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Analgesic Effects of 3-Substituted Derivatives of 1,4-Benzodiazepines and their Possible Mechanisms

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In experiments on mice, we studied the analgesic activity of some 3-substituted derivatives of 1,4-benzodiazepines, including 3-propoxy-7-bromo-5-(2'-chloro)phenyl-1,2-dihydro-3H-1,4-benzodiazepin-2-one (compound 6). This compound demonstrated clearly pronounced anti-inflammatory and antinociceptive properties in the acetic acid-induced writhing test (induction of visceral pain) in mice, test with carrageenan-induced paw edema in rats, and formalin test in mice. On multicellular preparations of circular smooth muscles from the fundal part of the rat stomach, we estimated the value of affinity of compound 6 (pK_B = 6.41). We hypothesize that the mechanism underlying inhibition of bradykinin (BK) receptors by compound 6 is mostly competitive. Therefore, compound 6 can be considered a promising basis for the development and synthesis of antagonists of BK receptors, which can be applied in clinical practice.

KEYWORDS: 3-substituted 1,4-benzodiazepines, analgesia, smooth muscles of the stomach, edema, tensometric studies, bradykinin (BK) receptors.

INTRODUCTION

The development of techniques for prophylaxis and treatment of diseases that are accompanied by acute and/or chronic pain is an important medical/biological problem. Its solution is related, first of all, to the successful search for highly active and safe analgesics. The published experimental data are indicative of the analgesic properties of both some well-known agents [1] and novel derivatives of 1,4-benzodiazepines [2, 3]. Some representatives of this class with substituent in the third position showed a significant analgesic activity and high affinity with respect to bradykinin (BK) receptors [4-6].

Nonapeptide bradykinin (BK) is a potent natural algogen that belongs to the kallikrein-kinin system. This system participates in the control of important functions in the human organism, namely contractile activity and tone of smooth muscles of the vessels and gastrointestinal tract and pain sensitivity [7, 8]. It is also a key link whose abnormal activation causes the development of acute and chronic inflammation of the corresponding tissues [9-11]. Cell responses underlying these reactions are mediated by activation of BK receptors of two subtypes (B_1 and B_2) [12, 13].

In smooth muscles of the airways, gastrointestinal tract, and myometrium, activation of BK receptors induces contraction. *Vice versa*, smooth muscles of the vessels mostly respond to the action of BK by relaxation [14].

Over the last decades, studies devoted to the synthesis of antagonists of BK receptors have been carried out very actively. Inhibitors of the kallikrein-kinin system of peptide and non-peptide nature (HOE 140, desArg^9 [Leu^8] BK, R-954, etc.) are at present known [14, 15]; nonetheless, further development, synthesis, and analysis of the properties of compounds capable of selectively suppressing pathological (pain-related and inflammatory) processes mediated by activation of BK receptors remain urgent. We believe that the synthesis of novel highly-effective analgesics, which are antagonists of BK receptors, should solve many problems related to pain relief.

We studied the analgesic properties of a series of novel 3-alkoxy-1,2-dihydro-3H-1,4-benzodiazepine-2-ones, whose molecular structure is indicative of their potential ability to interact

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with BK receptors. Bioassays on the antagonistic action of the most active compound ("leader") were performed on multicellular preparations of circular smooth muscles of the fundal part of the rat stomach.

METHODS

Experiments were carried out on mongrel albino rats weighing 240-260 g and mongrel albino mice weighing 20-25 g; the animals were kept under standard vivarium conditions with water and food allowance *ad libitum*. At the end of the experiment, the animals were injected with propofol (anesthetic drug; Sigma, USA) in a lethal dose.

Acetic Acid-Induced Writhing Test (Induction of Visceral Pain). Analgesic activity was studied using an experimental model of visceral pain. Chemical stimulation of nociceptors of the abdominal cavity in mice was induced by i.p. injection of acetic acid solution (0.75%); this resulted in the appearance of involuntary contractions of the abdominal muscles ("writhings") accompanied by extension of the hindlimbs and arching of the back. Injections of acetic acid solution were performed 40 min after i.p. injection of the tested compounds in doses of 0.03 to 3.00 mg/kg. The animals were under observation for 20 min; the number of writhings was calculated for each mouse. The analgesic activity (AA) was estimated by the ability of the tested compounds to decrease the number of such writhings in the experimental animal group *vs* the control group and was expressed in percentage; its normalized value was calculated as follows:

$AA = (N_c - N_e / N_c) \cdot 100\%$,

where N_c and N_e are the mean numbers of writhing in the control and experimental groups, respectively.

