Review

THE TOXICOLOGY OF BIOREGULATORS AS POTENTIAL AGENTS OF BIOTERRORISM*

Slavko BOKAN

Ministry of Defence of the Republic of Croatia, Croatian Military Academy, Zagreb, Croatia

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Bioregulators or modulators are biochemical compounds such as peptides, that occur naturally in organisms. Advances in biotechnology create the potential for the misuse of peptide bioregulators in offensive biological weapons programmes. Bioregulators are a new class of weapons that can damage the nervous system, alter mood, trigger psychological changes and kill. Over the last twenty years, neuroscience has produced an explosion of knowledge about receptor systems in the nerve cells that are of critical importance in receiving chemical transmitter substances released by other nerve cells. Bioregulators are closely related to substances normally found in the body that regulates normal biological processes. The potential military or terrorist use of bioregulators is similar to that of toxins. Together with increased research into toxins, the bioregulators have also been studied and synthesized. This paper presents a review of bioregulators that could be used in terrorist or other hostile activities.

KEY WORDS: *algogens, criteria for the selection of bioregulators, peptide bioregulators, physical incapacitation, public health preparedness, terrorism*

Many biological agents have the capacity to cause diseases and can be used to threaten civilian populations (1). The purpose of this paper is to provide information on bioregulators to health-care providers at all levels, to help them make informed decisions on protection from these agents. Bioregulators can act as neurotransmitters and can modify neural response. Bioregulators can be used to cause pain, as anaesthetics and to affect blood pressure.

These substances can also be modified synthetically, whereupon they may obtain new properties. It is feasible to produce some of these compounds by chemical synthesis. The past decade has brought an enormous new knowledge about the pharmacology and structural biology of receptors (2).

In the last ten years, considerable advances have taken place in this *in vitro* synthesis of peptides and large quantities of various pharmaceutical peptides are already available commercially. Synthetic derivates or slightly modified forms of these compounds can have drastically altered toxic effects, and these could be important in the development of new agents. Recent years have seen a rapid advance in the discovery of new bioregulators, especially of the incapacitating ones, in the understanding of their mode of action and synthetic routes for manufacture. Some of these compounds may be many hundreds of times more potent than the traditional chemical warfare agents. Some very important characteristics of new bioregulators that would offer significant military advantages are novel sites of toxic action; rapid and specific effects; penetration of protective filters and equipment, and militarily effective physical incapacitation. Peptide bioregulators are interesting regulatory molecules for many reasons. Their range of activity covers the entire living system, from mental processes (e.g. endorphins) to many aspects of health such as control of mood, consciousness, temperature control, heart rate, immune responses, sleep, or emotions, exerting regulatory effects on the body. As

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such, they are produced in very small quantities that are essential for the normal homeostatic functions of the body. They are also capable of regulating a wide range of physiological activities, such as bronchial and vascular tone and muscle contraction. This opens an unprecedented possibility to use toxic substances that could not be traced in human body. In any case, a clandestine application of such substances can lead to death - "killing without a trace".

It seems that we have discovered the majority of the "most toxic" (LD_{50}<0.0025 mg kg^{-1}) naturally occurring toxins and bioregulators. However, bioregulators are still considered to be less suitable for dispersal on a large scale. Nonetheless, they could be used for sabotage or in or for special purposes, e.g., against key persons. Since bioregulators have low volatility, they are dispersed as aerosols and then taken up mostly through inhalation. The new microencapsulation technology, which is easy to use, makes it possible to protect unstable toxins when dispersed. In recent years, discussions have started on the risk of bioregulators being used as chemical warfare agents. These types of substances do not belong to the group of toxins but are, nonetheless, grouped with them since their possible use is similar. They are closely related to substances normally found in the body and may be algogenic (causing pain), anaesthetic, or affecting blood pressure. They are active at extremely low doses and frequently have rapid effect.

There are still many unknowns regarding bioregulators and their weaponisation. A Mass Casualty Biological (Toxin) Weapon (MCBTW) is any biological or toxin weapon capable of causing death or disease on a large scale, such that the military or civilian infrastructure of the state or organization being attacked is overwhelmed. A militarily significant (or terrorist) weapon is any weapon capable of affecting - directly or indirectly, physically or through psychological impact - the outcome of a military operation. The advances in the use of viral and bacterial vectors enhance the possibility for direct delivery of a toxin or bioregulator to the human target or they could be used to transfer the toxin or bioregulator genes to the target.

