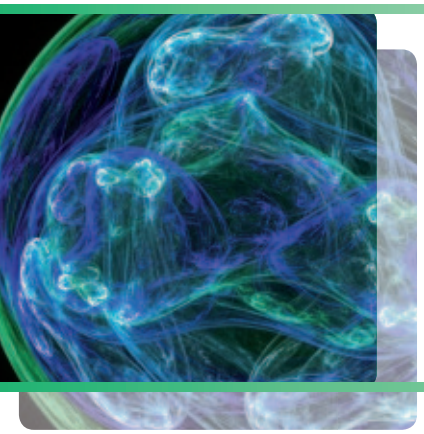

Necrosis & Necroptosis Inhibitors

Necrosis

Cell death has traditionally been subdivided into regulated (apoptosis) and unregulated (necrosis) forms. While apoptosis has always been recognized to be a pathway of highly orchestrated signaling events, necrosis or necrotic cell death is known as a fortuitous and unregulated means of cell death that is induced by nonspecific and nonphysiological stress. Due to the absence of a common biochemical denominator, necrosis is still largely identified in negative terms by the absence of apoptotic or autophagic markers. Nowadays, necrosis is morphologically characterized by a gain in cell volume (oncosis), swelling of organelles, plasma membrane rupture and subsequent loss of intracellular contents. For a review see [1].

Necroptosis

Necroptosis is considered as a specialized biochemical pathway of programmed necrosis that depends on the serine/threonine kinase activity of RIP1 (receptor-interacting protein 1) [1, 2, 3]. It can be avoided by inhibiting RIP1 [1]. Stimulation of death receptors upon ligation by TNF-α, FasL and TRAIL under apoptosis-deficient conditions, in particular in the presence of caspase inhibitors, may induce necroptosis [2, 4]. Furthermore, phosphorylation-driven assembly of the RIP1-RIP3 necrosis complex seems to regulate necroptosis [5]. Although it occurs under regulated conditions, the cell morphology of necroptosis is very similar to that of necrosis [6]. Necroptosis is an important cellular death mechanism likely to be involved in many human pathologies from viral infections to neurodegenerative diseases [4].

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[2] Identification of a molecular signaling network that regulates a cellular necrotic cell death pathway: J. Hitomi, et al.; Cell **135,** 1311 (2008)

[3] Necroptosis: a specialized pathway of programmed necrosis: L. Galluzzi, et al.; Cell **135,** 1161 (2008)

[4] Necroptosis as an alternative form of programmed cell death: D. E. Christofferson & J. Yuan; Curr. Opin. Cell Biol. **Epub ahead of print**

[5] Phosphorylation-driven assembly of the RIP1-RIP3 complex regulates programmed necrosis and virus-induced inflammation: Y. S. Cho, et al.; Cell **137,** 1112 (2009)

[6] Chemical inhibitor of nonapoptotic cell death with therapeutic potential for ischemic brain injury: A. Degterev, et al.; Nat. Chem. Biol. **1,** 112 (2005)

Special Feature

NecroX™ – A Novel Class of Small Molecule Necrosis Inhibitors

- Orally available small molecules
- Good *in vivo* safety profile
- Low cellular toxicity
- Broad application spectrum against necrotic insults including chemicals, fatty acids, free radicals and ethanol

SEE NEXT PAGE

NecroX™ – A Novel Class of Small Molecule Necrosis Inhibitors

There is extensive evidence that necrosis plays a prominent role in a wide range of human pathological conditions, such as myocardial infarct, ischemic injury and neurodegeneration. Therefore, development of necrosis inhibitors is of high interest. Very little attempt, however, has been made to develop therapeutic agents to specifically target necrosis because of the conventional notion that, unlike apoptosis, necrosis is unregulated.

LG Life Sciences, Inc. recently identified a series of necrosis inhibitors, called NecroX™, a novel class of small molecules with strong potential as therapeutic candidates. Possible target indications are necrosis related diseases, liver diseases & fibrosis, ischemia & reperfusion injury and neurodegenerative diseases. In addition, the compounds are investigated in other diverse applications, like cell therapy, research reagents and skin care.

