### Chemical reagents

We used Z-Phe-Arg-N-Mec (N-alpha-benzyloxycarbonyl-L-phenyl-alanyl-L-arginine-7-amido-4-methylcoumarin), Mec (7-amino-4-methylcoumarin), E-64 inhibitor L-epoxysuccinylleucyl-amido (4-guanidino) butane (Fluka BioChemika, Buchs, Switzerland), and the enzymes papain (3.4.22.1) and cathepsin B (3.4.22.2) (Sigma-Aldrich, St. Louis, USA). The other reagents were chemically pure. Chicken egg white cystatin was isolated using affinity chromatography on a carboxymethylpapain Sepharose 4B column.<sup>15</sup>

### Clinical material (recruitment of human subjects)

Serum samples were taken from patients diagnosed with breast cancer. The patients had been referred for routine tests to the Department of Oncology of the Regional Hospital in Świdnica, Poland, between 2007 and 2009. Blood for the biochemical tests was taken in parallel with blood taken for routine follow-up tests. For the study, we recruited 108 patients with breast cancer (age:  $56.36 \pm 9.90$  years), and the decision of whether to include each patient was based on preliminary chest radiographs, mammography, ultrasonography, and blood tests – in which the levels of CEA and CA 15-3 markers were measured. The patients were diagnosed with cancer (stages IIA–IIIA of the TNM system), but had not undergone surgical treatment, radiotherapy or chemotherapy. The blood samples were centrifuged and the resulting serum was placed in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ . Biochemical tests were

performed at the laboratory of the Department of Pharmaceutical Chemistry and Microbiology at the University of Wrocław.

### Determination of cysteine peptidase-like activity in the serum

Each 100- $\mu\text{L}$  serum sample was augmented with 700  $\mu\text{L}$  of 0.4 M phosphate buffer (pH 6.0) – containing 4 mM EDTA and 2.5 mM DTT – and a 200- $\mu\text{L}$  solution of diluted Z-Phe-Arg-N-Mec substrate in order to achieve a final substrate concentration of 40  $\mu\text{M}$ . Enzymatic hydrolysis of the substrate in each sample was carried out for 60 min at  $37^{\circ}\text{C}$ . The hydrolysis was terminated by adding 2.0 mL of 1.0 mM iodoacetic acid. In a parallel experiment, a control solution was prepared for each sample by adding 2.0 mL of iodoacetic acid before adding the substrate. Apart from that, other reagents were the same. These samples were marked as controls. After the inhibition of substrate hydrolysis, we measured the fluorescence of the 7-amino-4-methylcoumarin (Mec) released at an excitation wavelength of 370 nm and an emission wavelength of 440 nm. The results are expressed in units of activity for cysteine endopeptidases after conversion into the amount of protein present in 1 mL of serum. One unit of enzymatic activity (U) was defined as the amount of enzyme able to catalyze the release of 1 nmol of Mec in 1 min under the conditions described above.<sup>16</sup>

### Inhibition of cysteine peptidase-like activity in serum

One hundred microliters of the tested serum was added to 700  $\mu\text{L}$  of 0.01 M phosphate buffer (pH 6.8) containing 2.0 mM EDTA and 2.0 mM glycine. Then, 100  $\mu\text{L}$  of the inhibitor solution was added in relevant concentrations: 50.0 nM CEWC or 5.0 nM E-64. The sample was incubated at  $37^{\circ}\text{C}$  for 10 min; then, 100  $\mu\text{L}$  of the Z-Arg-AMC substrate solution was added and the sample was incubated again for 30 min. After 30 min, the hydrolysis of the substrate was terminated by adding 2.0 mL of 1.0 mM iodoacetic acid to each sample. The amount of 7-AMC released was measured against a sample containing the same reagents, but the reaction was interrupted immediately after adding the substrate. In a parallel test, the enzyme activity was measured for samples that contained 0.01 M pH 6.8 phosphate buffer instead of the inhibitor. Inhibition or activation units were not converted. Instead, the observed changes in inhibition/activation were expressed as a percentage of change in autogenic CP-like activity in the serum samples.

