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Mikulski et al.

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| [54] | COMPOSITIONS COMPRISING ONCONASE™ AND CISPLATIN, MELPHALAN, OR DOXORUBICIN HCL | 4,888,172 12/1989 Szebenyi et al. 424/105 | |
| | | 5,175,005 12/1992 Fukushima et al. 424/583 | |

OTHER PUBLICATIONS

- | | | |
|------|---|---|
| [75] | Inventors: Stanislaw M. Mikulski , Essex Fells, N.J.; Wojciech J. Ardelt , New City, N.Y. | Abstract (Dialog file 155 Accession No. 89146182) Legha, S. S. 1989, <i>Semin Oncol.</i> 16: 34-44. |
| [73] | Assignee: Alfacell Corporation , Bloomfield, N.J. | Hird et al. 1990. <i>Gener and Cancer</i> , (P. Carney et al. eds.) John Wiley & Sons Ltd., N.Y. pp. 184-189. |
| [21] | Appl. No.: 283,971 | Ardelt et al. 1991. <i>J. Biol. Chem.</i> 266(1):245-251. |
| [22] | Filed: Aug. 1, 1994 | Mikulski et al. 1990. <i>Cell Tissue Kinet.</i> 23:237-246. |

Related U.S. Application Data

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| [63] | Continuation of Ser. No. 921,180, Jul. 30, 1992, abandoned, which is a continuation-in-part of Ser. No. 436,141, Nov. 13, 1989, abandoned, which is a continuation-in-part of Ser. No. 178,118, Apr. 6, 1988, Pat. No. 4,882,421. | |
| [51] | Int. Cl.⁶ | A61K 38/43 ; C12N 9/22 |
| [52] | U.S. Cl. | 424/94.6 ; 435/199; 530/350; 514/12 |
| [58] | Field of Search | 530/350; 424/94.6; 435/199; 514/12 |

References Cited

U.S. PATENT DOCUMENTS

- | | |
|------|--|
| [56] | 4,882,421 11/1989 Shogen et al. 530/350 |
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[57] ABSTRACT

A pharmaceutical known by the trademark ONCONASE, as described in pending commonly owned application application Ser. No. 07/436,141 filed Nov. 13, 1989, is combined with drugs sold under the names cisplatin, Melphalan and Doxorubicin HCl. The combinations of ONCONASE with these drugs has unexpected bioactivity in vitro against OVCAR-3 human ovarian adenocarcinoma cells.

3 Claims, No Drawings

**COMPOSITIONS COMPRISING
ONCONASE^(TM) AND CISPLATIN,
MELPHALAN, OR DOXORUBICIN HCL**

This application is a continuation of application Ser. No. 07/921,180, filed Jul. 30, 1992, now abandoned, which is a continuation-in-part of application Ser. No. 07/436,141, filed Nov. 13, 1989, now abandoned, which is a continuation-in-part of application Ser. No. 07/178,118, filed Apr. 6, 1988, now U.S. Pat. No. 4,882,421.

BACKGROUND OF THE INVENTION

The invention relates to pharmaceuticals, and more particularly relates to pharmaceuticals for use in treating cells which cause cancer tumor in humans.

The above-referenced patent application discloses a pharmaceutical which will be referred to herein by the trademark ONCONASE. It has not been determined that when this pharmaceutical is used in vitro in a combined therapy with three other drugs, the results of the combined therapy are, in certain instances, much more bioactive than would be expected.

These other drugs are known as Cisplatin, Melphalan and ADRIAMYCIN.

**DETAILED DESCRIPTION OF PREFERRED
EMBODIMENTS**

In vitro data indicate that a combination of ONCONASE with a drug sold under the name Cisplatin is much more bioactive against human ovarian OVCAR-3 adenocarcinoma than would be expected, given the separate activities of ONCONASE and Cisplatin. In vitro also data indicate that a combination of ONCONASE with a drug sold under the name Melphalan is much more bioactive against human ovarian OVCAR-3 adenocarcinoma than would be expected, given the separate activities of ONCONASE and Melphalan. In vitro data indicate that a combination of ONCONASE with a drug sold under the trademark ADRIAMYCIN is much more bioactive against human ovarian OVCAR-3 adenocarcinoma than would be expected given the separate activities of ONCONASE and ADRIAMYCIN.

