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Synthesis and biological evaluation of amino analogs of Ludartin: Potent and selective cytotoxic agents

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ABSTRACT

Diverse amino analogs of Ludartin, a cytotoxic guaianolide and a position isomer of an anticancer drug, Arglabin were prepared through Michael type addition at its highly active α-methylene-γ-lactone motif. The semisynthetic derivatives were subjected to sulphorhodamine B cytotoxicity assay against a panel of four different human cancer cell lines viz. lung (A-549), leukemia (THP-1), prostate (PC-3) and colon (HCT-116) to look into structure–activity relationship. Few of the analogs displayed potent selective cytotoxicity compared to the parent molecule-Ludartin (**1**). (11*R*)-13-(Diethyl amine)-11,13-dihydroludartin (**6**) and (11*R*)-13-(piperidine)-11,13-dihydroludartin (**10**) showed almost same cytotoxicity against leukemia cell lines (THP-1) as that of parent molecule-Ludartin, but were more active against colon (HCT-116) cancer cells. (11*R*)-13-(Morpholine)-11,13-dihydroludartin (**11**) displayed selectively better cytotoxicity against Leukemia cancer cells (THP-1) exhibiting IC_{50} of 2.8 μM. (11*R*)-13-(6-Nitroindazole)-11,13-dihydroludartin (**17**) was four times more potent than Ludartin with selective cytotoxic effects against prostate cancer cells (2.2 μM) while as (11*R*)-13-(6-nitroindazole)-11,13-dihydroludartin (**18**) exhibited three-fold selective cytotoxicity for Lung (A-549) cancer cell lines exhibiting IC_{50} of 2.6 μM.

Sesquiterpene lactones (SLs) display a wide array of biological activities like antiviral, antibacterial, antiulcer, cytotoxic, anti-inflammatory, antifungal and effects on the central nervous system and cardiovascular system.[1] The unique chemical properties of SLs like presence of alkylating center reactivity (α-methylene-γ-lactone), lipophilicity along with molecular geometry and electronic features make them highly bioactive with in the living systems.[2] However the clinical translation of these lactones is hampered due to their nonselective binding at undesired targets via the highly reactive Michael acceptor, that is, α-methylene-γ-lactone.[3] The high lipophilicity allows their facile penetration via the cell membranes increasing their cytotoxicity in vitro, but higher lipophilicity dictates their lower drug bioavailability in vivo.[2] These queries have been addressed by synthetic chemists and one such approach has been the amino-prodrug approach, where in the aza-Michael adducts display the enhanced aqueous solubility, improved pharmarmacokinetic potential and even retention or augmentation in bioactivity.[3] These amino based Michael adducts have been prepared for various bioactive sesquiterpene lactones such as alantolactone,[4] costunolide,[5] parthenolide,[6–9] α-santonin,[10] helenalin[11] and ambrosin[12] and effective structure–activity relationships have been developed. It is however assumed that the amino analogs serve as prodrugs which in presence of thiol groups of proteins, enzymes and glutathione release the amine group to generate the parent enone motif that defines their mechanism of action at the desired targets.[9] Approaches have been made to devise such synthetic methods that make SLs highly target specific to cancerous cells while sparing the normal cells. At present the SL drugs in clinical trials are artemisinin from *Artemisia annua* L., thapsigargin from *Thapsia* and parthenolide from *Tanacetum parthenum* and/or many of their synthetic derivatives.[2]

Ludartin, a bioactive natural product of the widely distributed class of guaianolides was previously isolated as a mixture along with 11,13-dihydroderivative from *A. caruthii*[13] and then in pure form from *Stevia yaconensis* var. *subeglandulosa*[14] and from *A. filatovae*.[15] The heart of this molecule consists of a cycloheptane ring with five contiguous stereocenters, to which two five membered rings are *trans*-annulated. One of the five membered rings is a γ-butyrolactone with an exocyclic double bond. Ludartin shows gastric cytoprotective effect[16] and also inhibits aromatase enzyme which is involved in hormone-dependent breast cancer.[17] So far there are no reports on the SAR studies of this molecule, and keeping in view the bioactivity of the molecule along with the literature precedent that reductive amination at exocyclic double bond of a cytotoxic agent can enhance selectivity for malignant cell lines,[18]

