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(54) **Title:** N-GUANYL DERIVATIVES OF 9-AMINO-5,11-DIMETHYL-5H-INDOLO[2,3-B]QUINOLINE HAVING CYTOTOXIC ACTIVITY

(57) **Abstract:** New N-guanyl derivatives of 9-amino-5,11-dimethyl-5H-indolo[2,3-b]quinoline, their preparation process and the pharmaceutical preparations thereof are provided. N-Guanyl derivatives of 9-amino-5,11-dimethyl-5H-indolo[2,3-b]quinoline possess a selective cytotoxic activity and may be useful for the treatment of neoplastic diseases.



N-GUANYL DERIVATIVES OF 9-AMINO-5,11-DIMETHYL-5H-INDOLO[2,3-b]QUINOLINE HAVING CYTOTOXIC ACTIVITY

5 **Field of the Invention**

This invention relates to N-guanyl derivatives of 9-amino-5,11-dimethyl-5H-indolo[2,3-b]quinoline, their preparation process and the pharmaceutical preparations thereof. N-Guanyl derivatives of 9-amino-5,11-dimethyl-5H-indolo[2,3-b]quinoline possess a
10 selective cytotoxic activity and may be useful for the treatment of neoplastic diseases.

Background of the Invention

Cancers are one of the most challenging problems in modern medicine.

A basic method for metastatic cancer treatment and one of the methods used in the
15 combination therapy of solid carcinoma is chemotherapy. However, it is considered one of the most difficult anti-cancer treatments, mainly due to the unknown tumour-causing factors, minimal biochemical differences between cancer and healthy cells, non-specific anti-cancer activity of cytostatic drugs, their low therapeutic index and toxic effect on healthy cells.

20 Modern medicine has been using hundreds of anticancer drugs which may be classified according to different criteria. Classification according to the chemical structure and the source of drugs comprise cytotoxic drugs, drugs altering hormonal milieu, monoclonal antibodies and biological response modifiers.

However, there is still a need for new drugs with a selective anti-tumor, non-toxic
25 activity which would destroy tumor cells without harming healthy cells.

A great group of commonly used anticancer drugs are cytostatics which interact with the DNA helix and induce the mechanisms leading to cell death. Cytostatics can bound to the DNA as alkylating agents (eg. cis-platine), intercalate into the DNA strands (eg. anticancer antibiotics) or interact with the minor or major DNA groove (doxorubicin,
30 mitoxantron, actinomycin D).

A group of natural-origin compounds interacting with the DNA comprises, *inter alia*, indoloquinoline alkaloids. Indoloquinoline alkaloids isolated from an African plant

species, *Cryptolepis sanguinolenta*, have a broad spectrum of biological activities such as antibacterial, antifungal and cytotoxic activities. A high cytotoxic activity against many cancer cells is displayed, *inter alia*, by cryptolepine (5-methyl-5H-indolo[3,2-b]quinoline), neocryptolepine (5-methyl-5H-indolo[2,3-b]quinoline, and also by isocryptolepine (5-methyl-5H-indolo[3,2-c]quinoline) (Lavrado, J. et al., Indoloquinolines as Scaffolds for Drug Discovery, Current Med. Chem. 2010, 17, 2348-2370]. The chemical structure of the main indoloquinoline alkaloids is shown in Figure 1.

As a result of years of research on α -carbolins, a new generation of cytostatics having a benzo-iso- α -carbolin (indolo[2,3-b]quinoline) structure has been selected. These compounds are considered as neocryptolepine analogues (Kaczmarek Ł., Nantka-Namirski P., *Pol. J. Pharmacol. Pharm.* 1981, 33, 121-127; Peczyńska-Czoch, W. et al., Eds. Bushel, M.E., Graefe U. *Progress in Industrial Microbiology* 1989, 27, 333-353. Kaczmarek Ł. et al., *Archiv. Pharm.* 1988, 321, 463-467).

5,11-Dimethyl-5H-indolo[2,3-b]quinoline (DiMIQ) is the neocryptolepine analogue that shows the highest cytotoxic activity against cervical cancer KB cell lines, $IC_{50} = 1 \mu M$. 5,11-Dimethyl-5H-indolo[2,3-b]quinoline (DiMIQ) has become the lead compound for potential anticancer agents among different derivatives of indoloquinolines. Successive investigations *in vitro* have proved that DiMIQ intercalates the DNA strongly and stabilizes the drug-DNA-topoisomerase II complex, while the potency of its bonding with the DNA is dependent on pH, i.e. the occurrence of DiMIQ in a protonated form (Peczyńska-Czoch W. et al., *J. Med. Chem.* 1994, 37, 3503-3510). However, due to the toxicity of DiMIQ and its low solubility in aqueous media, the search for derivatives with a high cytotoxic activity accompanied by low toxicity and improved bioavailability is continued.

In Polish patent PL 206855 B1 specification, some synthetic derivatives having dialkylaminoalkyl substituents connected with 5,11-dimethyl-5H- or 6,11-dimethyl-6H-indolo[2,3-b]quinoline chromophores via amide, amine or ether bonds, were disclosed. Their cytotoxic activity against KB cell was also studied.

Cytotoxic activity studies of 6H-indolo[2,3-b]quinoline derivatives with (dialkylamino)alkyl substituents in N-6 position, and (dimethylamino)alkylamino- or (dimethylamino)alkoxy in C-2 or C-9 positions described in the publications Kaczmarek

Ł. et al., *Bioorg. and Med. Chem.* 1999, 7, 2457-2464; Osiadacz J. et al., *Anticancer Res.* 1999, 19, 3333-3342; Humeniuk R. et al., *Oncol. Res.* 2003, 13, 269-277; Kaczmarek Ł. et al., *Acta Polon. Pharm.* 2002, 59, 199-207; Godlewska J. et al., *Anticancer Res.* 2005, 25, 2857-2868) found that the derivatives containing alkyl substituents in position N-6
5 did not exhibit cytotoxic activity against cervical cancer KB cell lines. On the other hand, the change of these substituents for the alkyl-amino-alkyl group resulted in the pronounced cytotoxicity of the obtained conjugates and the values of IC₅₀ ranged from 2 to 9 μM.

The effect of the chromophore and substituent structures on the cytotoxic activity of
10 the investigated indolo[2,3-b]quinolines is depicted in Figure 2.

Polish patent specifications PL 202545 B1 and PL 206681 B1 as well as the publication of Godlewska J. et al., *Radiol. Oncol.* 2004, 38, 137-144 also revealed glycosyl derivatives of 5,11-dimethyl-5*H*-indolo[2,3-b]quinoline and 6,11-dimethyl-6*H*-indolo[2,3-b]quinoline. The glycosyl groups, such as deoxy-glucose, deoxy-rhamnose,
15 deoxy-lactose or aminoglycosyl (derivatives of daunosamine and acosamine) are situated in the positions 2 and 9. These derivatives are characterized by a better solubility in water as compared to the unsubstituted chromophores and by a moderate cytotoxic activity against KB cell lines, ranging from a couple of μM to a dozen μM.