The activity of the tested compounds was compared with that of a reference compound, diclofenac sodium, used in its generally adopted dose ED_{50} = 10 mg/kg. The ED_{50} value was calculated according to Prozorovskii [16].

Carrageenan-Induced Paw Edema. Anti-exudative activity was studied on the model of carrageenan-induced edema of the rat hind pow. The edema was induced by injection of 0.1 ml of a 1% water solution of λ-carrageenan under the foot aponeurosis of the animals of the experimental and control groups [17, 18]. The tested compound and the reference agent diclofenac sodium were injected i.p. 40 min prior to induction of inflammation. Control rats received an equivalent amount of Tween-80 emulsion in physiological saline. The degree of edema was estimated by measuring the volume of the foot with the help of a mechanical oncometer [19] before injection of the phlogogen and 2 and 4 h after injection of the latter. Anti-exudative activity was estimated by the ability of the tested compound to suppress the inflammatory reaction in experimental animals *vs* control ones. Calculation was performed according to the following formula:

$A = (\Delta V_c - \Delta V_e / \Delta V_c) \cdot 100\%$,

where A is the anti-exudative activity; ΔV_e and ΔV_c are the differences between volumes of the edematous and normal feet in the experiment and control, respectively.

Formalin Test. The antinociceptive activity was studied with the use of the formalin test on mice. Behavioral manifestation of somatic pain in this test is licking of the limb injected with formalin. The pain reaction, in this case, includes two phases, namely the early one lasting 5 min after phlogogen injection and the longer late phase that begins from about the 11th min and is completed in 40-50 min. Phase I is related to direct formalin-mediated stimulation of nociceptors, while phase II is induced by inflammatory factors and can be considered *per se* as primary hyperalgesia.

The test was performed with the help of subplantar injection of 0.01 ml of 3% water solution of formalin into the hind paw of animals of the experimental and control groups. The tested compounds and the reference agent diclofenac sodium were i.p. injected 40 min prior to induction of the pain reaction. Mice of the control group received an equivalent amount of physiological solution with emulsified Tween-80. After injection of formalin solution, the animal was placed into a separate cage (8×8×8 cm) and was under observation for 40 min; the total time interval of licking the affected hind paw by the animal was measured. The antinociceptive activity of the tested compounds was estimated by their ability to decrease the duration of the pain behavioral reaction.

Contractile Activity of Muscles of the Stomach. Experiments were carried out on mucosa-free preparations of circular smooth muscles of the antral part of the rat stomach. Such strips (mean size 1.5×10 mm) were placed into a working chamber (volume 2 ml) perfused with Krebs solution (perfusion rate 5 ml/min); the chamber was thermostated at 37°C. The preparation was stretched with a force of 10 mN for 1 h (up to the appearance of spontaneous contractions with constant amplitude and frequency and contractions with constant

mechanokinetic parameters, which were induced by the action of BK or hyperpotassium solution). The contractile activity was studied in an isometric mode using a force transducer and an electrical potentiometer.

In our experiments, we used Krebs solution of the following composition (mM): NaCl, 20.4; KCl, 5.9; $NaHCO_3$, 15.5; NaH_2PO_4, 1.2; $MgCl_2$, 1.2; $CaCl_2$, 2.5; glucose, 11.5; pH of the solution was 7.4. A hyperpotassium solution containing K^+ in the concentration of 80 mM was prepared by means of isotonic replacement of the necessary part of Na^+ by an equimolar amount of K^+ in the initial Krebs solution. In the case where we used BK, we initially prepared a concentrated water solution of the latter, and necessary amounts of this 1% solution of the substance were added to the Krebs solution to obtain the necessary final concentrations.

We plotted the cumulative "concentration–effect" curves for the above-mentioned BK concentrations (from 1 nM to 10 μM). After plotting the control "concentration–effect" curve, the preparations were washed out with Krebs solution (on average, for 20 min), and then they were subjected to preliminary incubation for 60 min in the Krebs solution with the addition of the tested compound (concentrations of 1, 10, and 50 μM were used). Since the tested substances were hydrophobic, they were preliminarily dissolved in DMSO (mother solution), and necessary amounts of 1% of mother solution were added to the final volume. Analogous control solutions also contained 1% DMSO.