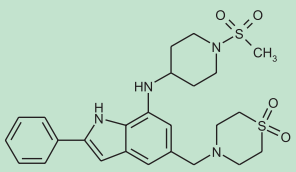
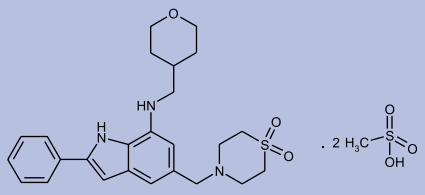
Key Features of NecroX™-Compounds

- **Orally available small molecules**
- **Good *in vivo* safety profile**
- **Low cellular toxicity**
- **Broad application spectrum against necrotic insults including chemicals, fatty acids, free radicals and ethanol**

Research Areas/Applications

- ***In vitro* studies: neurotoxic, cold stress, hypoxic injury and oxidative stress (ROS/RNS)-induced necrosis**
- ***In vivo* studies: necrosis-related pathology such as ischemia/reperfusion injury-inducing organ failure, stroke, myocardial infarct and neurodegenerative diseases**
- ***Ex vivo* studies: protection of organ damage in cold storage conditions**

Overview on Selected NecroX™-Compounds

Product	NecroX™-2	NecroX™-5
Product Nr. / Size	ALX-430-166-M001 1 mg ALX-430-166-M005 5 mg	ALX-430-167-M001 1 mg ALX-430-167-M005 5 mg
Structure		
Purity	≥96% (HPLC)	≥96% (HPLC)
Application	*In vitro* studies	*In vivo* studies

Applications for the NecroX™ - Series of Compounds

- ***In vitro* Cell-based Assays:**
  - Cold Stress
  - Hypoxia
  - Oxidative Stress
- ***In vivo* Necrosis Animal Models for Liver Diseases:**
  - DILI (Acetaminophen, CCl_4)
  - CCl_4 induced Liver Fibrosis/Cirrhosis
- **Ischemia & Reperfusion Injury:**
  - Stroke
  - Myocardial Infarction
  - Acute Pancreatitis & Acute Tubular Necrosis
- **Cell Media:**
  - Cell Therapy
  - CHO Growth

Overview on Selected Results

Protective Effect against Cold Stress-induced Necrotic Cell Death

Procedure:

a) LB-HEL (human lung fibroblast) cell plating in DMEM + 10% FBS

b) Compounds pre-incubation for 1h

c) Cold stress (4°C) for 24 h and rewarming (37°C) for 24 h

d) Viability test by WST-1 assay

Cell type	Pan-caspase Inhibitor	Necrostatin-1*	NecroX™-2	NecroX™-5
LB-HEL	>10	10>	0.243	0.14

* Necrostatin-1: necroptosis inhibitor

NecroX™-2 / NecroX™-5

Anti-oxidant Activity Profiles - DPPH & DHR123 Assay & $ONOO^-$ Scavenging

	NecroX™-2	NecroX™-5
DHR123 oxidation assay using H9C2 (IC_{25}/μM)**	<0.1	0.04
LDH assay using H9C2 (IC_{25}/μM)***	<0.2	0.05
In vitro DPPH assay (IC_{50}/μM)*	17	22
In vitro $ONOO^-$ scavenging activity (IC_{50}/μM)	0.9	n.d.

* Trolox was used as reference in DPPH assay (Trolox IC_{50}=15.5)
** 1.25μM DHR123 used. DMEM +0.1% FBS used for H9C2 culture (DHR123 - Used as an indicator of peroxynitrite formation - one of the most widely used ROS probes for intracellular measurement)
*** DMEM + 10% FBS used for H9C2 culture.

NecroX™-2 / NecroX™-5

Additives in Organ Preservation Solution: Protection against Cold Stress in HTK using Hepatocytes

Procedure:

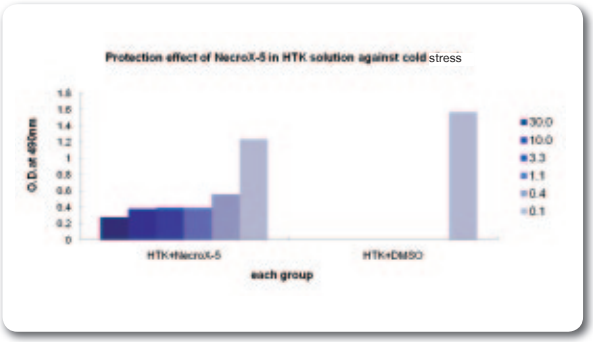
a) Attachment of rat hepatocytes for 24 h

b) Medium change into HTK solution containing NecroX™-5

c) Cold stress (4°C) for 20 h

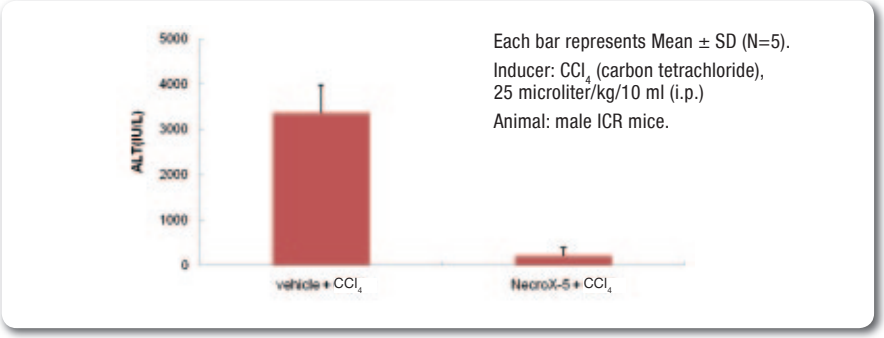
d) Viability assay by LDH

NecroX™-5 prevented necrosis induced by cold stress *in vitro.* This experiment done in HTK solution which is cold organ preservation solution.