Table 1 *Rating of the listed bioregulators according to criteria explained in the text*

Bioregulator	Rating criteria										
	(1) High morbidity	(2) High level of mortality/ incapacity	(3) Stability in the environment	(4) Ease of production	(5) High level of dissemination	(6) High toxicity	(7) High level of intoxication	(8) No effective prophylaxis and antidote therapy	(9) Difficulty of detection/ identification	(10) Public health preparedness	Total score
Endorphins (α, β, and δ-Endorphin)	++	+++	+	+	+++	+++	+++	+	+	+++	21
Substance P (SP) (Neurokinin)	++	+++	+	+	+++	+++	+++	+	+	+++	21
Endothelins (ET-1, ET-2, ET-3) or Sarafotoxins (S6a, S6b)	++	+++	+	+	+++	+++	+++	+	+	+++	21
Bradykinin (Kinin-9, Kallidin)	++	+++	+	+	+++	+++	+++	+	+	+++	21
Vasopressin (VP)	++	+++	+	+	+++	+++	+++	+	+	+++	21
Angiotensins (I, II, III)	++	+++	+	+	+++	+++	+++	+	+	+++	21
Enkephalins (Leu- and Met-enkephalin)	++	+++	+	+	+++	+++	+++	+	+	+++	21
Somatostatin (SS, SRIF)	++	++	+	+	+++	+++	+++	+	+	+++	20
Bombesin (BN)	++	++	+	+	+++	+++	+++	+	+	+++	20
Neurotensin	++	++	+	+	+++	+++	+++	+	+	+++	20

RATING AND CRITERIA QUALIFYING BIOREGULATORS AS POTENTIAL TERRORIST AGENTS

From the point of view of public health, bioregulators which are less known must be evaluated and prioritised in order to assure appropriate allocation of limited funds and resources within public health systems. The criteria for rating bioregulators as potential terrorist agents have been adopted from the existing criteria for toxin warfare and terrorism agents**)**. Table 1 shows the rating of individual bioregulators discussed in this paper, which is based on the following criteria (3):

1. High level of morbidity: higher rating (++) if clinical disease requires hospitalisation for treatment including supportive care, and lower rating (+) if outpatient treatment is possible for most cases;
2. High level of mortality or incapacity: agents with an expected mortality of ≥50 % were rated higher (+++), and those with the expected mortality of 21-49 % and <21 % were rated lower (++ and +, respectively);
3. Stability in the environment after release (+);
4. Ease of production and transportation (+);
5. Likely methods of terrorist usage and high level of dissemination or contamination by aerosol for respiratory exposure (+++), contamination in quantities that could affect large populations (++), and dissemination potential for sabotage in food and water supply (+);
6. High toxicity or potency or low toxic dose: LD_{50} <0.000025 mg kg^{-1} (+++), LD_{50} from 0.000025-0.0025 mg kg^{-1} (++), and LD_{50} >0.0025 mg kg^{-1} (+);
7. High level of intoxication by route: oral (+), respiratory (++), or both (+++);
8. No effective prophylactics and/or antidote therapy (+);
9. Difficult early diagnosis or identification, even by advanced laboratory diagnostics (+).
10. Public health preparedness required, including stockpiling of therapeutic drugs (+), enhanced surveillance and education (+), and advanced laboratory diagnostics (+).

Endorphins

Endorphins are small-chain peptides which activate opiate receptors, producing a feeling of wellbeing and increasing tolerance to pain. These compounds are hundreds or even thousands of times more potent than morphine on a molar basis. Because of this potency, their concentrations *in vivo* are low, and it has taken recent advances in experimental neuroscience to elucidate the chemistries of these hormones. A term used to denote endorphins is *opioid peptides*. Proopiomelanocortin - POMC (pro-ACTH-Endorphin) - is a glycosylated 31 kDa protein precursor posttranslational processing of which yields several neuroactive peptides upon specific cleavage and possibly a great number of as yet unidentified small peptides that may be pharmacologically active. Endorphins can further decompose to small fragments (oligomers) which are still active, and which pass the blood-brain barrier more readily. Their high activity and specificity make endorphins attractive compounds from a clinical point of view, but most are active only if injected into the blood (or the cerebrospinal fluid). This is because peptides are digested in the stomach, decomposed by proteolytic and other enzymes. Moreover, they have difficulty passing into the brain because of their size and structure. Thus, despite the low oral to parenteral ratio of many morphine derivatives, they will probably not be replaced by small-chain peptides anytime soon. Dipeptidyl carboxypeptidase, enkephalinases, angiotensinases, and other enzymes accomplish enzymatic degradation of small-chain endorphins.