### Inhibition of pure cathepsin B in 9 serum samples with CEWC

Ten microliters of cathepsin B solution from Sigma-Aldrich, enzyme activity 6000 U/mL, was added to 690  $\mu\text{L}$

of 0.01 M phosphate buffer (pH 6.8); after 10 min, 100 mL of 100 nM CEWC was added. Nine serum samples were measured. The inhibition of autogenic CPs was found in 3 samples, and increased fluorescence was found in 6 samples.

## Statistics

A standard statistical Student's t-test was conducted to compare the 2 variables. The values are presented as the means  $\pm$  standard deviation (SD), and the level of statistical significance was set at  $p < 0.05$ . Statistical differences between the groups were assessed using one-way analysis of variance (ANOVA). Tukey's test was used for the post hoc analysis.

## Results

In this paper, we tried to explain the effect of CEWC on the blood components of patients with breast cancer, focusing on autogenic CP-like activity in vitro. After adding CEWC to the samples of serum from patients with breast cancer, we found inhibition of CP-like activity in 36 out of 108 samples (Table 1a), and increased fluorescence in the remaining samples (Table 1b).

To confirm the results, we repeated the tests using a specific inhibitor of these enzymes (E-64). In the 2<sup>nd</sup> experiment we found inhibition of the activity of the same enzymes for 14 out of 22 serum samples; in the remaining samples, the fluorescence increased. Chicken egg white cystatin added to the same serum samples inhibited the enzyme activity in 8 samples, and in 14 caused an increase in the level of fluorescence. The purpose of the next stage of the study was to clarify the increase in fluorescence after the addition of CEWC. We used pure, commercially available cathepsin B in the tests to compare its reaction with CEWC and to obtain additional information on the similar reaction with autogenic CP-like activity in the serum of cancer patients (Table 2).

The same amount of pure cathepsin B was added to 9 randomly selected samples (Table 3). Chicken egg white cystatin inhibited CP-like activity in 3 of these samples, while in 6 samples, an increase in fluorescence was observed. We found that CEWC completely inhibited the activity of the commercially available cathepsin B, but there was no change in its effect on autogenic cysteine peptidases.

## Discussion

The involvement of cysteine peptidases, including cathepsins B, L and K in cancer has inspired scientists to investigate changes in CP-like activity in the serum of patients with breast cancer in relation to changes

**Table 1a.** Effects of CEWC on CP-like activity in the sera of patients with breast cancer (samples with inhibited activity)

Sample No.	CP-like activity in serum [U/mL]	CP-like activity in serum + cystatin [U/mL]	Remaining activity + cystatin (%)
1	11.56	9.8	84.78
4	6.45	6.12	94.88
6	5.01	4.16	83.03
9	0.44	0.25	56.82
14	7.82	4.45	56.91
21	2.75	1.71	62.18
22	3.3	2.69	81.52
24	9.5	7.4	77.89
25	6.97	5.2	74.61
37	6.46	5.9	91.33
38	6.38	5.8	90.91
40	5.2	3.48	66.92
43	6.33	5.64	89.10
44	11.56	5.92	51.21
45	21.93	6.38	29.09
46	8.08	5.5	68.07
47	6.45	6.12	94.88
49	5.01	4.16	83.03
52	0.44	0.25	56.82
57	7.82	4.45	56.91
64	2.75	1.71	62.18
65	3.3	2.69	81.52
67	9.5	7.4	77.89
68	6.97	5.2	74.61
80	6.46	5.9	91.33
81	6.38	5.8	90.91
83	5.2	3.48	66.92
86	6.33	5.64	89.10
88	7.0	5.52	78.86
90	7.6	5.98	78.68
92	7.86	5.09	64.76
95	10.77	6.86	63.7
97	2.34	1.24	52.99
103	4.99	4.43	88.78
104	8.8	4.06	46.14
106	7.9	6.17	78.1

in the activity of these enzymes. There are a number of papers presenting the inhibition of CPs using various substances, including CEWC. In our study, we decided to demonstrate the effects of CEWC on blood components, including autogenic CPs (Tables 1a,b). Findings on the efficiency of this inhibitor, E-64, and other synthetic inhibitors generated interest in them as potential components in next-generation anticancer drugs. They have been reported to be efficient inhibitors in some processes that play