Protective Effect of NecroX™-5 against CCl_4-induced Liver Injury in 24h-acute Mice Model

Oral administration of NecroX™-5 (50 mpk), 3 hours prior to treatment of CCl_4, showed hepatic protective effect in 24h-acute mice model. No protective effect was observed by administration of caspase inhibitor (data not shown).



In Vitro Studies: Protective effect on tBHP-induced Necrotic Cell Death

NecroX™-5 inhibits necrosis induced by tBHP.

	Cell Death Inhibition IC_{50} (μM)			
Cell types	**Pan-caspase Inhibitor**	**Necrostatin-1**	**3-MA**	**NecroX™-5**
Primary rat chondrocytes**	>30	20	>30	<0.1
LLC-PK1 (renal cells)***	>30	>30	>30	0.45
SK-N-MC (neuronal cells)***	>30	16	>30	<0.1
RINm5F (pancreatic beta cells)**	>30	6.1	n.d.	<0.1

, * were assessed by SRB and LDH method, respectively
tBHP: tert. butyl hydroperoxide
3-MA: 3-methyladenine (autophagy inhibitor)
Necrostatin-1: necroptosis inhibitor
SK-N-MC: human neuroblastoma cell line

NecroX™-5

Other Necrosis & Necroptosis Inhibitors

Necrostatins

A specific and potent small-molecule inhibitor of necroptosis, necrostatin-1 (Nec-1) has been identified by screening for chemical inhibitors of necrotic cell death in U937 cells induced by TNF-α in the presence of caspase inhibitors [1]. Nowadays, several structurally and functionally distinct necrostatins and corresponding modifications [2] have been reported. The initial study showed that Nec-1 inhibits RIP kinase-induced necroptosis. This finding was confirmed later, when RIP1 kinase was identified as a specific cellular target of Nec-1, Nec-3 [3] and Nec-5 [4], but through distinct mechanisms [5]. Nec-1 was shown to inhibit RIP1 kinase in a T-loop-dependent manner, binding preferentially to and stabilizing the inactive conformation of RIP1 kinase, thereby shifting the equilibrium toward the inactive state [5]. In contrary to the mechanism of Nec-1 it was shown that Nec-5 inhibits RIP1 kinase indirectly [5]. Those findings underline the critical role of active RIP1 kinase for necroptosis.

Compound	EC_{50}*
Necrostatin-1	0.49 µM
Necrostatin-5	0.24 µM
Necrostatin-7	10.6 µM

*EC_{50} determinations for inhibition of necroptosis in FADD-deficient Jurkat T cells treated with TNF-α [2, 4, 6]

As for Nec-5, the biological activity of Nec-7 is different from that of Nec-1 as Nec-7 does not inhibit RIP1 kinase [6]. This suggests the possibility that Nec-7 may target an additional regulatory molecule in the pathway of necroptosis [6].

Nec-1i has been described as an inactive control compound [1].

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[1] Chemical inhibitor of nonapoptotic cell death with therapeutic potential for ischemic brain injury: A. Degterev, et al.; Nat. Chem. Biol. **1,** 112 (2005)

[2] Structure-activity relationship study of novel necroptosis inhibitors: X. Teng, et al.; Bioorg. Med. Chem. Lett. **15,** 5039 (2005)

[3] Structure-activity relationship study of tricyclic necroptosis inhibitors: P. G. Jagtap, et al.; J. Med. Chem. **50,** 1886 (2007)

[4] Structure-activity relationship analysis of a novel necroptosis inhibitor, Necrostatin-5: K. Wang, et al.; Bioorg. Med. Chem. Lett. **17,** 1455 (2007)

[5] Identification of RIP1 kinase as a specific cellular target of necrostatins: A. Degterev, et al.; Nat. Chem. Biol. **4,** 313 (2008)

[6] Structure-activity relationship study of a novel necroptosis inhibitor, necrostatin-7: W. Zheng, et al.; Bioorg. Med. Chem. Lett. **18,** 4932 (2008)

Necrostatin-1

[Nec-1; 5-(1H-Indol-3-ylmethyl)-(2-thio-3-methyl)hydantoin; MTH-Trp; Methyl-thiohydantoin tryptophan]

BML-AP309-0020	20 mg
BML-AP309-0100	100 mg

Necroptosis inhibitor.