For the majority of the above DiMIQ analogues their cytotoxic activity *in vitro* was high
20 and in the case of many derivatives their cytotoxic activity was similar to the one of doxorubicine (0.8 μM against the KB cell lines). However, in the *in vivo* study (mouse leukaemia model P388) both DiMIQ and its alkylamino, alkylaminoalkyl, as well as aminoglycosyl derivatives proved ineffective. In low doses the tested compounds did not show any anti-cancer activity, while in high doses they were too toxic (unpublished
25 data).

The influence of the amino acids bonding to the inactive 6*H*-indolo[2,3-b]quinolines on the cytotoxic activity was also investigated. In the case of hybrid conjugates in which the indoloquinoline skeleton was conjugated with the amino acids or peptides residue
30 *via* nitrogen atom in the peptide bond, a significant cytotoxic activity and better solubility in water as compared with the parent 6,11-dimethyl-6*H*-indolo[2,3-b]quinoline was demonstrated (K. Sidoryk et al., *Polish J. Chem.*, 82, 2095, 2008; Polish Patent Application P.385006). The highest cytotoxic activity with the IC₅₀ range from 3

to 5 μM was displayed by the conjugates formed by glycine, L-proline and L-histidine as the substituents.

On the other hand, bonding of the amino acids to the more cytotoxic chromophore 5,11-dimethyl-5*H*-indolo[2,3-*b*]quinoline resulted in the hybrid derivatives with a
5 higher or equal cytotoxic activity *in vitro*, better selectivity and bioavailability than it was observed in the case of the unsubstituted DiMIQ (K. Sidoryk et al., *J. Med. Chem.* 55 (11), pp. 5077–5087, 2012; Pol. App. P-390846). The derivatives substituted with glycine and L- and D-proline exhibited the highest cytotoxic activity. The values of IC_{50} for these compounds ranged from 0.42 to 0.73 μM against KB cell lines. The cytotoxic

10 activity of dipeptide derivatives which were designed on the basis of the cytotoxic activity study results for the amino acid derivatives, was similar or lower than the activity of the unsubstituted DiMIQ (IC_{50} values between 1.54 - 4.65 μM). The cytotoxic activity of the amino acid and peptide derivatives of DiMIQ was similar or higher than the cytotoxic activity of doxorubicin.

15 The above review teaches that many of the so far obtained analogues of indolo[2,3-*b*]quinoline show a high cytotoxic activity against cancer cell lines *in vitro*. However, the possibilities of their clinical application are limited because of their high cytotoxic activity against normal BALB 3T3 cells, as can be seen in Table 1.

The lack of selectivity of action of the so far obtained indolo[2,3-*b*]quinoline
20 derivatives entails the necessity of further study aimed at obtaining more selective anti-tumour with a high cytotoxic activity against cancer cells as well as a low cytotoxic activity against normal cells.

This aim was successfully accomplished by the introduction of the guanyl (amidine) group into indolo[2,3-*b*]quinoline conjugates according to the present invention.

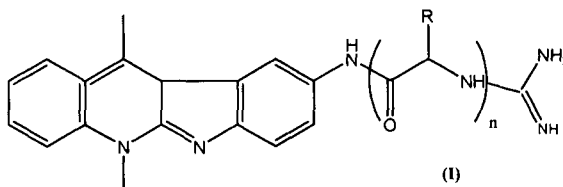
25 The guanyl group may play an important role in the delivery of the drug to cancer cells and also due to its strong basic properties (pK_a 12.5) may strongly interact with the phosphate residues of the minor groove of the DNA helix (Orner B.P., Hamilton A.D., *J. Incl. Phen Macrocycl. Chem.* 2001, 41, 141-147). The guanyl group can be found in many naturally occurring substances as well as in the metabolites of different living
30 organisms. A good example is ptilomycalin A isolated from the Red Sea and Caribbean Sea sponges, exhibiting antimicrobial, antifungal, antiviral as well as cytotoxic activity.

The introduction of guanyl substituents can increase the DiMIQ-DNA interaction and, as a result, advantageously affect the cytotoxic activity of DiMIQ.

Apart from stabilizing the drug-DNA complex, the guanyl group may also improve the delivery of the substance inside the cancer cells by increasing the hydrophilicity and water solubility of the formed molecule as well as lowering its toxicity (Kralova J. et al., *J. Med. Chem.* 2003, 46, 2049-2056; Hau V.S. et al., *J. Pharm. Sciences* 2002, 91, 2140-2149; Liu H. Mei. et al., *J. Pharmacol Exp. Ther.* 2006, 319, 308-316).

Summary of the Invention

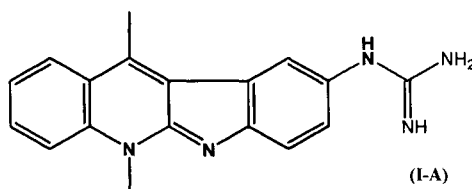
- The present invention provides new N-guanyl derivatives of 9-amino-5,11-dimethyl-5H-indolo[2,3-b]quinoline with the hybrid structure, where the guanyl group is directly bonded to the chromophore through a nitrogen atom of the amino group in position 9 or to the N^α-amino group of the amino acid residue of the acylating 9-amino-5,11-dimethyl-5H-indolo[2,3-b]quinoline.
- Thus, the present invention provides the N-guanyl derivatives of 9-amino-5,11-dimethyl-5H-indolo[2,3-b]quinoline of Formula (I)



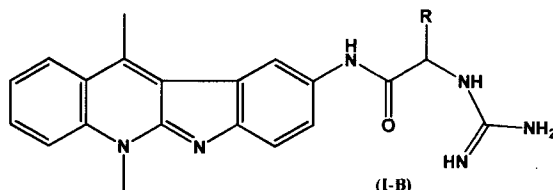
wherein:

- n is 0 or 1, and
when n = 1, R is a side chain of the acylating α -amino acid residue,
and their pharmaceutically acceptable salts.

In one embodiment, the present invention provides the compound of the formula (I), wherein n = 0 having the structure represented by Formula (I-A):



In another embodiment, the present invention provides the compound of the formula (I), wherein $n = 1$ and R is a side chain of the acylating α -amino acid residue having the structure represented by Formula (I-B):

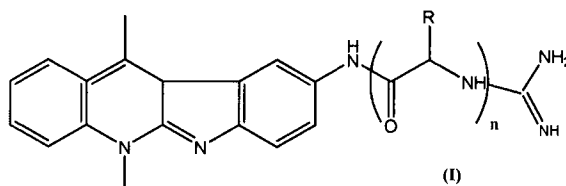


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The term "side chain of the acylating α -amino acid residue", as used herein, relates to naturally occurring L- and D- α -amino acids.