**Table 1b.** Effects of CEWC on CP-like activity in the sera of patients with breast cancer (samples with increased fluorescence)

Sample No.	CP-like activity in serum [U/mL]	CP-like activity in serum + cystatin [U/mL]	Remaining activity + cystatin (%)	Sample No.	CP-like activity in serum [U/mL]	CP-like activity in serum + cystatin [U/mL]	Remaining activity + cystatin (%)
2	21.96	26.76	121.86	56	10.56	13.05	123.58
3	8.08	7.63	94.43	58	4.6	6.89	149.78
5	4.47	5.84	130.65	59	3.7	3.8	102.70
7	2.65	4.69	176.98	60	7.9	17.83	225.70
8	8.19	9.70	118.44	61	1.55	6.61	426.45
10	5.12	5.15	100.59	62	10.78	11.44	106.12
11	4.55	8.80	193.41	63	8.82	12.69	143.88
12	1.42	2.78	195.77	66	4.65	4.69	100.86
13	10.56	13.05	123.58	69	5.48	7.18	131.02
15	4.6	6.89	149.78	70	5.9	7.26	123.05
16	3.7	3.8	102.70	71	5.8	6.42	110.69
17	7.9	17.83	225.70	72	8.34	10.08	120.86
18	1.55	6.61	426.45	73	4.5	4.84	107.56
18	10.78	11.44	106.12	74	4.88	5.74	117.62
20	8.82	12.69	143.88	75	4.73	6.09	128.75
23	4.65	4.69	100.86	76	10.35	12.38	119.61
26	5.48	7.18	131.02	77	5.55	7.71	138.92
27	5.9	7.26	123.05	78	5.3	8.43	159.06
28	5.8	6.42	110.69	79	1.57	2.46	156.69
29	8.34	10.08	120.86	82	15.36	16.08	104.69
30	4.5	4.84	107.56	84	2.33	7.73	331.76
31	4.88	5.74	117.62	85	3.64	7.78	213.74
32	4.73	6.09	128.75	87	7.7	12.58	163.38
33	10.35	12.38	119.61	89	7.9	8.1	102.53
34	5.55	7.71	138.92	91	8.9	10.93	122.81
35	5.3	8.43	159.06	93	6.41	14.42	224.96
36	1.57	2.46	156.69	94	4.77	12.02	251.99
39	15.36	16.08	104.69	96	4.86	7.44	153.09
41	2.33	7.73	331.76	98	5.02	7.75	154.38
42	3.64	7.78	213.74	99	2.35	3.57	151.91
48	4.47	5.84	130.65	100	5.73	7.27	126.88
50	2.65	4.69	176.98	101	5.16	5.39	104.46
51	8.19	9.7	118.44	102	8.23	10.19	123.82
53	5.12	5.15	100.59	105	3.63	5.1	140.5
54	4.55	8.8	193.41	107	4.12	6.8	165.05
55	1.42	2.78	195.77	108	15.36	17.18	111.85

key roles in cancer development. Our research is focused on finding CP inhibitors that would be non-toxic and could replace or supplement the relevant autogenic analogues, including cystatins, in a patient's body.<sup>2,17–19</sup> A deficiency of autogenic cysteine peptidase inhibitors with simultaneous overexpression of these enzymes are key factors responsible for neoplastic processes. Because of this disturbed balance, researchers have suggested supplementing the level of active CP inhibitors in patients' bodies by using exogenous inhibitors that are able to block overexpression

in vivo. So far, the efficiency of chemically synthesized inhibitors has been best investigated, particularly CA-074Me and E-64. However, they were found to be too toxic to humans to be used for scheduled anticancer therapy, and so far they have only been tested on cell lines and experimental animal models.<sup>18–22</sup>

Our choice of research topic was inspired by findings about the use of specific cysteine peptidase inhibitors for the in vitro regulation of neoplastic processes. Two of these inhibitors were found to be very effective. It was found that



**Table 2.** Changes in cysteine peptidase-like activity induced by CEWC and E-64 in selected serum samples from patients with breast cancer