POMC cleavage products include a large N-terminal fragment, which yields γ-MSH (melanocyte stimulating hormone-gamma) and possibly other unidentified cleavage products, ACTH (corticotropin, 39 amino acids), lipotropin, α-MSH (melanocyte stimulating hormone-alpha, melanotropin, acetylated and amidated ACTH 1-13), β-MSH (melanocyte stimulating hormone-beta) and β-endorphin. Individual products of the POMC protein act on immune cells and can be produced by them, thus establishing close links between immune cells and the nervous system. Endorphin molecules have a separate nomenclature (α, β, γ) that denotes their stereochemistry.

Beta-endorphin (and its derivates α-endorphin and γ-endorphin) is also produced by macrophages and lymphocytes. Beta-endorphin appears to act differentially; its C-terminal moiety enhances T-cell proliferation whereas this stimulatory effect can be prevented by peptides that possess the N-terminal enkephalin sequence. Human β-endorphin is the most potent of three stereoscopic variants, and has the same sequence as the C-terminal end of β-lipotropin.

Endorphins enhance the natural cytotoxicity of lymphocytes and macrophages to tumor cells, stimulate human peripheral blood mononuclear cell chemotaxis and inhibit the production of T-cell chemotactic factors. Opiate receptors presynaptically inhibit the transmission of excitatory pathways including acetylcholine, catecholamines, serotonin, and substance P, a neuropeptide active in pain neurons. Endorphins may also be involved in glucose regulation.

Substance P

Substance P (P stands for powder, the designation originating from early studies using powdered extracts of equine brain and intestines) is also known as neurokinin-1. It is a member of a family of proteins known as tachykinins. This neuropeptide was found in the gut as well as in the brain. It is responsible for a number of excitatory effects on both central and peripheral neurons. It contracts smooth muscles, constricts bronchioles and increases capillary permeability. When released from afferent nerves, it causes a neurogenic inflammatory response, including mast cell degranulation. Substance P is a polypeptide (molecular weight: 1350 Da) active in doses of less than one microgramme. It causes a rapid loss of blood pressure, which in turn may cause unconsciousness (4).

Endothelins (ET) or endothelium-derived contracting factors (EDCFs)

Endothelins are a family of closely related peptides of 21 amino acids with two disulfide bonds. The four known species are isoforms encoded by four different genes. The isoforms are called endothelin-1 (ET-1), endothelin-2 (ET-2), endothelin-3 (ET-3) and vasoactive intestinal contractor (VIC).

Endothelin is a highly potent vasoconstrictor peptide first isolated from porcine endothelial cell supernatant. Varying amounts of ET are also produced in other cell types such as smooth muscle, neuron, mesangium, melanocyte, parathyroid and amnion. Individual ET may posses different physiological or pathophysiological roles in different target tissues. The secretion of ET is stimulated by epinephrine, angiotensin II, arginine vasopressin, transforming growth factor-beta, thrombin, interleukin-1, and hypoxia. Endothelins stimulate the contraction of many smooth muscles including blood vessels, uterus, bladder, and intestines. ET-1 is the most potent vasoconstrictor peptide yet discovered.

Numerous studies implicate endothelins in cardiovascular diseases such as hypertension, heart failure, and atherosclerosis. Endothelin levels are elevated in atherosclerosis, congestive cardiac failure and renal insufficiency. ET may play an important role in homeostatic hemodynamic balance. Endogenous endothelins and ET receptor subtypes are present in various endocrine organs. ET appears to modulate the secretion of prolactin, gonadotropins, growth hormone (GH) and thyroid stimulating hormone (TSH). It may also act as a neurotransmitter. Among endothelins, ET-1 is the most studied compound. The therapeutic potential of endothelins has generated a tremendous interest in numerous laboratories around the world. The structure of a recently isolated snake venom sarafotoxin (namely, Sarafotoxin S6a and S6b) bears a striking resemblance to endothelins. They (endothelins and sarafotoxins) are vasoconstrictors and potent coronary constrictors. They can cause heart arrest in several minutes at an LD_{50} value of 15 mg kg^{-1} (5).