In the preferred embodiment of the present invention the acylating α -amino acid residue is glycine or L-proline.

- 10 A further aspect of the invention is the process for the preparation of N-guanyl derivatives of 9-amino-5,11-dimethyl-5H-indolo[2,3-b]quinoline of Formula (I)

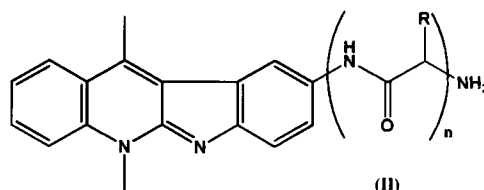


wherein:

n is 0 or 1, and

- 15 when $n = 1$, R is a side chain of the acylating α -amino acid residue,
and their pharmaceutically acceptable salts,
comprising the following steps:

- (i) guanylation of 9-amino-derivative of 5,11-dimethyl-5H-indolo[2,3-b]quinoline of Formula (II)



20

wherein:

n is 0 or 1, and

when $n = 1$, R is a side chain of the acylating α -amino acid residue,
using a guanylation reagent,

- (ii) isolation of the crude reaction product of step (i) and its purification by column chromatography,
- (iii) if necessary, the removal of the protecting groups under acidic conditions to obtain product (I) in the form of salt with the acid used in step (iii),
- 5 (iv) isolation and purification of product (I) obtained in step (iii),
- (v) optionally, conversion of product (I) into a desired pharmaceutically acceptable salt.

The starting derivatives of Formula (II) wherein R is the α -amino acid residue are prepared as described in PL 219085 B1 and in Sidoryk K. *et al.*, *J. Med. Chem.* 55 (11),
10 pp 5077–5087, 2012. The amino groups in the side chain of the acylating α -amino acid residue, if present, are protected in a manner known to the persons skilled in the art.

The methods of amine guanylation (amidination) are well known in organic chemistry. A review article, Katritzky A.R. *et al.* *Archive for Organic Chemistry*, 2005 (4), 49-87; online: 19 JUL 2005, DOI: <http://dx.doi.org/10.3998/ark.5550190.0006.406>,
15 describes many guanylating agents. Those widely used include: 1) thioureas (for example diBoc-thiourea or thiourea generating reagents such as carbamoyl-isothiocyanates, thioureas immobilized on resins; 2) isothioureas and especially S-methylisothioureas including diBoc- and diZ-derivatives, S-methylisothioureas immobilized on resins; 3) 1 *H*-pyrazole-1-carboxyamidines i.e. 1-amidine-1*H*-pyrazoles,
20 mainly not substituted 1*H*-pyrazole-1-carboxamide and its mono- or di-Boc, and mono- or di-Z-derivatives; 4) triflyl guanidine, and di-Boc- and diZ-triflylguanidines; and in special cases cabodiimides, cyanamides or oxidation products of thioureas (for example aminoiminosulfonic acids).

Recently, the use of new, differently substituted guanylating agents such as o-Cl-Z (o-chlorobenzyloxycarbonyl)- and o-Br-Z (o-bromobenzyloxycarbonyl)-S-methylisithioureas (Gers T. *et al.*, *Synthesis* 2004,1,7-42) as well as *N,N'*-(2-chlorobenzyloxycarbonyl)-1*H*-1-pirazole-carboxyamidine (Diss M. *et al.*, *J. Am. Chem. Soc.* 2008, 130, 1321) was proposed.

The use of some guanylating agents such as 1*H*-1-pirazole-carboxyamidine (PCA)
30 directly gives guanylated amines, although this reaction may be accompanied by the formation of some by-products. The use of substituted guanylating agents such as the

protected 1*H*-1-pirazole-carboxyamidines or protected *S*-methyl-isothiourea requires the removal of the protecting group after guanylation. Moreover, the guanylation with the protected thioureas and *S*-methyl-isothioureas runs slowly and the use of a mercuric (II) salt, for example HgCl₂, as a catalyst is recommended (Levallet C. i in.,
5 *Tetrahedron*, 1997, 53, 5291-5304). The use of other catalysts, such as 2-chloro-1-methylpyridinium iodide, was also suggested (Yong Y.F. et al., *J. Org. Chem.*, 1997, 62, 1540-1542) or *N*-iodosuccinimide (Ohara K. i in., *Tetrahedron Lett.*, 50 (2009)1463-1465).

In the best mode of carrying out the present invention, the guanylation reaction is
10 carried out with the *N*-protected guanylating agents in the presence of a catalyst, and subsequently the protecting group is removed from the reaction product.

Suitable *N*-protected guanylating agents include diBoc-, diZ-, o-Cl-Z-, o-Br-Z- substituted *S*-methyloisothioureas; mono-, di-Boc- di-o-Cl-Z- substituted 1*H*-1-pirazole-carboxyamidines or di-Boc- and diZ-triflylguanidines

15 The most preferred *N*-protected guanylating agent is *N,N'*-diBoc-*S*-methyloisothiourea.

The guanylation reaction is preferably carried out in the presence of HgCl₂ as a catalyst and a tertiary amine.

The process of guanylation using an *N*-protected guanylating agent such as *N,N'*-diBoc-*S*-methyloisothiourea in the presence of mercury (II) chloride requires the purification
20 of the resulting unstable diBoc-derivative by column or preparative plate chromatography, and then, after the removal of the protected Boc-protecting groups additional purification of the final product by, for example, recrystallization is necessary.

N-Protecting groups in the guanyl moiety and, if present, the protecting groups from
25 the side chain of the amino acid residue, are removed according to one of the well-known procedures, e.g. under acidic conditions such as by trifluoroacetic acid or hydrogen chloride treatment.

Deprotection is carried out in an alcohol selected from the aliphatic alcohols saturated with gaseous hydrochloride acid or in the neat trifluoroacetic acid.

30 The crude product is separated by concentrating the reaction mixture and purified by a typical procedure. Purification might include the treatment of the separated material

with basic solutions, typically an aqueous hydrogen carbonate solution, and extraction with an organic, water-immiscible solvent selected from the group comprising esters, ethers, cyclic ethers, aliphatic and aromatic halogenated hydrocarbons; the use of chloroform is preferred.

- 5 Alternatively, the crude product may be purified by several treatments with a hydroxy solvent and the removal of the solvent *in vacuo*.

The obtained product (I) is separated and purified. The reaction product is obtained in the form of the salt with the acid used in step (iii), namely hydrochloride and trifluoroacetate.

- 10 To prepare the desired salt of compound (I) with another pharmaceutically acceptable acid, the product of step (iv), for example trifluoroacetate, is reacted with a stronger acid, for example hydrochloric acid, or neutralized first and the free base is treated with another pharmaceutically acceptable acid.

- Pharmaceutically acceptable addition salts of N-guanyl derivatives of 9-amino-5,11-dimethyl-5H-indolo[2,3-b]quinoline of formula (I) formed with mineral or organic acids
15 are also included within the scope of the present invention.