Sample No.*	CP-like activity in serum [U/mL]	CP-like activity in serum M + E-64 [U/mL]	Remaining CP-like activity in serum + E-64 (%)	Remaining CP-like activity in serum + cystatin [U/mL]	Remaining CP-like activity in serum + cystatin (%)
87	7.7	6.8	<b>88.31</b>	12.58	163.38
<b>88</b>	<b>7.0</b>	<b>6.72</b>	<b>96</b>	<b>5.52</b>	<b>78.86</b>
89	7.9	9.12	115.44	8.1	102.53
<b>90</b>	<b>7.6</b>	<b>7.37</b>	<b>96.97</b>	<b>5.98</b>	<b>78.68</b>
91	8.9	11.51	129.33	10.93	122.81
<b>92</b>	<b>7.86</b>	<b>0.99</b>	<b>12.6</b>	<b>5.09</b>	<b>64.76</b>
<b>93</b>	<b>6.41</b>	<b>0.1</b>	<b>1.56</b>	14.42	224.96
94	4.77	5.17	108.39	12.02	251.99
<b>95</b>	<b>10.77</b>	<b>3.11</b>	<b>28.88</b>	<b>6.86</b>	<b>63.7</b>
96	4.86	10.5	216.05	7.44	153.09
<b>97</b>	<b>2.34</b>	<b>1.95</b>	<b>83.33</b>	<b>1.24</b>	<b>52.99</b>
<b>98</b>	<b>5.02</b>	<b>2.63</b>	<b>52.39</b>	7.75	154.38
99	2.35	3.06	130.21	3.57	151.91
<b>100</b>	<b>5.73</b>	<b>4.97</b>	<b>86.74</b>	7.27	126.88
<b>101</b>	<b>5.16</b>	<b>4.57</b>	<b>88.57</b>	5.39	104.46
102	8.23	10.15	123.33	10.19	123.82
<b>103</b>	<b>4.99</b>	<b>5.15</b>	<b>103.21</b>	<b>4.43</b>	<b>88.78</b>
<b>104</b>	<b>8.8</b>	<b>3.86</b>	<b>43.86</b>	<b>4.06</b>	<b>46.14</b>
<b>105</b>	<b>3.63</b>	<b>2.6</b>	<b>71.63</b>	5.1	140.5
<b>106</b>	<b>7.9</b>	<b>5.03</b>	<b>63.67</b>	<b>6.17</b>	<b>78.1</b>
<b>107</b>	<b>4.12</b>	<b>3.1</b>	<b>75.24</b>	6.8	165.05
108	15.36	15.5	100.91	17.18	111.85

\* Sample numbers in bold font were previously depicted in Table 1a; samples in standard font were previously depicted in Table 1b. Samples with inhibited activity are in bold font. Samples with increased fluorescence are in standard font.

the activity of cysteine peptidases associated with breast cancer cells was most effectively inhibited by CEWC and, to a lesser extent, by E-64 peptide (Table 2). This indicates that these inhibitors can be used as potential components of anticancer drugs targeting processes that regulate cancer progression.<sup>14</sup> Recent studies have also indicated that E-64 cannot be used in practice, not only because of its high price, but also its significant toxicity. The dose of E-64 used in vitro or in vivo in experimental studies on animals per kilogram of human body weight is highly toxic, and therefore cannot be used as a potential component of novel anticancer drugs.

The results of our study indicate that CEWC, or its derivatives obtained from other biological sources, may be a potential component of next-generation anticancer drugs. So far, numerous in vitro studies have also confirmed that this cystatin is highly efficient in inhibiting the activity of enzymes associated with major neoplastic processes. This suggests that inhibitors isolated from biological sources – chicken egg white protein, in particular – may be new targets for anticancer therapy. It is also known that CEWC shows more than 40% similarity to its autogenic analogues in the human body. This suggests that a new therapeutic target can be planned to employ this inhibitor as a potential component of next-generation anticancer drugs

for “inhibitor therapy”.<sup>23,24</sup> The results presented in this paper provide additional information suggesting the use of CEWC for the inhibition of CP overexpression in cancer patients (Table 1a). Surprisingly, CEWC caused an increase in fluorescence in about 60% of the serum samples from patients with breast cancer, which suggests the activation of autogenic cysteine peptidases (Table 1b).