Bradykinin (Kinin-9, Kallidin)

Bradykinin is the final product of the kinin system and is split from a serum α-2-globulin precursor by kallikreins and also by trypsin or plasmin. Bradykinin reduces blood pressure by dilating blood vessels. In bronchial smooth muscles and also in the intestines and the uterus, bradykinin leads to muscle contraction. Bradykinin is also one the most potent known substances inducing pain. It causes hypotension and the contraction of smooth muscles, and increases vascular permeability. It also plays a role in pain pathways and inflammation. Bradykinin antagonists are used for treating inflammations, pain, rheumatic arthritis, osteoarthritis, pancreatitis, rhinitis, asthma and gout. Bradykinin is a potent stimulator of smooth muscle contraction, inducing hypotension, increasing blood flow and the permeability of capillaries (6-8).

Vasopressin

This protein is also called antidiuretic hormone (ADH), adiuretin, vasotocin, pituitrin P and pitressin. It is a cyclic nonapeptide synthesized in the hypothalamus and stored in the posterior lobe of the pituitary, from which it is released into the circulation. Vasopressin stimulates ATCH release, improves memory and learning capacity, reduces the

pressure in the pulmonary arteries and reduces renin and angiotensin-converting enzyme (ACE) activity. Vasopressin regulates the osmotic pressure in body fluids via a specific vasopressor receptor (V1). It has a direct antidiuretic effect on the kidney, mediated by the antidiuretic receptor V2, and promotes re-adsorption of water in the distal convoluted tubules of the kidney. It also causes vasoconstriction in peripheral small blood vessels by stimulating smooth muscle cells in the cell walls to contract.

Angiotensins

Angiotensin is a decapeptide originally found to be produced by kidney-derived renin from an α-2 hepatic globulin. It is mainly known for its potent pharmacological activities. Angiotensin elevates blood pressure through its direct vasoconstricting, sympathomimetic, and (through release of aldosterone) sodium-retaining action.

Angiotensins are formed in biological fluids by the enzymatic cleavage of proteins. The species-specific enzyme renin, which can be generated by kallikrein from inactive prorenin, is responsible for the formation of angiotensin I (AT I) from globulin angiotensinogen (ATG). AT I, which has no effect on the blood pressure, is split by the membrane bound ACE to form angiotensin II (AT II).

Angiotensin II is a very potent vasoconstrictor and acts directly on the adrenal gland to stimulate the release of aldosterone. The inhibition of ACE results in a double hypotensive effect because both the formation of blood-pressure-raising AT II and the degradation of the blood-pressure-lowering kinin are inhibited. AT II agonists are used for the treatment of shock and collapse in which normal blood pressure could be restored as quickly as possible, while ACE inhibitors and AT II antagonists are applied for the treatment of hypertension (9, 10).

Enkephalins

These compounds comprise the basis for the body's own pain fighting mechanisms. The enkephalins are found in many areas of the body. Changes in these compounds and their metabolism have been associated with different headache disorders.

Two 5-peptide enkephalins have been identified. One terminates in a leucine, and is known as Leu-enkephalin; the other terminates in a methionine, and is called Met-enkephalin. The enkephalins are relatively weak analgesics, which activate all opioid receptors, but appear to have the highest affinity for the d-receptor. Apart from the nervous tissue, enkephalins have been identified in many other organ systems, including the gut, sympathetic nervous system, and adrenal glands. In the central nervous system, enkephalins have been found in many areas, but predominantly in those associated with nociception (e.g. periaquaductal grey matter and dorsal horn). Their precursor molecule is proenkephalin and they are rapidly degraded by enkephalinase. An investigation into why the human brain should have receptor sites for alkaloids from the opium poppy led to the discovery of a family of natural painkillers, the endorphins (from endogenous morphines). These substances are oligopeptides, containing from 5 to 30 amino acids.