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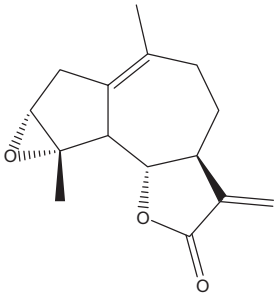
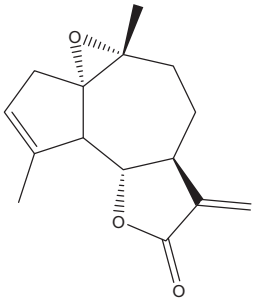
we designed the synthesis of various Michael adducts at the highly electrophilic α-methylene-γ-lactone moiety with the hope to develop analogs with enhanced bioavailability, lesser toxicity and better activity. This however would constitute an important step towards the rationalization of lead properties of Ludartin (**1**).



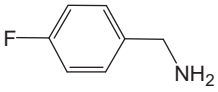
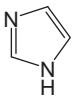
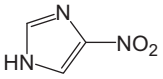
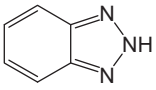
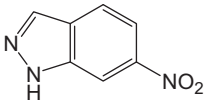
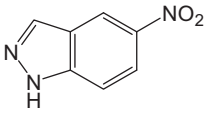
Ludartin (**1**)



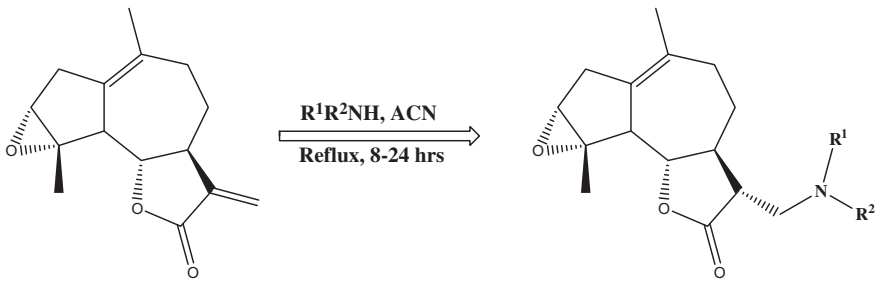
Arglabin (**19**)

Our synthetic efforts were mainly focussed towards Michael addition at exocyclic double bond of α-methylene-γ-lactone motif (Scheme 1). Therefore Ludartin was subjected to synthetic modifications (Michael addition) using a diversity of amines as Michael donors. Different analogs were prepared by refluxing a solution of Ludartin (**1**) in acetonitrile and an appropriate amine for 8–24 h[19] (Scheme 1). The reaction was completely clean furnishing products with satisfactory yields (70–92%) (Table 1). Unlike primary amines cyclic secondary amines like pyrollidine, piperidine, morpholine reacted quickly without using any base. On the other hand azole type Michael donors like imidazole, benzotriazole, required DBU as base for the successful completion of the reaction owing to lesser reactivity of *N*-containing heterocycles. A library of 17 analogs was prepared whose formation could easily be confirmed by the disappearance of two diagnostic proton resonances at δ 5.38 ppm (d, J = 3.5 Hz) and δ 6.21 ppm (d, J = 3.5 Hz) of α-methylene-protons (13-H_2) of Ludartin (**1**). Since the molecule Ludartin in itself bears five contiguous chiral centers (C-3–7), the Michael addition creates one more chiral center at C-11 position whose configuration was determined on the basis of correlations deduced from NOESY experiment. In the NOE experiment of compound **9**, the NOESY correlation observed between H-6 and H-11 unambiguously established the H_7–H_{11} anticonfiguration (Fig. 1). Since the configuration of already existing stereocenter at C-7 is *S*, the stereocenter at C-11 was unambiguously put as *R*, hence staying in tune with the literature precedent[4,6,18,20] that the Michael addition at exocyclic double bond of sesquiterpene lactones proceeds with high diastereoselectivity at position-11 giving preferentially the less hindered *R* isomer.