To elucidate the pattern of interaction between the tested substance and BK, we used the Shield's regression equation [20]. The antagonist-induced parallel shift of the "concentration–effect" curves was estimated as the ratio of the equieffective concentrations of the agonist (CRs) responsible for 50% contraction by the agonist in the control and in the presence of the antagonist. The affinity of the antagonist (pK_B) was calculated by the equation: $pK_B = \lg [CR - 1] - \lg [C_a]$, where C_a is the antagonist concentration.

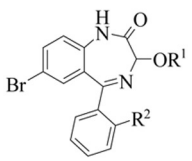
Experimental data were processed using OriginPro 8 software. We checked the correspondence of samplings to the normal law using the Shapiro–Wilk criterion. To estimate the significance of differences between two mean values of samplings, we used the paired Student's *t*-test; the differences were considered significant with $P < 0.05$. Analysis of significance of the data approximation by a linear function was performed using the Fisher's F-criterion; the coefficients of determination (R^2) were not greater than 0.9. Normalized data are presented below as arithmetic means ± s.e.m.; *n* is the number of experiments.

RESULTS AND DISCUSSION

Based on the published data that some 3-substituted 1,2-dihydro-3H-1,4-benzodiazepines demonstrate a considerable analgesic activity [1-6], we, in the search for novel analgesics, have synthesized earlier a series of 3-alkoxy-1,2-dihydro-3H-1,4-benzodiazepine-2-ones (compounds 1-15) [21-23] (Fig. 1).

All the tested compounds 1-15 demonstrated a high analgesic activity in *in vivo* experiments under conditions of the acetic acid-induced writhing test (visceral pain in mice induced by i.p. injection of acetic acid solution). The intensity of analgesia significantly exceeded in all cases the effect of the reference compound (diclofenac sodium). The range of ED_{50} values for the tested compounds varied from 0.03 to 1.77 mg/kg; for diclofenac sodium, the ED_{50} was 10.00 ± 1.80 mg/kg.

The maximum analgesic activity with ED_{50} = 0.030 ± ± 0.007 mg/kg was demonstrated by compound 6;



1) R^1 =CH_3 ; R^2 = H;
2) R^1 = CH_3 ; R^2 = Cl;
3) R^1 = C_2H_5 ; R^2 = H;
4) R^1 = C_2H_5 ; R^2 = Cl;
5) R^1 = $(CH_2)_2CH_3$; R^2 = H;
6) R^1 = $(CH_2)_2CH_3$; R^2 = Cl;
7) R^1 = $(CH_2)_3CH_3$; R^2 = H;
8) R^1 = $(CH_2)_3CH_3$; R^2 = Cl;
9) R^1 = $(CH_2)_4CH_3$; R^2 = H;
10) R^1 =  ; R^2 =H;
11) R^1 =  ; R^2 =Cl;
12) R^1 = $(CH_2)_2OH$; R^2 = H;
13) R^1 = $(CH_2)_2OH$; R^2 = Cl;
14) R^1 = $(CH_2)_2OCH_3$; R^2 = H;
15) R^1 = $(CH_2)_2OCH_3$; R^2 = Cl;

Fig. 1. Structure of the molecules of the tested compounds 1-15 (3-alkoxy-1,2-dihidro-3H-benzodiazepin-2-ones).

its analgesic efficacy exceeded that observed for the comparison drug (diclofenac sodium) more than 30 times.

The pharmacological profile of many analgesic drugs includes anti-inflammatory and antinociceptive activities. In this regard, we have carried out experiments on revealing anti-inflammatory and antinociceptive activity of the compound 6. Our data showed that administration of compound 6 [3-propanoxy-7-bromo-5-(2'-chloro)phenyl-1,2-dihydro-3H-1,4-benzodiazepin-2-one] resulted in a moderate decrease in the size of edema induced by injection of carrageenan into the rat hind paw (by 36.7 and 25.0 % after 2 and 4 h, respectively).