Somatostatin

Somatostatin, known also as somatotropin release inhibiting hormone (SIH), is a peptide of 14 amino acids found in the hypothalamus and central and peripheral nervous system. Angiopeptin is a stable analogue of SRIF. Somatostatin (SRIF) is formed as prepro-SRIF. The main product of gene expression is pro-SRIF-(1-64), which is processed at the C-terminus to form SRIF-28 and SRIF-14. SRIF and SRIF-like substances have been found in the hypothalamus, central and peripheral nervous system and gastrointestinal tract. The main biological effect of SRIF is to inhibit the release of the growth hormone, thyroid stimulating Hormone (TSH), prolactin, corticotropin-releasing hormone (CRH), insulin, glucagon, vasoactive intestinal peptide (VIP), secretin, pancreatic polypeptide, gastrin-releasing peptide, gastrin, cholecystokinin (CCK) and motilin. A possible role for SRIF in affective disorders is suggested by its low concentration in cerebrospinal fluid of patients with depression. Somatostatin in the brain might be involved in the therapeutic effects of some antidepressant drugs.

Bombesin

Bombesin is a tetrapeptide originally isolated from the skins of the amphibians *Bombina bombina* and *Bombina variegata variegata*. It is distributed in the central nervous system, gastrointestinal tract and peripheral tissues. Bombesin and bombesin-like factors show a wide spectrum of biological activities. These include regulating contraction of smooth muscle cells and inducing secretion of neuropeptides

and hormones. Bombesin increases the plasma levels of gastrin, CCK, glucagon, insulin, pancreatic peptide, VIP and many other gastrointestinal peptides. The C-terminal nonapeptide of bombesin has the minimum length with the maximum effect. Bombesin is used as a diagnostic aid in the gastrin stimulation test. It is a potent stimulant of gastric acid secretion and exerts strong biological activity in the central nervous system.

Neurotensin

Neurotensin is a 13 amino acid peptide isolated from bovine hypothalamus. It causes hypotension in the rat and its smooth muscle actions include the relaxation of the rat duodenum and the contraction of guinea pig ileum and rat uterus. Neurotensin may also act as a CNS neurotransmitter. Certain regions involved with the memory function in the brains of the Alzheimer's disease patients have been found to be neurotensin deficient. This peptide may also be involved in the pathophysiology of the Parkinson's disease and schizophrenia.

CONCLUSION

Although many biological agents such as bioregulators can be used to cause illness, there are only a few that can truly threaten civilian populations on a large scale. If released, these agents could pose the most significant challenge for public health and medical responses. The criteria for rating bioregulators could be used to prioritise threats in national public health preparedness against bioterrorism. Having a defined method for evaluating biological threat agents allows for a more objective evaluation of new potential threat agents, as well as for a continued re-evaluation of established threat agents. Using this prioritisation method could provide a focus to public health activities related to detection and response to bioterrorism, and could help to make the best use of the limited public health resources.

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Sažetak

TOKSIKOLOGIJA BIOREGULATORA KAO POTENCIJALNIH BIOLOŠKIH AGENSA

Bioregulatori ili modulatori su biokemijski spojevi kao što su peptidi koji se prirodno pojavljuju u raznim organizmima. Razvoj biotehnologije omogućava zloporabu peptidnih bioregulatora u ofenzivnim biološkim ratnim programima. Bioregulatori su nova vrsta oružja koja mogu oštetiti živčani sustav, mijenjati raspoloženje i ponašanje, uzrokovati ozbiljne psihološke promjene i na kraju ubiti. Posljednjih dvadeset i više godina s razvojem znanosti o neurologiji nastupa prava eksplozija znanja o sustavima receptora živčanih stanica koja su od velike važnosti u otkrivanju kemijskih transmiterskih tvari što oslobađaju druge živčane stanice. Bioregulatori su tvari slične onima koje se prirodno nalaze u organizmu a koje reguliraju biološke procese. Mogućnost uporabe bioregulatora u ratne ili terorističke svrhe slična je kao kod toksina. Zajedno s porastom istraživanja toksina istraživani su i sintetizirani bioregulatori. Ovaj rad prikazuje evaluaciju bioregulatora koji bi se mogli upotrijebiti u nekom od terorističkih napada ili kao biološki ratni agens u nemiroljubivim aktivnostima.

KLJUČNE RIJEČI: *algogeni, fizičko onesposobljavanje, javnozdravstvena pripravnost, kriteriji za selekciju bioregulatora, terorizam*

REQUESTS FOR REPRINTS:

Slavko Bokan, M.D., Specialist in Occupational Health, Toxicologist
Ministry of Defense of the Republic of Croatia, Croatian Military Academy,
Laboratory for NBC Protection
Ilica 256 b, HR-10000 Zagreb, Croatia
E-mail: *slavko.bokan@morh.hr*