Table 1
Preparation of different amino analogs of Ludartin (**1**)

S. no	R^1R^2HN[a]	Product	Yield[b] (%)
1		**2**	80
2		**3**	76
3		**4**	64
4		**5**	72
5		**6**	67
6		**7**	78
7		**8**	80
8		**9**	90
9		**10**	92
10		**11**	90
11		**12**	73
12		**13**	64
13		**14**	60
14		**15**	66
15		**16**	75
16		**17**	78
17		**18**	84

[a] Here R^1R^2NH refers to any primary, secondary or heteroaromatic amine.
[b] Refers to the yield after isolation of products.



Ludartin (1) **17 amino Ludartin analogs**

Scheme 1.

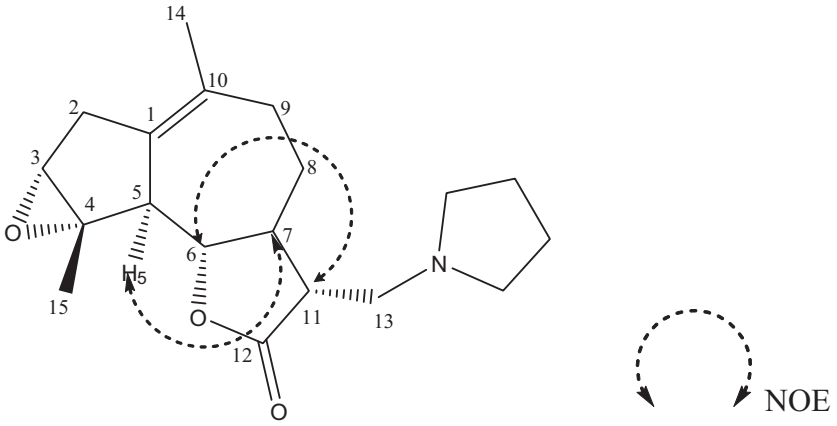


Figure 1. NOE correlations of compound **9**.

Ludartin along with its 17 amino analogs were then studied in a calorimetric sulphorhodamine B (SRB) cytotoxicity assay[21] against four human cancer cell lines viz. lung (A-549), leukemia (THP-1), prostate (PC-3) and colon (HCT-116). Preliminary cytotoxic screening of the analogs was carried out at 50 μM concentration. The analogs which exhibited greater than 50% growth inhibition at that concentration were further assayed at different concentrations (10–100 μM) to generate the IC_{50} values given in Table 2. From the cytotoxicity profile it is clear that Ludartin exhibited a broad spectrum cytotoxic activity against all the tested cancer cell lines displaying IC_{50} values of 3.1 (THP-1), 7.4 (A-549), 6.9 (HCT-116) and 7.5 (PC-3) μM. However certain analogs prepared from Ludartin exhibited a bit selective and improved cytotoxicity towards particular cancer cell lines with some others showing complete loss of activity. The secondary amino analogs of Ludartin in general exhibit better activity as compared to the primary analogs. Both the diethyl (**6**) and piperidine (**10**) amino analogs exhibited twofold increase in cytotoxicity against the colon cells (HCT-116) and retained same cytotoxicity level against leukemia (THP-1) compared to Ludartin (**1**). However incorporation of O-atom at the position 4 of piperidine affording Morpholino analog (**11**) increased both cytotoxity as well as selectivity of this analog towards leukemia cell line (THP-1) exhibiting IC_{50} value of 2.8 μM. However the same Morpholino analog was weakly active against other three cancer cell lines. Among the heteroaromatic amines used imidazole (**13**), 4-nitroimidazole (**14**) and benzotriazole (**16**) analogs exhibited complete loss of cytotoxicity displaying very low growth inhibition at the preliminary screening concentration. However 1,2,4-triazole analog (**15**) exhibited moderate cytotoxicity profile against all the tested cancer cell lines. An interesting difference was observed between 6-nitroindazole (**17**) and 5-nitroindazole (**18**) analogs against the cancer cell lines. 6-Nitroindazole analog (**17**) of Ludartin was cytotoxic against prostate cancer cells (PC-3) and displayed 3 to 4 fold increase in cytotoxicity (IC_{50} = 2.2 μM) compared to Ludartin while its 5-nitro analog exhibited the three fold increase in cytotoxicity but human lung cancer cell (A-549) specificity (IC_{50} = 2.6 μM).