This observation was confirmed by replacing CEWC with another inhibitor, E-64, which also inhibited the CP activity in some serum samples, and increased fluorescence in other samples (Table 2). Other results indicated differences in inhibiting CP-like activity and pure cathepsin B by CEWC. The findings from our study are insufficient to suggest the activation of autogenic CP or differences between CP-like activity and pure cathepsin B (Table 3). Nevertheless, it is important that the same incomplete inhibitory effect on CP-like activity was obtained in about 30% of the samples, and that fluorescence increased in the remaining samples, which was similar to serum samples from patients with prostate cancer (unpublished data). Similar results were reported 20 years ago in a paper on a peptide isolated from the urine of patients with colorectal cancer which was able to activate autogenic CP-like activity. This study from 20 years ago and the findings of our research

**Table 3.** Inhibitory effect of commercially available cathepsin B on autogenic CP-like activity in 9 serum samples from patients with breast cancer

Sample no.	CP-like activity in serum [U/mL]	CP-like activity in serum + 10 µl cathepsin B [U/mL]	CP-like activity in serum + cystatin [U/mL]	Remaining activity + cystatin (%)
2	21.96	<b>81.79</b>	28.13	128.10
13	10.56	<b>77.39</b>	13.11	124.15
33	10.35	<b>73.15</b>	11.97	115.65
42	3.64	<b>59.67</b>	8.18	224.73
51	8.19	<b>65.93</b>	10.23	124.91
76	10.35	<b>73.14</b>	11.97	115.65
64	<b>2.75</b>	<b>62.12</b>	<b>1.97</b>	<b>71.64</b>
81	<b>6.38</b>	<b>69.02</b>	<b>6.03</b>	<b>94.51</b>
86	<b>6.33</b>	<b>67.80</b>	<b>5.58</b>	<b>88.15</b>

Samples with inhibited activity are in bold font. Samples with increased fluorescence are in standard font.

seem to be complementary, but require additional studies to find a detailed explanation.<sup>25</sup>

The results presented herein provide important additional information, not only regarding CEWC tested in vitro on cell lines, but also about the use of CEWC for the inhibition of autogenic CP-like activity in the ascitic fluid of patients with pancreatic cancer, tumor tissue homogenates of the stomach, colon, and tongue, and ovarian cancer inhibited by a CP inhibitor isolated from human placenta. In these tests, CEWC showed an inhibitory effect on the activity of cysteine peptidases in almost 100% of samples. However, this experiment was not aimed at the complete inhibition of CPs; the target range was 50–80% in order to assess differences between the samples depending on the patients' cancer stage. The relevant results have been published.<sup>26–31</sup> Chicken egg white cystatin and its analogues isolated from placentas were also used in the experimental anticancer therapies, where they inhibited neoplastic processes in experimental animals with a grafted human inhibitor, or in combined inhibitor therapy, and photodynamic therapy.<sup>32–35</sup> Current research on the effects of CEWC on blood components in cancer patients have provided useful information for the assessment of this inhibitor as a potential component of new generation anticancer drugs for inhibitor therapy. The effects of CEWC on autogenic CP-like activity require additional studies in order to evaluate its suitability for intravenous administration.

## Limitations

The limitations of this study were caused by the lack of some reagents and limited access to medical equipment.

## Conclusions

The results suggest that there may be a potential difference between the commercially available cathepsin B and its autogenic analogues found in the serum of cancer patients. The increase in fluorescence induced in the reaction

between the inhibitors and autogenic CPs is still unexplained. There was no relationship between the observed inhibition/activation of CPs and any of the available indicators of the health of the patients examined.