The preferred embodiment of the invention was tested using a cell culture assay. In such an assay, a cell line of known growth rate over a predetermined period is treated with the substance under test and the growth of the treated cells is compared with the growth which would ordinarily be expected from untreated cells.

ONCONASE, described in the above-referenced pending patent application and manufactured in accordance with the methodology described in U.S. Pat. No. 4,882,421 (which methodology is hereby incorporated herein by reference as if fully set forth herein) was dissolved in phosphate buffered saline (PBS) to obtain 1 mg/ml stock solution.

Cisplatin, also known as cis-diamminedichloroplatinum, is a heavy metal complex containing a central atom of platinum surrounded by two chloride atoms and two ammonia molecules in the cis position. It has the molecular formula $Pt Cl_2 H_6 N_2$ and a molecular weight of 300.1.

Melphalan, also known as L-phenylalanine mustard, phenylalanine mustard, L-PAM, or L-sarcosylsin, is 4-[bis(2-chloroethyl)amino]-L-phenylalanine.

ADRIAMYCIN is a trademark for Doxorubicin HCl, USP. Doxorubicin is an anthracycline antibiotic isolated from cultures of *Streptomyces peucetius* var. *caesius*. Doxo-

rubicin consists of a naphthacenequinone nucleus linked through a glycosidic bond at ring atom 7 to an amino sugar, daunosamine.

The assay system utilized the OVCAR-3 human ovarian adenocarcinoma cell line, obtained from the American Type Culture Collection (accession number ATCC HTB 161). The cell line was cultured in RPMI 1640 medium and supplemented with 20% heat-inactivated fetal bovine serum, 200 mM L-glutamine, 10,000 units per 1 ml penicillin, 10 mg per 1 ml streptomycin, 25 μ g per 1 ml fungizone, 10 mM HEPES buffer and 10 μ g per 1 ml bovine insulin. This was the complete growth medium.

The cells were seeded into 96-well tissue culture plates at a density of 6000 viable cells (50 μ l) per well. The cells were allowed to settle for 24 hours and then 50 μ l of appropriate ONCONASE and/or Cisplatin, Melphalan or ADRIAMYCIN solutions were added per well. The following final concentrations were used:

- a) ONCONASE, 20 ng to 10 μ g/ml;
- b) Cisplatin, 10 to 500 nM;
- c) Melphalan, 0.25 to 5 μ M; and
- d) ADRIAMYCIN, 5 to 100 nM.

The plates were incubated for an additional six days at 37° C. and 5% carbon dioxide atmosphere. The total assay time was consequently seven days (one day in which the cells are allowed to settle, and six days of incubation). Percentages of viable cells were then determined using the MTT colorimetric assay using the Bio-Rad EIA microtiter plate reader.

The number of cells was determined by a direct count in an AO-Spencer "Brightline" hemocytometer with a Neubauer ruling. Attached cells were washed three times with Hanks' Balanced Salt Solution and treated with 2 ml of a 0.25% Trypsin—0.02% EDTA solution in buffered saline for about thirty seconds. The solution was removed and the cells were left at 37° C. for 10 minutes, then suspended in 10 ml of the complete growth medium. The 0.25 ml of the cell suspension was diluted with 0.75 ml of the complete growth medium and then 1 ml of 0.5% Trypan Blue solution was added and viable cells were counted.

Tables 1, 2 and 3 present the result of the above experiments. Except for the ED₅₀ values, these tables are expressed in the Interaction Index developed by Berenbaum. In this Interaction Index, a result of 1.0 indicates that the two drugs do not interact, i.e. that their combined effectiveness against a particular cell line is unchanged from what would be expected from using them individually. An Interaction Index result which is greater than 1.0 indicates that the two drugs are antagonistic, i.e. that their combined effectiveness against a particular cell line is less than what would be expected from using them individually. Progressively higher Interaction Index results indicate progressively greater antagonism. An Interaction Index result which is less than 1.0 indicates that the two drugs are synergistic, i.e. that their combined effectiveness against a particular cell line is greater than what would be expected from using them individually. As the Interaction Index approaches 0 (maximum synergism), this indicates progressively greater synergism. Thus, a lower number indicates a higher bioactivity against the cell line used in the experiment. The ED₅₀ values represent isoeffective doses; they indicate the dose required to halve the growth rate of the cells undergoing the assay. Thus, the lower the ED₅₀ figure, the smaller the dose required to achieve the same bioactivity.