- Among suitable mineral acids are hydrochlorides, hydrobromides, sulphates and phosphates. Among suitable organic acids are malic, fumaric, succinic, itaconic, citraconic, oxalic, benzoic, p-aminobenzoic, ascorbic, acetic, propionic, tartaric,
20 salicylic, citric, gluconic, lactic, mandelic, cinnamic, aspartic, methanesulfonic, ethanesulofonic, benzenesulfonic, p- toluenesufonic, glycolic, glutamic, stearic or palmitic acid.

- In the preferred embodiment of the present invention, N-guanyl derivatives of 9-amino-5,11-dimethyl-5H-indolo[2,3-b]quinoline of formula (I) are acetate or
25 hydrochloride salts.

N-Guanyl derivative of 9-amino-5,11-dimethyl-5H-indolo[2,3-b]quinoline of formula (I) or its salt can be, if necessary, purified by column gel chromatography or by recrystallization.

- N-Guanyl derivatives of 9-amino-5,11-dimethyl-5H-indolo[2,3-b]quinoline and their
30 salts can occur in non-solvated form or as solvates with pharmaceutically acceptable solvents such as water, alcohols and others. This invention includes all forms of N-guanyl derivatives of formula (I): solvated forms as well as free forms without solvents.

N-Guanyl derivatives of 9-amino-5,11-dimethyl-5H-indolo[2,3-b]quinoline of formula (I) exhibit potentially valuable pharmacological properties which are demonstrated by the *in vitro* studies of their activity against selected cancer cells including: human cancer cell lines KB (cervix carcinoma), non-small cell lung cancer A549, breast cancer MCF-7, and colon cancer LoVo.

The following compounds of the invention are most preferred as regards their cytotoxic activity:

N-guanyl-*N*-(5,11-dimethyl-5*H*-indolo[2,3-b]quinolyl-9-yl)-amine its hydrochloride (**1a**), *N*^α-guanyl-glycyl-*N*-(5,11-dimethyl-5*H*-indolo[2,3-b] quinolyl-9-yl)amide, optionally in

the hydrochloride form (**2a**),

N^α-guanyl-L-prolyl-*N*-(5,11-dimethyl-5*H*-indolo[2,3-b]quinol-9-yl)amide, optionally in the hydrochloride form (**3a**).

Among the tested *N*-guanyl derivatives of 9-amino-5,11-dimethyl-5*H*-indolo[2,3-b]quinoline of formula (I), the most selective one is *N*-guanyl-*N*-(5,11-dimethyl-5*H*-indolo[2,3-b]quinolyl-9-yl)-amine (**1a**) which shows the highest cytotoxic activity against cancer cells and simultaneously the lowest inhibiting effect on the growth of normal cells. Its cytotoxic activity against normal cells is up to 1000 times lower than against A549 and MCF-7 cancer cells.

Due to their advantageous physicochemical and pharmacological properties, *N*-guanyl derivatives of 9-amino-5,11-dimethyl-5*H*-indolo[2,3-b]quinoline according to the present invention may be used as the active substances in the pharmaceutical formulations for the prevention or therapy of different cancers in humans, including, but not limited, to cervix carcinoma, prostate, breast, cancer, colon or lung cancer.

The pharmaceutical formulation for the treatment and/or prevention of cancers comprises *N*-guanyl derivative of 9-amino-5,11-dimethyl-5*H*-indolo[2,3-b]quinoline of Formula (I) or its pharmaceutically acceptable salt and at least one pharmaceutically acceptable carrier and/or excipient.

The pharmaceutical formulation of the present invention comprising the therapeutically effective amount of *N*-guanyl derivative of 9-amino-5,11-dimethyl-5*H*-indolo[2,3-b]quinoline amine of Formula (I) or its pharmaceutically acceptable addition salt is administrated to the patient in need in a suitable pharmaceutical dosage form

and by any acceptable route, including intravenous, subcutaneous, intramuscular or oral administration.

The choice of the dosage strength and regimen depend on a particular disease, patient age, weight and condition, and may be determined by a clinician based on the clinical studies and the well-known therapeutic and prophylactic methods for the treatment of cancer. In the treatment of cancers, a suitable dosage strength of the compound according to the present invention is from 0.1 to 100 mg/kg daily, preferably from 0.5 to 10 mg/kg daily. The suitable dose may be administrated to the patient in one or a few dosage units per day, as monotherapy or in combination with other therapeutic agents. Such compounds may be administrated simultaneously as one preparation or in separate preparations, or one after another, in the order and intervals determined by a specialist.

The pharmaceutical formulation of the present invention may be prepared in various pharmaceutical dosage forms, well-known to those skilled in the art, such as those cited in Remington's Pharmaceutical Sciences, 18th Ed. , Mack Publ.Co. 1990.

The pharmaceutical formulations suitable for injections and infusions comprise sterile aqueous, aqueous-organic and non-aqueous suspensions, dry substances and tablets for solution preparations, as well as implants. The excipients are used for the suspension preparation to ensure an even distribution of the active ingredient in the liquid phase and they include polysorbates, lecithine, polyoxyethylene-polyoxypropylene co-polymers, peptizing agents, such as phosphoranes, polyphosphoranes and citrates of water-soluble polymers such as carboxymethyl cellulose, methyl cellulose, polyvinylpyrrolidone, gums and gelatin. Formulations for injections may contain pharmaceutically acceptable excipients, such as pH regulators, buffers, tonicity agents and preservatives. Dry substances are used for the preparation of the solutions or suspensions *ex tempore*, by dilution with a suitable solvent.

The pharmaceutical forms for oral administration include tablets, pills, powders, granules, pellets or capsules containing pharmaceutically acceptable solid carriers such as corn starch, lactose, sucrose, sorbitol, hydrous magnesium silicate, stearic acid, magnesium stearate, dicalcium phosphate or gums. Tablets or granules may be coated or otherwise processed in order to achieve a dosage unit ensuring preferable extended release. For the formation of such protective layers or coatings a number of various

substances may be used, including polymeric acids and mixtures of polymeric acids with other substances, such as shellac, cetyl alcohol or cellulose acetate.

The invention is further illustrated by the following examples.

Examples

5 Abbreviations:

BSTU- *N,N'*-bis-(*tert*-butyloxycarbonyl)-*S*-methyl-thiourea,

DMF - dimethylformamide,

DIPEA - *N,N*-diisopropylethylamine;

MeOH – methanol,

10 TFA - trifluoroacetic acid,

DMSO - dimethylsulfoxide

The purity of new compounds was determined by high-performance liquid chromatography (HPLC) using a Waters system consisting of a multisolvent delivery
15 system, column thermostat, autosampler, detector PDA, Chromatography Manager Empower 2 software for PC computations. For the evaluation a C8 column 150 x 4,6 mm with 3.5 μ m particles was used. The mobile phases consisting of 0.1% TFA (phase A), and 0.1% TFA in acetonitrile (phase B) were used at the flow rate of 1mL/min. Gradient elution was used in the gradient system 98% - 5% phase A, and
20 2% - 95% phase B. The concentration of the injected sample was about 0.2 mg/mL and water was used as the solvent. Detection was performed at 275 nm.