The position isomer of Ludartin (**1**), that is, Arglabin (**19**), was approved for its use in several countries to treat lung, liver and ovarian cancers.[23] Derivatives of Arglabin prepared from the reaction of exocyclic double bond show similar or slightly reduced but cell line dependent cytotoxicity.[23] Among all the analogs of Arglabin (**19**), piperizine analog is the selective but potent cytotoxic agent displaying IC_{50} = 3.75 μM towards ovarian cancer cells

Table 2
Cytotoxicity profile of Ludartin and its amino analogs against four different cancer cell lines

Compound	Lung (A-549)		Leukemia (THP-1)		Prostate (PC-3)		Colon (HCT-116)	
	% Inhibition[a]	IC_{50}	% Inhibition[a]	IC_{50}	% Inhibition[a]	IC_{50}	% Inhibition[a]	IC_{50}
1	97	7.4	98	3.1	97	7.5	97	6.9
2	10	nd	25	nd	12	nd	16	nd
3	20	nd	68	29	43	nd	36	nd
4	39	nd	23	nd	17	nd	02	nd
5	00	nd	28	nd	14	nd	04	nd
6	73	22	98	3.5	56	32	95	3.7
7	0	nd	39	nd	16	nd	28	nd
8	36	nd	40	nd	33	nd	04	nd
9	00	nd	67	26	11	nd	15	nd
10	87	17	93	3.9	85	23	90	4.0
11	42	nd	97	2.8	59	nd	58	nd
12	16	nd	26	nd	64	nd	00	nd
13	00	nd	36	nd	53	nd	42	nd
14	00	nd	32	nd	15	nd	30	nd
15	69	32	83	15	66	35	70	20
16	25	nd	15	nd	40	nd	13	nd
17	84	35	63	29	99	**2.2**	86	17
18	99	**2.6**	72	31	52	34	57	40

IC_{50} values are expressed in μM concentration.
nd = Not determined in the given concentration range.
[a] % Inhibition refers to growth inhibition measured at 50 μM concentration.

(A2780) compared to the parent Arglabin (**19**) with IC_{50} of 12.55 μM against the same cell line. On the other hand the most selective derivative of Ludartin is Morpholino analog which shows an IC_{50} = 2.8 μM towards leukemia (THP-1) and the most potent analog is 6-nitroindazole (**17**) with IC_{50} = 2.2 μM towards prostate (PC-3) compared to parent Ludartin with IC_{50} of 3.1 and 6.9 μM towards leukemia (THP-1) and prostate (PC-3), respectively. Thus our study is in fine tune with those of *R*. Csuk that modification at exocyclic double bond leads to analogs with either modified selectivity towards particular cell lines or similar/reduced cytotoxicity.

Dimethyl amino analog of Arglabin is a registered antitumor substance in the Republic of Kazakstan.[24]

The amino analogs of Arglabin act by impeding the protein farnesylation especially those of RAS proteins. The drug inhibits the incorporation of [(3)*H*]farnesylpyrophosphate into human H-RAS protein by FTase.[24] Farnesylation of RAS is required for proteolytic processing and tight binding of RAS to cellular membranes. In the absence of farnesylation oncogenic forms of RAS cannot oncogenically transform cells. Thus inhibitors of farnesyl-protein transferase are considered to be useful anticancer therapeutics for many types of cancers.[25] Since Ludartin is a position isomer of Arglabin, it is likely that the amino analogs of Ludartin may also follow the same mechanism of action. However further studies are needed to explore the exact pathway and the selectivity in action of the amino analogs of Ludartin. We have already started the work in this direction.