TABLE 1

Interaction Index and ED ₅₀ Values for varying doses of ONCONASE together with Cisplatin for OVCAR-3 human ovarian adenocarcinoma cells and ED ₅₀ values for ONCONASE and Cisplatin individually					
ONCONASE Dose (µg/ml)	0	0.02	0.2	2.0	ED ₅₀
ONCONASE Alone					1.447
ONCONASE + 10 mM Cisplatin		1.159	0.891	0.482	0.697
ONCONASE + 25 mM Cisplatin		1.098	0.830	0.421	0.609
ONCONASE + 50 mM Cisplatin		1.132	0.864	0.455	0.658
ONCONASE + 100 mM Cisplatin		0.858	0.590	0.181	0.262
ONCONASE + 250 mM Cisplatin		0.700	0.432	0.023	0.034
ONCONASE + 500 mM Cisplatin		0.685	0.417	0.008	0.011
ED ₅₀ Values*:	316.509	214.299	129.616	<0.001	

*for Cisplatin in combination with respective concentrations of ONCONASE (vertically); ED₅₀ value for Cisplatin at 0 µl ONCONASE is for Cisplatin alone, OVCAR-3 cells being considered resistant to Cisplatin alone

TABLE 2

Interaction Index and ED ₅₀ Values for varying doses of ONCONASE together with Melphalan for OVCAR-3 human ovarian adenocarcinoma cells and ED ₅₀ values for ONCONASE and Melphalan individually					
ONCONASE Dose (µg/ml)	0	0.02	0.2	2.0	ED ₅₀
ONCONASE Alone					
ONCONASE + 0.25 µM Melphalan		1.701	1.492	0.747	1.705
ONCONASE + 0.5 µM Melphalan		1.778	1.569	0.824	1.881
ONCONASE + 0.75 µM Melphalan		1.746	1.537	0.792	1.808
ONCONASE + 1.0 µM Melphalan		1.142	0.934	0.189	0.411
ONCONASE + 2.5 µM Melphalan		0.987	0.779	0.034	0.051
ONCONASE + 5.0 µM Melphalan		0.975	0.766	0.021	0.022
ED ₅₀ Values*:	4.786	4.623	3.624	0.056	

*for Melphalan in combination with respective concentrations of ONCONASE (vertically); ED₅₀ value for Melphalan at 0 µl ONCONASE is for Melphalan alone, OVCAR-3 cells being considered resistant to Melphalan alone

TABLE 3

Interaction Index and ED ₅₀ Values for varying doses of ONCONASE together with ADRIAMYCIN for OVCAR-3 human ovarian adenocarcinoma cells and ED ₅₀ values for ONCONASE and ADRIAMYCIN individually					
ONCONASE Dose (µg/ml)	0	0.02	0.2	2.0	ED ₅₀
ONCONASE Alone					1.166
ONCONASE + 5 nM ADRIAMYCIN		1.744	1.305	0.881	0.957
ONCONASE + 10 nM ADRIAMYCIN		1.702	1.263	0.839	0.908
ONCONASE + 25 nM ADRIAMYCIN		1.208	0.769	0.346	0.332
ONCONASE + 50 nM ADRIAMYCIN		0.923	0.484	0.061	0.001
ED ₅₀ Values*:	37.340	34.473	18.090	2.265	

TABLE 3-continued

Interaction Index and ED ₅₀ Values for varying doses of ONCONASE together with ADRIAMYCIN for OVCAR-3 human ovarian adenocarcinoma cells and ED ₅₀ values for ONCONASE and ADRIAMYCIN individually					
ONCONASE Dose (µg/ml)	0	0.02	0.2	2.0	ED ₅₀