LIT: A. Degterev, et al.; Nat. Chem. Biol. **1,** 112 (2005) ▪ X. Xu, et al.; J. Neurochem. **103,** 2004 (2007) ▪ B. R. Stockwell & N. M. Gangadhar; Curr. Opin. Chem. Biol. **11,** 83 (2007) ▪ C. C. Smith, et al.; Cardiovasc. Drugs Ther. **21,** 227 (2007) ▪ O. Takikawa; Int. Congr. Ser. **1304,** 290 (2007) ▪ A. Fontanini, et al.; J. Biol. Chem. **284,** 8369 (2009)

Necrostatin-5

[Nec-5; 3-p-Methoxyphenyl-5,6-tetramethylenothieno[2,3-d] pyrimidin-4-one-2-mercaptoethylcyanide]

ALX-430-169-M005	5 mg
ALX-430-169-M025	25 mg

Necroptosis inhibitor. In contrary to the mechanism of necrostatin-1 (Prod.No. BML-AP309) it was shown that necrostatin-5 inhibits RIP1 kinase indirectly. A potent inhibitor of immunoprecipitated, but not recombinant RIP1.

LIT: Structure-activity relationship analysis of a novel necroptosis inhibitor, Necrostatin-5 : K. Wang, et al.; Bioorg. Med. Chem. Lett. **17,** 1455 (2007) ▪ Idenfication of RIP1 kinase as a specific cellular target of necrostatins: A. Degterev, et al.; Nat. Chem. Biol. **4,** 313 (2008)

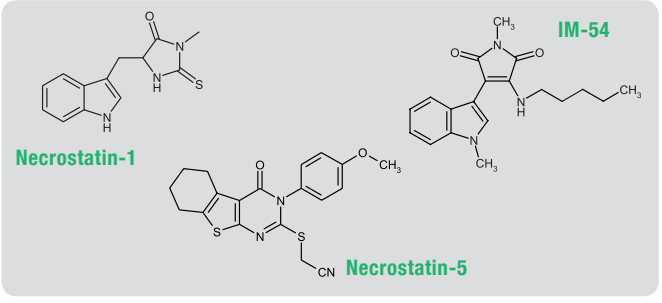
IM-54

[2-(1H-Indol-3-yl)-3-pentylamino-maleimide]

ALX-430-137-M001	1 mg
ALX-430-137-M005	5 mg
ALX-430-137-M025	25 mg

Cell permeable necrosis inhibitor. Selectively blocks oxidative stress-induced necrotic cell death (~3µM). Does not protect against etoposide-induced apoptosis.

LIT: K. Dodo, et al.; Bioorg. Med. Chem. Lett. **15,** 3114 (2005)



NecroX™ Crowdsourcing Program
(Short Paper Writing Competition with NecroX™)

What is NecroX™

It refers to a new class of small molecules that has a strong potential as therapeutic candidates. Possible target indications are necrosis-related diseases, liver diseases & fibrosis, ischemia & reperfusion injury and neurodegenerative diseases. In addition, the compounds are investigated in other diverse applications such as in cell therapy and skin care.

What is Crowdsourcing?

Crowdsourcing is an act of outsourcing tasks, traditionally performed by an employee or contractor to a large group of people or community (a crowd), through an open call.

The most famous crowdsourcing case study can be found in the example of Goldcorp.

Goldcorp is a Toronto-based gold mining company which was at a risk of failing from its 50 year old gold mine. Most analysts assumed that without discovery of substantial new gold deposits, Goldcorp was likely to fold up the business. New CEO was in need of a miracle. He attempted his searching for a miracle from the crowds through an open-call, crowdsourcing. The new CEO revealed his geological data on the web for the public to see and challenged the world to carry out the prospection. The attempt led to a triumph. From the crowds that consisted of more than 1,000 prospectors, Goldcorp emerged from the grave and continued its gold-mine legacy from once told as `dying gold mine`.

New drug development is very similar to gold mining, especially if it is related to innovative compounds such as NecroX™. We consider that implementing crowdsourcing into pharmaceutical industry would resolve a dilemma that is associated with low R&D productivity problems that many of modern pharmaceutical companies suffer.

Now, LG Life Sciences, Ltd. is inviting scientists around the globe to NecroX™ Crowdsourcing Program. Scientists are welcomed to apply NecroX™ into their existing system and if the end results are practical, researchers may send us the short paper using the template from website. Our intension is solely limited to the evidence of NecroX™ in the application areas and not expanded to further research without correspondents' agreements. Scientists are not restricted from publishing and chartering patent with any impediments.

For more information please visit **NecroX.lgls.com**.

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