Example 1. *N*-bis(*tert*-butyloxycarbonyl)guanyl-*N*-(5,11-dimethyl-5*H*-indolo[2,3- b]quinolin-9-yl)-amine

25 5,11-Dimethyl-5*H*-indolo[2,3-*b*]quinolin-9-yl)-amine (250 mg; 0.95 mM) and 412 mg of BSTU (1.42 mM) was dissolved in 5 mL of dry DMF, then 0.5 mL of DIPEA (2.85 mM) and 385.5 mg of HgCl₂ (1.42 mM) were added. Stirring was continued for 3 hours (for the reaction monitoring the TLC method was used in the CHCl₃:MeOH = 6:1 system, showing completion of substrate III). After evaporation *in vacuo* (< 40°C) the crude
30 product was purified by column chromatography, and CHCl₃:MeOH 20:1, 10:1 was used as the eluent. Then, the fractions were evaporated and 280 mg of the product was obtained with a 58% yield. The result of the ESI MS analysis: calcd. for C₂₈H₃₃N₅O₄

M= 503.59, found $[(M+H)^+] = 504.4$; $[(M+Na)^+] = 526.3$; $[(2M+H)^+] = 1008.6$; $[(2M+Na)^+] = 1029.6$; the correct ^1H - and ^{13}C -NMR spectra.

Example 2. *N*-guanyl-*N*-(5,11-dimethyl-5*H*-indolo[2,3-*b*]quinolin-9-yl)-amine dihydrochloride (1a)

IV (158 mg; 0.31 mM) was treated with 3 mL TFA, and mixed overnight at room temperature. Then, TFA was evaporated *in vacuo* (< 40°C) and the residue was treated with 10 mL of MeOH and evaporated to dryness. The procedure was repeated three times. The solution of HCl/MeOH was added to the residue and evaporated to dryness. Product **1a** 120 mg was obtained as a yellow solid (90%) with 99.7% purity according to the HPLC method. The result of the ESI MS analysis: calcd. for $\text{C}_{18}\text{H}_{17}\text{N}_5$ M= 303.3, found $[(M+H)^+] = 304.4$; $[(2M+H)^+] = 607.3$; the correct ^1H - and ^{13}C -NMR spectra.

Example 3. *N* $^{\alpha}$ -bis(*tert*-butoxycarbonyl)guanyl-glycyl-*N*-(5,11-dimethyl-5*H*-indolo[2,3-*b*]quinolin-9-yl)-amide

N-(5,11-Dimethyl-5*H*-indolo[2,3-*b*]quinolin-9-yl)-glycylamide (50 mg; 0.157 mM), which was obtained according to the methods previously described (K. Sidoryk et al., *J. Med. Chem.* 55 (11), pp. 5077–5087, 2012), and 70 mg of BSTU (0.24 mM) were dissolved in 2 mL of dry DMF, next 0.2 mL of DIPEA (1.4 mM) and 65.1 mg of HgCl_2 (0.24 mM) were added. Stirring was continued for 1 hour (for the reaction monitoring the TLC method was used in the CHCl_3 :MeOH = 6:1 system, it shows completion of the substrate). After the evaporation *in vacuo* (< 40°C) the crude product was purified by preparative TLC chromatography in CHCl_3 :MeOH = 6:1 system. The main band corresponding to the product was removed and washed with MeOH. Then the filtrate was evaporated and 39 mg of the pure product was obtained (44 % yields). The result of the ESI MS analysis: calcd. for $\text{C}_{30}\text{H}_{33}\text{N}_6\text{O}_5$ M= 560.64, found $[(M+H)^+] = 561.4$; $[(M+Na)^+] = 583.4$; $[(2M+H)^+] = 1121.7$; $[(2M+Na)^+] = 1143.7$; the correct ^1H - and ^{13}C -NMR spectra.

Example 4. *N* $^{\alpha}$ -guanyl-glycyl-*N*-(5,11-dimethyl-5*H*-indolo[2,3-*b*]quinolin-9-yl)-amide hydrochloride (2a)

The compound obtained in the example 3 (20 mg; 0.035 mM) was treated with 3 mL of TFA and stirred for 2 hours at room temperature. TFA was evaporated *in vacuo* (< 40°C), and the obtained product was treated with 10 mL of MeOH, then evaporated to dryness. The procedure was repeated three times. The solution of HCl/MeOH was added to the residue and evaporated to dryness. 10 mg of the product was obtained as a yellow solid (80 % yields). The result of the ESI MS analysis: calcd. for $C_{20}H_{20}N_6O$ $M = 360.4$, found $[(M+H)^+] = 361.4$; $[(2M+H)^+] = 721.5$; the correct 1H - and ^{13}C -NMR spectra. The purity according to HPLC was 97.80 %.

The product in the form of a free base was obtained from hydrochloride which was purified by preparative TLC chromatography in methanol : chloroform : 25 % ammonia solution (80:40:1). The isolated amid was dark orange in colour.

Example 5. N^α -bis(*tert*-butyloxycarbonyl)guanyl-*L*-prolyl-*N*-(5,11-dimethyl-5*H*-indolo[2,3-*b*]quinolin-9-yl)-amide

N-(5,11-Dimethyl-5*H*-indolo[2,3-*b*]quinolin-9-yl)-*L*-prolylamide (50 mg; 0.139 mM), which was obtained according to the methods previously described (K. Sidoryk et al., *J. Med. Chem.* 55 (11), pp. 5077–5087, 2012), and 61 mg BSTU (0.21 mM) were dissolved in 2 mL of dry DMF, next 0.2 mL of DIPEA (1.4 mM) and 57 mg of $HgCl_2$ (0.21 mM) were added. Stirring was continued for 2 hours (for the reaction monitoring the TLC method was used in the $CHCl_3$:MeOH = 6:1 system, showing the completion of the substrate). After the evaporation *in vacuo* (< 40°C) the crude product was purified by preparative TLC chromatography in the $CHCl_3$:MeOH = 6:1 system. The main band corresponding to the product was removed and washed with MeOH. Then the filtrate was evaporated and 77 mg of the pure product was obtained (92 % yields). The result of the ESI MS analysis: calcd. for $C_{33}H_{40}N_6O_5$ $M = 600.7$, found $[(M+H)^+] = 601.5$; $[(M+Na)^+] = 623.5$; $[(2M+H)^+] = 1201.8$; $[(2M+Na)^+] = 1223.8$; the correct 1H - and ^{13}C -NMR spectra.