The data obtained in the formalin test were also indicative of the high antinociceptive activity of 3-propanoxy-1,2-dihydro-3H-1,4-benzodiazepine-2-one (compound 6) used in rather low doses. The intensity of the antinociceptive activity of the tested compound demonstrated a distinct dose dependence. With dose reduction from 3 to 0.1 mg/kg, the normalized value of inhibition of the pain response decreased from 91.1 and 98.4 % to 76.0 and 54.0% for phases I and II, respectively. With decrease in the dose by 300 times, i.e., in the case where compound 6 was injected in a dose of only 0.01 mg/kg, the ability to suppress appreciably the pain reaction was still preserved; the above indices for phases I and II under conditions of the formalin test were 15.0 and 32.1%, respectively.

For the analysis of probable antagonistic activity with respect to BK receptors, exactly the compound 6 was chosen among derivatives of 1,2-dihydro-3H-1,4-benzodiazepine-2-ones.

To characterize the interaction between the tested compound and BK, we recorded the contractile activity of smooth muscles of the stomach of mongrel albino rats using a tensometric technique. The 50% value of the maximum contractile force of these muscles was observed in the presence of $(1.78 \pm 0.14) \cdot 10^{-8}$ M BK; this EC_{50} value agrees *in toto* with the data obtained by Cabrindi et al. on the fundal part of the stomach [24].

The action of compound 6 in the concentration of 1.0 μM was accompanied by suppression of BK-mediated contractions of smooth muscles of the stomach by 37%, on average. Under analogous conditions, contractions of the *cecum* smooth muscles were inhibited, on average, by 45% (n = 3). Since the amplitude of BK-mediated contractions of these muscles was, in general, relatively small (forces about 5-7 mN were developed), further experiments on *cecum* preparations were not performed.

To elucidate the pattern of inhibition of BK receptors by compound 6, we plotted the cumulative "concentration–effect" curves in the presence of 1, 10, and 50 μM of the tested agent (Fig. 2).

Based on the obtained data, we initially constructed the Shield's plot (Fig. 3), estimated the values of the slope (1.28) and coefficient R^2

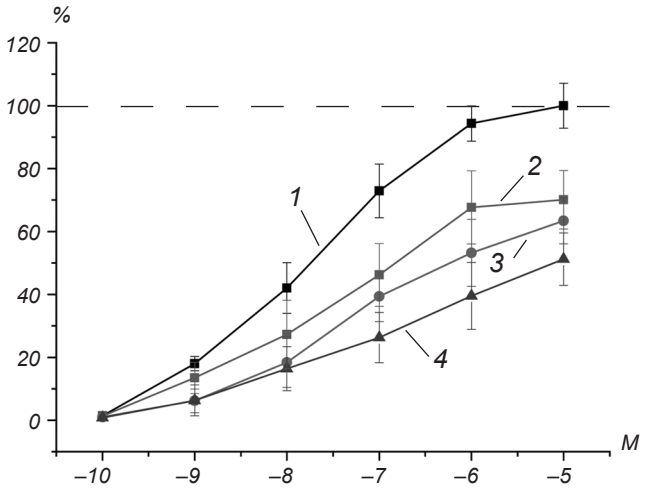


Fig. 2. Cumulative "concentration–effect" curves for bradykinin-induced contractions in the control (1) and in the presence of 3-propoxy-7-bromo-5-(2'-choro)phenyl-1,2-dihydro-3H-1,4-benzodiazepin-2-one (compound 6) in concentrations of 1.0 (2), 10 (3), and 50 μM (4). Abscissa) Decimal logarithm of the bradykinin concentration, M; ordinate) normalized amplitude of the contractions, %.

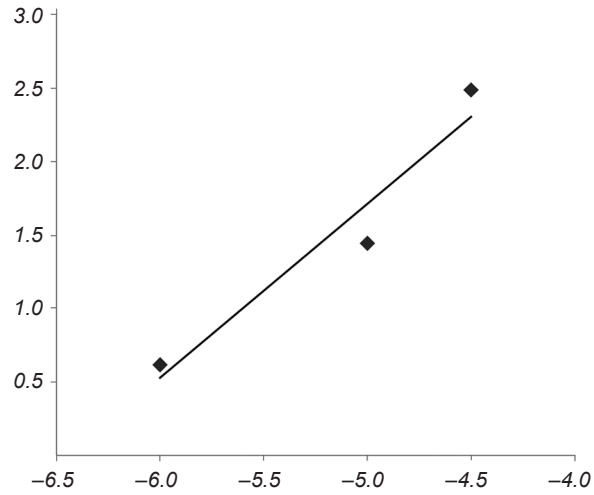


Fig. 3. Shield's plot for the action of 3-propoxy-7-bromo-5-(2'-chloro)phenyl-1,2-dihydro-3H-1,4-benzodiazepin-2-one (compound 6) in concentrations of 1, 10, and 50 μM on smooth muscles of the fundal part of the rat stomach. Abscissa) Logarithm of the concentration of the compound, M; ordinate) value of log (DR-1).