*for ADRIAMYCIN in combination with respective concentrations of ONCONASE (vertically); ED₅₀ value for Adriamycin at 0 µl ONCONASE is for ADRIAMYCIN alone, OVCAR-3 cells being considered resistant to ADRIAMYCIN alone

These results demonstrate that, in the instances shown, the bioactivities of ONCONASE combined with Cisplatin, Melphalan and ADRIAMYCIN on OVCAR-3 human ovarian adenocarcinoma are much greater than would be expected from the bioactivities of the individual drugs alone. (Indeed, the OVCAR-3 cell line originated from a patient who was clinically resistant to Cisplatin, Melphalan and ADRIAMYCIN.) This may be seen from the ED₅₀ figures which are along the right edge of the Tables. These figures represent computed isoeffective doses; the figure shown is the amount of material which would be required to halve the growth rate of the cells undergoing the assay. Thus, the lower the ED₅₀ figure, the smaller the dose required to achieve the same bioactivity.

Chemical Analysis and Composition of ONCONASE

ONCONASE has been well characterized chemically. While ONCONASE is a protein isolated from *Rana pipiens*, it is believed that ONCONASE may be produced using genetic engineering techniques, as long as the end result has the following chemistry and structure:

ONCONASE is a pure protein (i.e. homogeneous, as established by standard tests which are used to assay the homogeneity of proteins). By electrophoresis, the molecular weight of ONCONASE is approximately 14,500 Daltons. Calculation of the molecular weight based upon the below listed amino acid sequence indicates that the molecular weight should be 11,819 Daltons. However, because metal ions may have bonded to the protein despite all efforts to remove them, and because different isotopes may be involved, the molecular weight of ONCONASE as determined by mass spectroscopy is 12,430 Daltons. In view of this discrepancy, the molecular weight of ONCONASE as determined by mass spectrometry will be considered to be approximately 12,000 Daltons. ONCONASE has an isoelectric point pI which is at least 9.5 and may be as high as 10.5. ONCONASE has a blocked amino terminal group and is essentially free of carbohydrates (as determined by anthrone and orcinol methods). ONCONASE has the following amino acid composition:

Amino Acid Analysis	
AMINO ACID RESIDUE	MOL % (24 HOUR ACID HYDROLYSIS)
Aspartic acid/Asparagine	13.39
Threonine	9.84 (Note 1)
Serine	8.08 (Note 1)
Glutamic acid/Glutamine	5.88
Proline	3.98
Glycine	2.98
Alanine	2.92

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Amino Acid Analysis	
AMINO ACID RESIDUE	MOL % (24 HOUR ACID HYDROLYSIS)
Cystine/2	7.77
Valine	7.77
Methionine	0.94
Isoleucine	5.29 (Note 2)
Leucine	4.95
Tyrosine	2.85
Phenylalanine	5.73
Histidine	2.99
Lysine	11.78
Arginine	2.85
Tryptophan	Not Determined (Note 3)
Approximate Total	99.99%

Note 1: Threonine and serine are partially destroyed during hydrolysis and this value is corrected for such partial destruction.

Note 2: This value is corrected for incomplete hydrolysis.

Note 3: Tryptophan cannot be detected in acid hydrolysis of proteins because it is destroyed and is consequently shown as Not Determined. However, analysis of the ultraviolet spectrum revealed the presence of one tryptophan residue per molecule.

Amino Acid Composition (as calculated from amino acid sequence)	
AMINO ACID	NUMBER OF RESIDUES PER MOLECULE OF MATERIAL
Aspartic acid	6
Asparagine	8
Threonine	10
Serine	8
Glutamic acid	3
Pyroglutamic acid	1
Glutamine	2
Proline	4
Glycine	3
Alanine	3
Cystine/2	8
Valine	8
Methionine	1
Isoleucine	6
Leucine	5
Tyrosine	3
Phenylalanine	6
Histidine	3
Lysine	12
Arginine	3
Tryptophan	1
Approximate Total	104

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ONCONASE has been sequenced. As is shown below, the total length of the sequence is 104 residues. The N-terminus of the protein is pyroglutamic acid (<Glu). This is a cyclized derivative of glutamic acid which is devoid of the free amino group necessary for direct sequencing and which therefore "blocks" the N-terminus of the protein.