Example 6. N^α -guanyl-*L*-prolyl-*N*-(5,11-dimethyl-5*H*-indolo[2,3-*b*]quinolin-9-yl)-amide hydrochloride (3a)

The compound obtained in the example 5 (56 mg; 0.093 mM) was treated with 3 mL of TFA and stirred for 2 hours at room temperature. TFA was evaporated *in vacuo* (< 40°C) and the obtained product was treated with 10 mL of MeOH, then evaporated to

dryness. The procedure was repeated three times. The solution of HCl/MeOH was added to the residue and evaporated to dryness. 32 mg of product was obtained with 86 % yields. The result of the ESI MS analysis: calcd. for $C_{23}H_{24}N_6O$ $M = 400.4$, found $[(M+H)^+] = 401.4$; $[(M+Na)^+] = 423.4$; $[(2M+H)^+] = 801.5$; $[(2M+Na)^+] = 823.5$; the correct 1H - and ^{13}C -NMR spectra.

The product in the form of a free base was obtained from hydrochloride which was purified by preparative TLC chromatography in methanol : chloroform : 25 % ammonia solution (80:40:1). The isolated amid was red in colour.

Cytotoxic activity *in vitro* study

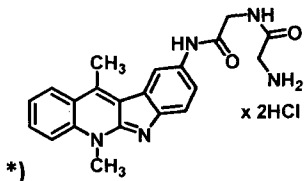
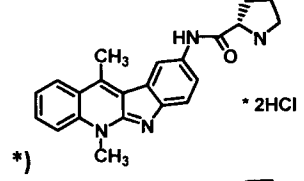
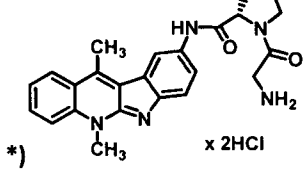
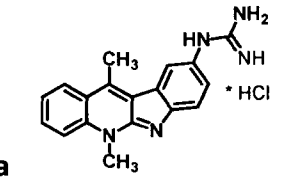
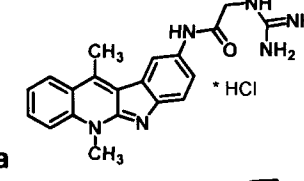
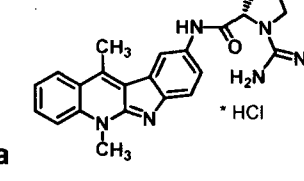
The selected N-guanyl derivatives of 9-amine-5,11-dimethyl-5*H*-indolo[2,3-*b*]quinoline obtained according to the invention were tested for their biological activity against the selected cancer cell lines. Human cancer cell lines: KB (cervix carcinoma), A549 (non-small cell lung cancer), MCF-7 (breast cancer) and LoVo (colon) were used in the study.

Selected derivatives were also tested for their cytotoxic activity against normal mice fibroblasts (BALB/3T3).

All cell lines are maintained at the Institute of Immunology and Experimental Therapy PAS in Wrocław, Poland. They are cultured in the mixture of RPMI 1649 + Opti-MEM (1:1) supplemented with 5% FBS, 100 $\mu g/ml$ streptomycin, 100 units/ml penicillin, 2 mM L-glutamine at 37°C with 5% CO₂ humidified atmosphere.

Test solutions of the studied compounds (1 mg/ml) were prepared by dissolving 1 mg of the substance in 100 μl DMSO completed with 900 μl of the tissue culture medium. Afterwards, the tested compounds were diluted in the culture medium to reach the final concentrations of 10, 1, 0.1 and 0.01 $\mu g/ml$. The biological study was performed using an SRB test which measures the inhibited proliferation of target cells in a 72-hour *in vitro* study (Skehan et al., J. Nat. Cancer. Inst., 82, 1107-1112, 1990). Each compound in a given concentration was tested in triplicate in each experiment. The experiments were repeated 3-5 times. The results of the cytotoxic activity study *in vitro* were expressed as ID₅₀ (the concentration of the compound in $\mu g/ml$ that inhibits the proliferation rate of the tumor cells by 50% as compared to the control untreated cells) and depicted in Table 1. The results of the cytotoxic activity assay of some representative compounds were presented, too.

Table 1. Cytotoxic activity in vitro of 5,11-dimethyl-5H-indolo[2,3-b]quinolin-9-yl-amine derivatives

Compound	IC ₅₀ [μg/mL]				
	BALB/ 3T3	A549	MCF-7	LoVo	KB
DIMIQ	1.42±0.23	0.54±0.12	0.38±0.13	0.05±0.10	0.21±0.04
DOX	0.58±0.01	0.18±0.05	0.24±0.08	0.05±0.01	0.45±0.01
Cisplatin	2.89±0.68	2.65±0.45	2.9±0.52	1.7±0.05	2.55±0.25
 *)	0.238±0.007	0.56±0.048	0.62±0.168	0.376±0.0	0.29±0.4
 *)	0.266±0.035	0.228±0.15	0.385±0.027	0.381±0.05	0.2±0.07
 *)	0.255±0.069	0.484±0.04	0.633±0.075	0.249±0.07	0.4±0.12
 1a	24.98±4.59	0.054±0.03	0.024±0.004	28.91±6.23	0.332±0.005
 2a	2.1±0.18	0.854±0.18	2.23±0.59	0.393±0.004	1.82±0.55
 3a	16.25±9.79	5.62±2.41	4.0±1.54	3.95±0.75	7.93±1.47

IC₅₀ – half maximal inhibitory concentration: non-small cell lung cancer **A549**, breast cancer **MCF-7**, colon cancer **LoVo**, cervix carcinoma **KB** and mice fibroblast **BALB/3T3**;

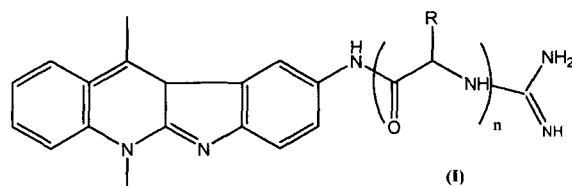
5 **DOX** – doxorubicin hydrochloride; *) prior art compound