## References

- Weidle UH, Tiefenthaler G, Georges G. Proteases as activators for cytotoxic prodrugs in antitumor therapy. *Cancer Genom Proteom*. 2014;11(2):67–79. PMID:24709544
- Turk V, Stoka V, Vasiljeva O, et al. Cysteine cathepsins: From structure, function and regulation to new frontiers. *Biochim Biophys Acta*. 2012;1824(1):68–88. doi:10.1016/j.bbapap.2011.10.002
- Cudic M, Fields GB. Extracellular proteases as targets for drug development. *Curr Protein Pept Sci*. 2009;10(4):297–307. doi:10.2174/138920309788922207
- Kos J, Lah TT. Cysteine proteinases and their endogenous inhibitors: Target proteins for prognosis, diagnosis and therapy in cancer. *Oncol Rep*. 1998;5(6):1349–1361. doi:10.3892/or.5.6.1349
- Gondi CS, Rao JS. Cathepsin B as a cancer target. *Expert Opin Ther Targets*. 2013;17(3):281–291. doi:10.1517/14728222.2013.740461
- Keppeler D. Towards novel anti-cancer strategies based on cystatin function. *Cancer Lett*. 2006;235(2):159–176. doi:10.1016/j.canlet.2005.04.001
- Zhong YJ, Shao LH, Li Y. Cathepsin B-cleavable doxorubicin prodrugs for targeted cancer therapy. *Int J Oncol*. 2013;42(2):373–383. doi:10.3892/ijo.2012.1754
- Magister S, Kos J. Cystatins in immune system. *J Cancer*. 2013;4(1):45–56. doi:10.7150/jca.5044
- Mullins SR, Sameni M, Blum G, Bogoy M, Sloane BF, Moin K. Three-dimensional cultures modeling premalignant progression of human breast epithelial cells: Role of cysteine cathepsins. *Biol Chem*. 2012;393(12):1405–1416. doi:10.1515/hsz-2012-0252
- Nomura T, Katunuma N. Involvement of cathepsins in the invasion, metastasis and proliferation of cancer cells. *J Med Invest*. 2005;52(1–2):1–9. doi:10.2152/jmi.52.1
- Szpadarska AM, Frankfater A. An intracellular form of cathepsin B contributes to invasiveness in cancer. *Cancer Res*. 2001;61(8):3493–3500. PMID:11309313
- Frlan R, Gobec S. Inhibitors of cathepsin B. *Curr Med Chem*. 2006;13(19):2309–2327. doi:10.2174/092986706777935122
- Tomoo K. Development of cathepsin inhibitors and structure-based design of cathepsin B-specific inhibitor. *Curr Top Med Chem*. 2010;10(7):696–707. doi:10.2174/15680261079113441
- Premzl A, Puizdar V, Zavasnik-Bergant V, et al. Invasion of ras-transformed breast epithelial cells depends on the proteolytic activity of cysteine and aspartic proteinases. *Biol Chem*. 2001;382(5):853–857. doi:10.1515/BC.2001.104
- Anastasi A, Brown MA, Kembhavi AA, et al. Cystatin, a protein inhibitor of cysteine proteinases. Improved purification from egg white, characterization, and detection in chicken serum. *Biochem J*. 1983;211(1):129–138. doi:10.1042/bj2110129