(0.93), and then estimated the value of affinity for the tested compound (pK_B = 6.41). Since the slope value of the Shield's plot is close to 1.0, we believe that antagonism of compound 6 with respect to BK is mostly competitive. The affinity of the tested compound for BK receptors corresponds to the higher range of indices of affinity of the antagonists, such as D-Arg-[Hyp^3,D-Phe^7]-BK and D-Arg-[Hyp^3,$Thi^{5,8}$,D-Phe^7]-BK. For the guinea-pig *taenia caeci*, the pK_B values are 5.89 and 5.81 [25]. At the same time, it should be noted that the affinity of this compound was relatively small as compared, e.g., with that of the well-known selective antagonist of BK_1 receptors des-Arg^9-NPC 17761 (pK_B = 8.47, although, in this case the slope of the Shield's plot was significantly smaller than 1.0) and of the antagonist of BK_2 receptors HOE 140 (pK_B was 9.05 for smooth muscles of the fundal part of the rat stomach studied in our experiments) [24].

Therefore, it is obvious that 3-propoxy-7-bromo-5-(2'-chloro)phenyl-1,2-dihydro-3H-1,4-benzodiazepine-2-one can be considered a promising basic compound for the development and synthesis of antagonists of BK receptors possessing analgesic and anti-inflammatory properties and, therefore, applicable in clinical practice.

Experiments were carried out in accordance with the International Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Strasbourg, 1985), as well as with the Law of Ukraine "On Protection of Animals from Inhumane Treatment."

The authors, V. I. Pavlovsky, O. V. Tsymbalyuk, V. S. Martynyuk, T. A. Kabanova, E. A. Semenishyna, E. I. Khalimova, and S. A. Andronati, confirm that they have no conflict of interest.