High cytotoxic activity was observed in the biological study *in vitro* of new N-guanyl derivatives of 5,11-dimethyl-5*H*-indolo[2,3-*b*]quinoline of formula (I). Compound **1a** showed the highest selectivity, displaying the highest cytotoxic activity against non-small cell lung cancer A549 (IC_{50} 0.054 ± 0.03 $\mu\text{g/ml}$), and against breast cancer MCF-7 (IC_{50} 0.024 ± 0.004 $\mu\text{g/ml}$). It showed cytotoxic activity against KB cervix carcinoma (IC_{50} 0.332 ± 0.005 $\mu\text{g/ml}$), however, it did not show any cytotoxic activity against colon cancer LoVo (IC_{50} $28,91 \pm 6,23$ $\mu\text{g/ml}$). The cytotoxic activity of compound **1a** against normal fibroblasts was up to 1000 times lower than against A549 and MCF-7 cancer cell lines. Such high selectivity was not observed in any of the standard anti-cancer drugs, such as doxorubicin and cisplatin, which showed comparative cytotoxic activity against cancer cells as well as against normal cell. The cytotoxic activity of DiMIQ against cancer cell lines is comparable to its cytotoxic activity against normal cell lines. A high cytotoxic activity was observed in derivatives **2a** and **3a**, however, a significant selectivity of action was not observed. The highest activity against the LoVo cell lines (IC_{50} 0.393 ± 0.004 $\mu\text{g/ml}$) and A549 cell lines (IC_{50} 0.854 ± 0.18 $\mu\text{g/ml}$) was exhibited by derivative **2a**, whereas its activity against the KB and MCF-7 cell lines was lower, 1.8 and 2.2 $\mu\text{g/ml}$, respectively. Compound **2a** exhibited a cytotoxic activity against normal cells - the IC_{50} value was 2.1 $\mu\text{g/ml}$. The cytotoxic activity of derivative **3a** against all cancer cell lines was in the range 3.95-7.93 $\mu\text{g/ml}$. However, its cytotoxic activity against normal BALB3T3 cell lines was lower, 16.25 ± 9.79 $\mu\text{g/ml}$.

Claims:

1. N-Guanyl derivative of 9-amino-5,11-dimethyl-5*H*-indolo[2,3-*b*]quinoline of Formula (I)

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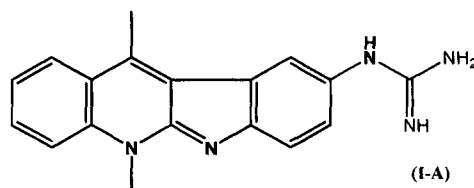
wherein:

n is 0 or 1, and

when *n* = 1, *R* is a side chain of the acylating amino acid residue,

10 and pharmaceutically acceptable salts thereof.

2. N-Guanyl derivative according to claim 1 having the structure represented by Formula (I) wherein *n* = 0

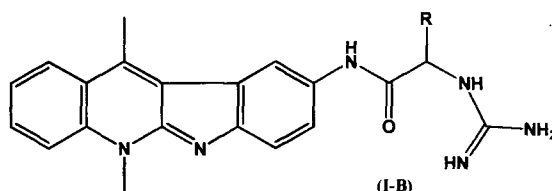


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3. N-Guanyl derivative according to claim 2 in a hydrochloride form.

4. N-Guanyl derivative according to claim 1 having the structure represented by Formula (I) wherein *n* = 1, and *R* is a side chain of the acylating α -amino acid residue

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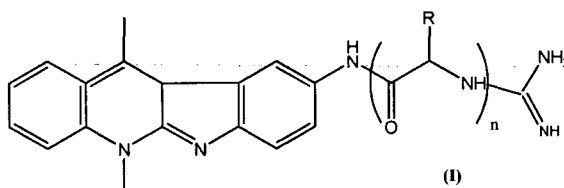
5. N-Guanyl derivative according to claim 1 wherein acylating α -amino acid residue is glycine or L-proline.

6. N-Guanyl derivative according to claim 4 selected from the group comprising:

N^{α} -guanyl-glycyl-*N*-(5,11-dimethyl-5*H*-indolo[2,3-*b*]quinolin-9-yl)amide, optionally in hydrochloride form;

N^{α} -guanyl-*L*-prolyl-*N*-(5,11-dimethyl-5*H*-indolo[2,3-*b*]quinolin-9-yl)amide, optionally in hydrochloride form.

7. A process for the preparation of N-guanyl derivatives of 9-amino-5,11-dimethyl-5*H*-indolo[2,3-*b*]quinoline of Formula (I)

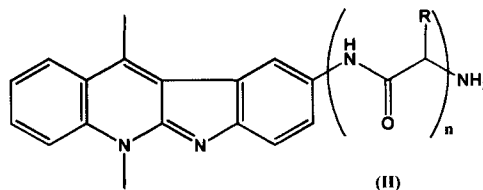


10 wherein:

n is 0 or 1, and

when $n = 1$, R is a side chain of the acylating α -amino acid residue, and their pharmaceutically acceptable salts, comprising the following steps:

15 (i) guanylation of 9-amino-derivative of 5,11-dimethyl-5*H*-indolo[2,3-*b*]quinoline of Formula (II)



wherein:

n is 0 or 1, and

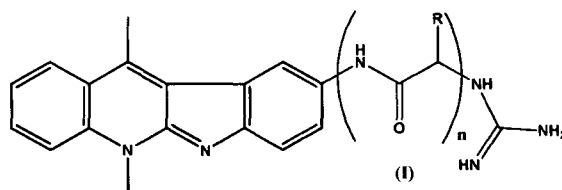
20 when $n = 1$, R is a side chain of the acylating α -amino acid residue, using a guanylation reagent,

(ii) isolation of the crude reaction product of step (i) and its purification by column chromatography,

(iii) if necessary, the removal of the protecting groups under acidic conditions to obtain product (I) in the form of salt with the acid used in step (iii),

(iv) isolation and purification of product (I) obtained in step (iii),

- (v) optionally, conversion of product (I) into a desired pharmaceutically acceptable salt.
8. The process according to Claim 7, characterized in that the *N*-protected guanylyating reagent is used for the guanylation reaction in step (i).
9. The process according to Claim 8, characterized in that the *N*-protected guanylyating reagent is *N,N'*-diBoc-*S*-methylisothiurea.
10. The process according to Claim 8, characterized in that the guanylation reaction using *N,N'*-diBoc-*S*-methylisothiurea is carried out in the presence of mercury (II) chloride as a catalyst and a tertiary amine.
11. The process according to Claim 7, characterized in that the protecting groups are removed by trifluoroacetic acid.
12. A pharmaceutical formulation for the treatment and/or prevention of cancer diseases comprising an active substance and pharmaceutically acceptable carriers and/or excipients, characterized in that the active substance is *N*-guanyl derivative of 9-amino-5,11-dimethyl-5*H*-indolo[2,3-*b*]quinoline of Formula (I)

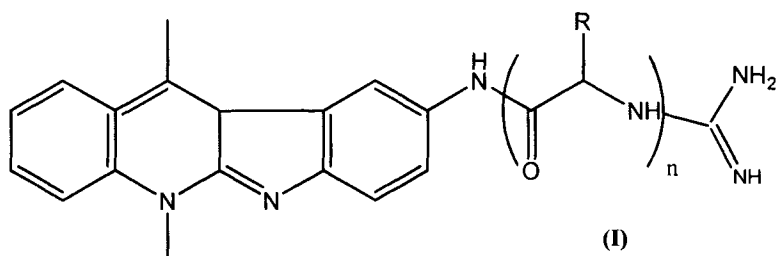


wherein:

n is 0 or 1, and

- when *n* = 1, *R* is a side chain of the acylating α -amino acid residue, or its pharmaceutically acceptable salt.