When the shorter fragment described in U.S. Pat. No. 4,882,421 was cleaved with pyroglutamate aminopeptidase, pyroglutamic acid was removed from the shorter fragment, permitting sequencing to commence at the second residue. Such cleavage is a strong indication that the N-terminus is pyroglutamic acid since pyroglutamate aminopeptidase only cleaves pyroglutamic acid. The presence of pyroglutamic acid was further confirmed by mass spectrometry of the referenced shorter fragment. The molecular weight of this shorter fragment determined by mass spectrometry agreed well with the weight as calculated assuming that pyroglutamic acid was present and disagreed with the weight as calculated assuming that glutamic acid was present.

ONCONASE has the following amino acid sequence:

1	2	3	4	5	6	7	8	9	10
<Glu	Asp	Trp	Leu	Thr	Phe	Gln	Lys	Lys	His
11									20
Ile	Thr	Asn	Thr	Arg	Asp	Val	Asp	Cys	Asp
21									30
Asn	Ile	Met	Ser	Thr	Asn	Leu	Phe	His	Cys
31									40
Lys	Asp	Lys	Asn	Thr	Phe	Ile	Tyr	Ser	Arg
41									50
Pro	Glu	Pro	Val	Lys	Ala	Ile	Cys	Lys	Gly
51									60
Ile	Ile	Ala	Ser	Lys	Asn	Val	Leu	Thr	Thr
61									70
Ser	Glu	Phe	Tyr	Leu	Ser	Asp	Cys	Asn	Val
71									80
Thr	Ser	Arg	Pro	Cys	Lys	Tyr	Lys	Leu	Lys
81									90
Lys	Ser	Thr	Asn	Lys	Phe	Cys	Val	Thr	Cys
91									100
Glu	Asn	Gln	Ala	Pro	Val	His	Phe	Val	Gly
101									104
Val	Gly	Ser	Cys						

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i i i) NUMBER OF SEQUENCES: 1

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 104 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(i i) MOLECULE TYPE: protein

-continued

(i i i) HYPOTHETICAL: N

(i v) ANTI-SENSE: N

(v) FRAGMENT TYPE: N-terminal

(v i) ORIGINAL SOURCE:

(A) ORGANISM: *Rana pipiens*

(D) DEVELOPMENTAL STAGE: Embryo

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Glu	Asp	Trp	Leu	Thr	Phe	Gln	Lys	Lys	His	Ile	Thr	Asn	Thr	Arg	Asp
1				5					10					15	
Val	Asp	Cys	Asp	Asn	Ile	Met	Ser	Thr	Asn	Leu	Phe	His	Cys	Lys	Asp
			20					25					30		
Lys	Asn	Thr	Phe	Ile	Tyr	Ser	Arg	Pro	Glu	Pro	Val	Lys	Ala	Ile	Cys
			35				40					45			
Lys	Gly	Ile	Ile	Ala	Ser	Lys	Asn	Val	Leu	Thr	Thr	Ser	Glu	Phe	Tyr
	50					55					60				
Leu	Ser	Asp	Cys	Asn	Val	Thr	Ser	Arg	Pro	Cys	Lys	Tyr	Lys	Leu	Lys
65					70					75					80
Lys	Ser	Thr	Asn	Lys	Phe	Cys	Val	Thr	Cys	Glu	Asn	Gln	Ala	Pro	Val
				85					90					95	
His	Phe	Val	Gly	Val	Gly	Ser	Cys								
			100												

30

Although a preferred embodiment has been described above, the scope of the invention is limited only by the following claims:

We claim:

1. A bioactive pharmaceutical comprising a protein having the following amino acid sequence:

1	2	3	4	5	6	7	8	9	10
<Glu	—Asp	—Trp	—Leu	—Thr	—Phe	—Gln	—Lys	—Lys	—His
11								20	
Ile	—Thr	—Asn	—Thr	—Arg	—Asp	—Val	—Asp	—Cys	—Asp
21								30	
Asn	—Ile	—Met	—Ser	—Thr	—Asn	—Leu	—Phe	—His	—Cys
31								40	
Lys	—Asp	—Lys	—Asn	—Thr	—Phe	—Ile	—Tyr	—Ser	—Arg
41								50	
Pro	—Glu	—Pro	—Val	—Lys	—Ala	—Ile	—Cys	—Lys	—Gly
51								60	
Ile	—Ile	—Ala	—Ser	—Lys	—Asn	—Val	—Leu	—Thr	—Thr
61								70	
Ser	—Glu	—Phe	—Tyr	—Leu	—Ser	—Asp	—Cys	—Asn	—Val
71								80	
Thr	—Ser	—Arg	—Pro	—Cys	—Lys	—Tyr	—Lys	—Leu	—Lys
81								90	
Lys	—Ser	—Thr	—Asn	—Lys	—Phe	—Cys	—Val	—Thr	—Cys
91								100	
Glu	—Asn	—Gln	—Ala	—Pro	—Val	—His	—Phe	—Val	—Gly
101		104							
Val	—Gly	—Ser	—Cys						

and Cisplatin.

2. A bioactive pharmaceutical comprising a protein having the following amino acid sequence:

1	2	3	4	5	6	7	8	9	10
<Glu	—Asp	—Trp	—Leu	—Thr	—Phe	—Gln	—Lys	—Lys	—His
11								20	
Ile	—Thr	—Asn	—Thr	—Arg	—Asp	—Val	—Asp	—Cys	—Asp
21								30	
Asn	—Ile	—Met	—Ser	—Thr	—Asn	—Leu	—Phe	—His	—Cys
31								40	
Lys	—Asp	—Lys	—Asn	—Thr	—Phe	—Ile	—Tyr	—Ser	—Arg
41								50	
Pro	—Glu	—Pro	—Val	—Lys	—Ala	—Ile	—Cys	—Lys	—Gly
51								60	
Ile	—Ile	—Ala	—Ser	—Lys	—Asn	—Val	—Leu	—Thr	—Thr
61								70	
Ser	—Glu	—Phe	—Tyr	—Leu	—Ser	—Asp	—Cys	—Asn	—Val
71								80	
Thr	—Ser	—Arg	—Pro	—Cys	—Lys	—Tyr	—Lys	—Leu	—Lys
81								90	
Lys	—Ser	—Thr	—Asn	—Lys	—Phe	—Cys	—Val	—Thr	—Cys
91								100	
Glu	—Asn	—Gln	—Ala	—Pro	—Val	—His	—Phe	—Val	—Gly
101		104							
Val	—Gly	—Ser	—Cys						

and 4-[bis (2-chloroethyl) amino]-L-phenylalanine.

3. A bioactive pharmaceutical comprising a protein having the following amino acid sequence:

1	2	3	4	5	6	7	8	9	10
<Glu	—Asp	—Trp	—Leu	—Thr	—Phe	—Gln	—Lys	—Lys	—His
11								20	
Ile	—Thr	—Asn	—Thr	—Arg	—Asp	—Val	—Asp	—Cys	—Asp

9
-continued

21 Asn—Ile—Met—Ser—Thr—Asn—Leu—Phe—His—Cys— 30
31 Lys—Asp—Lys—Asn—Thr—Phe—Ile—Tyr—Ser—Arg— 40
41 Pro—Glu—Pro—Val—Lys—Ala—Ile—Cys—Lys—Gly— 50
51 Ile—Ile—Ala—Ser—Lys—Asn—Val—Leu—Thr—Thr— 60
61 Ser—Glu—Phe—Tyr—Leu—Ser—Asp—Cys—Asn—Val— 70
71 Thr—Ser—Arg—Pro—Cys—Lys—Tyr—Lys—Leu—Lys— 80

10
-continued

81 Lys—Ser—Thr—Asn—Lys—Phe—Cys—Val—Thr—Cys— 90
5 91 Glu—Asn—Gln—Ala—Pro—Val—His—Phe—Val—Gly— 100
101 Val—Gly—Ser—Cys 104

10

and Doxorubicin HCl, USP.

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