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REFERENCES

1. H. U. Zeilhofer, D. Benke, and G. E. Yevenes, "Chronic pain states: pharmacological strategies to restore diminished inhibitory spinal pain control," *Annu. Rev. Pharmacol. Toxicol.*, **52**, 111-133 (2012).
2. E. Lattmann, P. Lattmann, Y. Boonprakob, et al., "*In vivo* evaluation of substituted 3-amino-1,4-benzodiazepines as anti-depressant, anxiolytic and anti-nociceptive agents," *Arzneimittelforschung*, **59**, No. 2, 61-71 (2009).
3. N. Nadzhafa, M. Pirali, R. Daulatobadi, et al., "Synthesis, analgesic, and anti-inflammatory activity of novel derivatives of benzodiazepine," *Khim.-Farmakol. Zh.*, **30**, No. 12, 21-23 (2005).
4. M. R. Wood, J. J. Kim, W. Han, et al., "Benzodiazepines as potent and selective bradykinin B_1 antagonists," *J. Med. Chem.*, **46**, 1803-1806 (2003).
5. WO/2006/071775 US, A2 C07D 403/14, *Novel Compounds Useful for Bradykinin B_1 Receptor Antagonism*, J. S. Tung, D. D. Dressen, R. J. Netiz, M. A. Pleiss, Published: 07.06.2006.
6. WO/02/099388 US, A2 G01N, *Benzodiazepine Bradykinin Antagonists*, M. R. Wood, M. G. Bock, Daishi SU. S. D. Kuduk, W. Han, B. D. Dorsey, Published: 12.12.2002.
7. A. H. Schmaier, "Assembly, activation, and physiologic influence of the plasma kallikrein/kinin system," *Int. J. Immunopharmacol.*, **8**, No. 2, 161-165 (2008).
8. J. W. Bryant and Z. Shariat-Madar, "Human plasma kallikrein-kinin system: physiological and biochemical parameters," *Cardiovasc. Hematol. Agents Med. Chem.*, **7**, No. 3, 234-250 (2009).
9. M. E. Moreau, N. Garbacki, G. Molinaro, et al., "The kallikrein-kinin system: current and future pharmacological targets," *J. Pharmacol. Sci.*, **99**, 6-38 (2005).
10. A. Stadnicki, "Intestinal tissue kallikrein-kinin system in inflammatory bowel disease," *Inflamm. Bowel. Dis.*, **17**, No. 2, 645-654 (2011).
11. A. Riad, J. L. Zhuo, H. P. Schultheiss, et al., "The role of the renal kallikrein–kinin system in diabetic nephropathy," *Curr. Opin. Nephrol. Hypertens.*, **16**, No. 1, 22-26 (2007).
12. F. Kuhr, J. Lowry, Y. Zhang, et al., "Differential regulation of inducible and endothelial nitric oxide synthase by kinin B_1 and B_2 receptors," *Neuropeptides*, **44**, No. 2, 145-154 (2010).
13. J. B. Calixto, R. Medeiros, E. S. Fernandes, et al., "Kinin B_1 receptors: key G-protein-coupled receptors and their role in inflammatory and painful processes," *Br. J. Pharmacol.*, **143**, 803-818 (2004).
14. L. M. F. Leeb-Lundberg, F. Marceau, W. M. U. Lller-Ester, et al., "International union of pharmacology. XLV. Classification of the kinin receptor family: from molecular mechanisms to pathophysiological consequences," *Pharm. Rev.*, **57**, No. 1, 27-77 (2005).
15. F. J. Hock, K. Wirth, U. Albus, et al., "Hoe 140, a new potent and long acting bradykinin-antagonist: *in vitro* studies," *Br. J. Pharmacol.*, **102**, 769-773 (1991).
16. V. B. Prozorovskii, "Statistical treatment of results of pharmacological studies," *Psikhopharmakol. Biol. Narkol.*, **7**, Nos. 3/4, 2090-2120 (2007).
17. F. P. Trinus, N. A. Mokhort, and B. M. Klebanov, *Nonsteroid Anti-Inflammatory Drugs* [in Russian], Zdorov'ya, Kyiv (1975).
18. C. A. Winter, E. A. Risely, and G. W. Nuss, "Carrageenan-induced oedema in hind paw of the rat as an assay for anti-inflammatory drugs," *Proc. Soc. Exp. Biol. Med.*, **3**, 544-547 (1962).
19. A. S. Zakharevskii, *Effects of Some Indole Derivatives on the Nervous System* [in Russian], Cand. Thesis, Med. Sci., Minsk (1969).

20. P. Cucchi, S. Meini, A. Bressan, et al., "MEN16132, a novel potent and selective nonpeptide antagonist for the human bradykinin B_2 receptor. *In vitro* pharmacology and molecular characterization," *Eur. J. Pharmacol.*, **528**, 7-16 (2005).
21. V. I. Pavlovsky, E. A. Semenishina, I. A. Kravchenko, et al., "3-Alkoxy-1,2-dihydro-3H-1,4-benzodiazepine-2-ones: synthesis, structure, and anticonvulsive activity," *Khim.-Farmakol. Zh.*., **46**, No. 9, 20-25 (2012).
22. Y. Simonov, P. Bourosh, V. Kravtsov, et al., "A comparative analysis of the crystal structure of R,S-racemate and R-enantiomer of 7-bromo-3-(2-methoxy) ethoxy-5-phenyl-l,2-dihydro-3H-l,4-benzodiazepin-2-one exhibiting a high analgesic activity," *Zh. Org. Farm. Khim.*, **9**, No. 4 (36), 70-73 (2011).
23. S. Andronati, E. Semenishyna, V. Pavlovsky, et al., "Structure and affinity of 3-alkoxy-1,2-dihydro-3H-1,4-benzodiazepin-2-ones for central benzodiazepine receptors," *Eur. J. Med. Chem.*, **45**, No. 4, 1346-1351 (2010).
24. D. Cabrindi, D. J. Kyle, and J. Calixto, "A pharmacological analysis of receptor subtypes and the mechanisms mediating the biphasic response induced by kinins in the rat stomach fundus *in vitro*," *J. Pharmacol. Exp. Ther.*, **277**, No. 1, 299-307 (1996).
25. J. L. Field, J. M. Hall, and I. K. M. Morton, "Bradykinin receptors in the guinea-pig *taenia caeci* are similar to proposed BK_3 receptors in the guinea-pig trachea, and are blocked by HOE 140," *Br. J. Pharmacol.*, **105**, 293-296 (1992).