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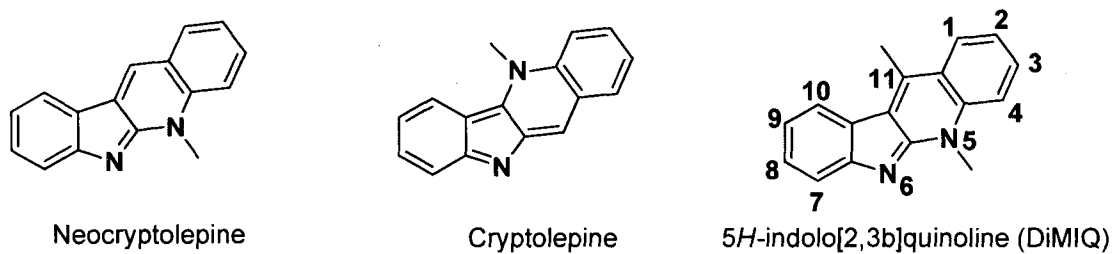
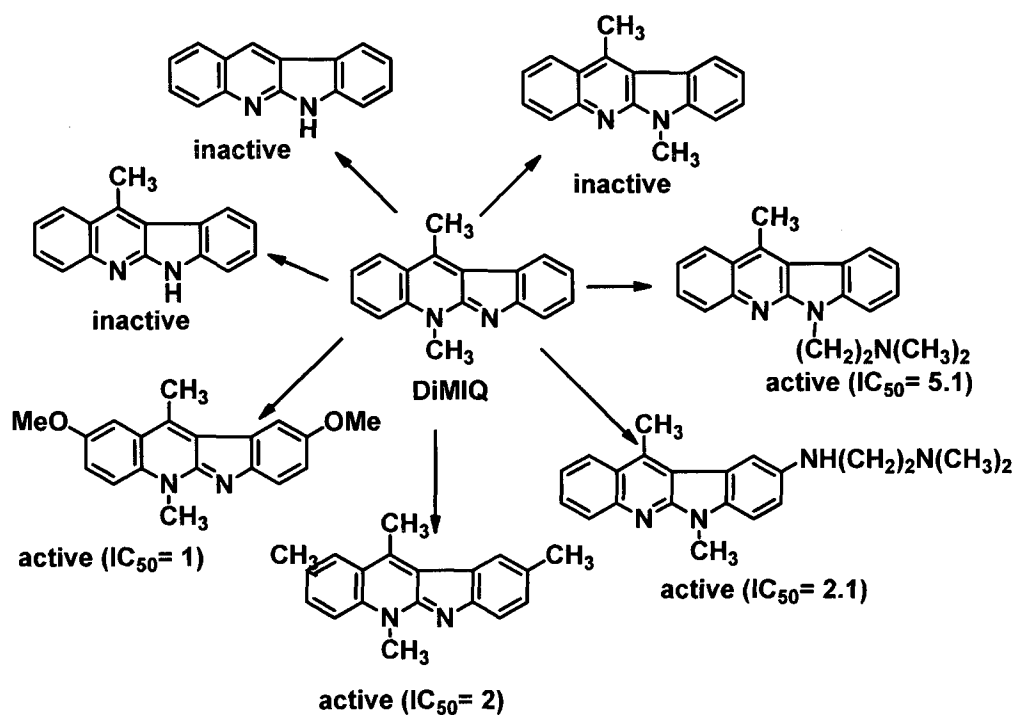


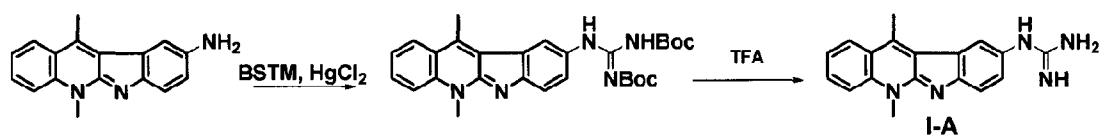
Fig. 1. Indoloquinoline alkaloids



10 Fig. 2. The effect of the type of a substituent on the cytotoxic activity of indolo[2,3-b]quinolines (values of IC_{50} are in μM).

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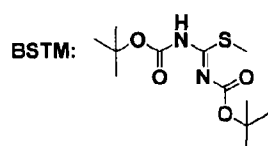
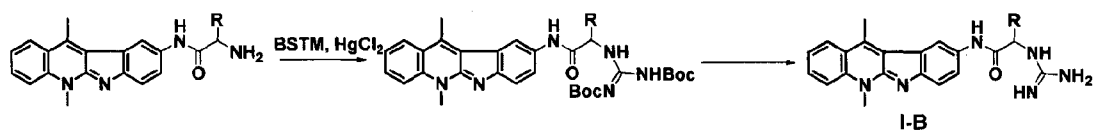
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Scheme 1. Preparation of the compounds of Formula (I-A)

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Scheme 2. Preparation of the compounds of Formula (I-B)

INTERNATIONAL SEARCH REPORT

International application No
PCT/PL2015/000053

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07D401/04 A61K31/437 A61P35/00
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07D

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BEILSTEIN Data, BIOSIS, CHEM ABS Data, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	KATARZYNA SIDORYK ET AL: "Synthesis and Biological Evaluation of New Amino Acid and Dipeptide Derivatives of Neocryptolepine as Anticancer Agents", JOURNAL OF MEDICINAL CHEMISTRY, vol. 55, no. 11, 14 June 2012 (2012-06-14), pages 5077-5087, XP055194483, ISSN: 0022-2623, DOI: 10.1021/jm300468t cited in the application	1,4-12
A	tables 1-3 ----- -/--	2,3



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

11 June 2015

Date of mailing of the international search report

24/06/2015

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
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Authorized officer

Stroeter, Thomas

INTERNATIONAL SEARCH REPORT

International application No
PCT/PL2015/000053

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SIDORYK KATARZYNA ET AL: "Searching for new derivatives of neocryptolepine: Synthesis, antiproliferative, antimicrobial and antifungal activities", EUROPEAN JOURNAL OF MEDICINAL CHEMISTRY, vol. 78, 20 March 2014 (2014-03-20), pages 304-313, XP028847869, ISSN: 0223-5234, DOI: 10.1016/J.EJMECH.2014.03.060	1,4-12
A	tables 1, 3	2,3
Y	----- CARUSO ANNA ET AL: "Synthesis and evaluation of cytotoxic activities of new guanidines derived from carbazoles", BIOORGANIC & MEDICINAL CHEMISTRY LETTERS, PERGAMON, AMSTERDAM, NL, vol. 24, no. 2, 17 December 2013 (2013-12-17), pages 467-472, XP028814533, ISSN: 0960-894X, DOI: 10.1016/J.BMCL.2013.12.047	1,4-12
A	table 2; compound 5f -----	2,3