16. Barrett AJ, Kirschke H. Cathepsin B, cathepsin H, and cathepsin L. *Methods Enzymol.* 1981;80(Pt C):535–561. doi:10.1016s0076-6879(81)80043-2.
17. Saleem M, Qadir MI, Perveen N, et al. Inhibitors of apoptotic proteins: New targets for anticancer therapy. *Chem Biol Drug Des.* 2013;82(3):243–251. doi:10.1111/cbdd.12176
18. Lankelma JM, Voorend DM, Barwari T, et al. Cathepsin L, target in cancer treatment? *Life Sci.* 2010;86(7–8):225–233. doi:10.1016/j.lfs.2009.11.016
19. Palermo C, Joyce JA. Cysteine cathepsin proteases as pharmacological targets in cancer. *Trends Pharmacol Sci.* 2008;29(1):22–28. doi:10.1016/j.tips.2007.10.011
20. Katunuma N. Structure-based development of specific inhibitors for individual cathepsins and their medical applications. *Proc Jpn Acad Ser B Phys Biol Sci.* 2011;87(2):29–39. doi:10.2183/pjab.87.29
21. Montaser M, Lalmanach G, Mach L. CA-074, but not its methyl ester CA-074Me, is a selective inhibitor of cathepsin B within living cells. *Biol Chem.* 2002;383(7–8):1305–1308. doi:10.1515/BC.2002.147
22. Szpaderska AM, Frankfater A. An intracellular form of cathepsin B contributes to invasiveness in cancer. *Cancer Res.* 2001;61(8):3493–3500. PMID:11309313
23. Saitoh E, Isemura S, Sanada K. Cystatin superfamily. Evidence that family II cystatin genes are evolutionary related to family III cystatin genes. *Biol Chem Hoppe Seyler.* 1988;369(Suppl):191–197. PMID:3202964
24. Agrawal AK, Ekonjo GB, Teterycz E, et al. Cysteine peptidases and their inhibitors in breast and genital cancer. *Folia Histochem Cytobiol.* 2010;48(3):323–327. doi:10.2478/v10042-10-0067-2
25. Siewinski M, Gutowicz J, Kielan W, Bolanowski M. Cysteine peptidase inhibitors and activator(s) in urine of patients with colorectal cancer. *Oncology.* 1994;51(5):446–449. <https://doi.org/10.1159/000227381>
26. Agrawal AK, Kielan W, Katib A, et al. Inhibition of cysteine peptidase activity in ascitic fluid in pancreatic cancer patients. *Folia Histochem Cytobiol.* 2010;48(4):513–517. doi:10.2478/v10042-010-0057-4
27. Saleh Y, Siewinski M, Kielan W, Ziolkowski P, Grybos M, Rybka J. Regulation of cathepsin B, L expression in vitro in gastric cancer tissues by egg white cystatin. *J Exp Ther Oncol.* 2003;3(6):319–324. doi:10.1111/j.1533-869x.2003.01105.x
28. Hap A, Kielan W, Grzebiński Z, et al. Control of active B and L cathepsins in tissues of colorectal cancer using cystatin isolated from chicken egg proteins: In vitro studies. *Folia Histochem Cytobiol.* 2011;49(4):670–676. doi:10.5603/FHC.2011.0075
29. Saleh Y, Wnukiewicz J, Trziszka T, Siewinski M, Ziolkowski P, Kopec W. Cathepsin B and cysteine protease inhibitors in human tongue cancer: Correlation with tumor staging and in vitro inhibition of cathepsin B by chicken cystatin. *J Cancer Molecules.* 2006;2:67–72.
30. Saleh Y, Siewinski M, Sebzda T, et al. Inhibition of cathepsin B activity in human breast cancer tissue by cysteine peptidase inhibitor isolated from human placenta – immunohistochemical and biochemical studies. *Folia Histochem Cytobiol.* 2003;41(3):161–167. PMID:13678335
31. Siewinski M, Saleh Y, Popiela A, Ziolkowski P, Jelen M, Grybos M. Expression of high molecular weight cysteine proteinase inhibitor in ovarian cancer tissues: Regulation of cathepsin B expression by placental CPI. *Biol Chem.* 2003;384(7):1103–1107. doi:10.1515/BC.2003.123
32. Saleh Y, Siewinski M, Sebzda T, Grybos M, Pawelec M, Janocha A. Effect of combined in vivo treatment of transplantable solid mammary carcinoma in Wistar rats using vitamin E and cysteine peptidase inhibitors from human placenta. *J Exp Ther Oncol.* 2003;3(2):95–102. doi:10.1046/j.1359-4117.2003.01077.x
33. Sebzda T, Saleh Y, Siewinski M, Rudnicki J, Ziolkowski P. The influence of vitamin E and human placenta cysteine peptidase inhibitor on the expression of cathepsin B and L implanted hepatoma Morris 5123 tumor model in the Wistar rats. *World J Gastroenterol.* 2005;11(4):587–592. doi:10.3748/wjg.v11.i4.587
34. Saleh Y, Ziolkowski P, Siewinski M, Milach J, Marszałik P, Rybka J. The combined treatment of transplantable solid mammary carcinoma in wistar rats by use photodynamic therapy and cysteine proteinase inhibitors. *In Vivo.* 2001;15(4):351–357. PMID:11695229
35. Zsebk B, Symonowicz K, Saleh J, Ziolkowski P, Bronowicz A, Verb G. Photodynamic therapy combined with a cysteine proteinase inhibitor synergistically decrease VEGF production and promote tumor necrosis in a rat mammary carcinoma. *Cell Profil.* 2007;40(1):38–49. doi:10.1111/j.1365-2184.2007.00420.x