In conclusion a diversity of amine analogs were prepared and studied for any structure–activity relationship. The SAR studies clearly indicate that modifications at α-methylene-γ-lactone moiety result in improved and selective cytotoxicity of the bioactive lactones. Moreover it is the leukemia cell line (THP-1) that forms the usual target of most of the analogs of Ludartin with enhanced cytotoxity compared to the parent molecule Ludartin (**1**). Above all this approach provides in one synthetic step, an opportunity to enhance pharmacokinetic properties to selectively target specific cells with either retention or augmentation of cytotoxic effect. Further efforts to expand the library of such amino analogs, to come up with a better lead molecule, are underway.

Acknowledgment

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References and notes

1. Chaturvedi, D.; Opportunity, Challenge and Scope of Natural Products in Medicinal Chemistry. 2011, pp 313–315.
2. Ghantous, A.; Muhtasib, H. G.; Vuorela, H.; Saliba, N. A.; Darwiche, N. *Drug Discovery Today* **2010**, *15*, 668.
3. Woods, J. R.; Mo, H.; Bieberich, A. A.; Alavanja, T.; Colby, D. *Med. Chem. Commun.* **2013**, *4*, 27.
4. Lawrence, N. J.; McGown, A. T.; Nduka, J.; Hadfield, J. A.; Pritchard, R. G. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 429.
5. Srivastava, S. K.; Abraham, A.; Bhatt, B.; Jaggi, M.; Singh, A. T.; Sanna, V. K.; Singh, G.; Agarwal, S. K.; Mukherjee, R.; Burman, A. C. *Bioorg. Med. Chem.* **2006**, *16*, 4195.
6. Hwang, D. R.; Wu, Y. S.; Chang, C. W.; Lien, T. W.; Chen, W. C.; Tan, U. K.; Hsu, J. T. A.; Hsieh, H. P. *Bioorg. Med. Chem.* **2006**, *14*, 83.
7. Neelakanthan, S.; Nasim, S.; Guzman, M. L.; Jordon, C. T.; Crooks, P. A. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 4346.
8. Nasim, S.; Crooks, P. A. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 3870.
9. Woods, J. R.; Mo, H.; Bieberich, A. A.; Alavanja, T.; Colby, D. A. *J. Med. Chem.* **2011**, *54*, 7934.
10. Klochkov, S. G.; Afanaseva, S. V.; Pushin, A. N.; Gerasimovr, G. K.; Vlasenkova, N. K.; Bulychew, Y. N. *Chem. Nat. Compd.* **2009**, *46*, 817.
11. Lee, M.; Furukawa, H.; Huang, E. S. *J. Med. Chem.* **1972**, *15*, 609.
12. Hejchman, E.; Haugwitz, R. D.; Cushman, M. *J. Med. Chem.* **1995**, *38*, 3407.
13. Geissman, T. A.; Griffin, T. S. *Phytochemistry* **1972**, *11*, 833.
14. Sosa, V. E.; Oberti, J. C.; Gil, R. R.; Ruveda, E. A.; Goedken, V. L.; Gutierrez, A. B.; Herz, W. *Phytochemistry* **1989**, *28*, 1925.
15. Jalmakhanbetova, R. I.; Adekenov, S. M. *Chem. Nat. Compd.* **2007**, *43*, 347.
16. Giordano, O. S.; Guerreiro, E.; Pestchanker, M. J.; Guzman, J.; Pastor, D.; Guardia, T. *J. Nat. Prod.* **1990**, *53*, 803.
17. Blanco, J. G.; Gil, R. R.; Alvarez, C. I.; Patrito, L. C.; Genti-Raimondi, S.; Flury, A. *FEBS Lett.* **1997**, *409*, 396.
18. Duvvuri, M.; Konkar, S.; Hong, K. H.; Blagg, B. S. J.; Krise, B. J. P. *ACS Chem. Biol.* **2006**, *1*, 309.
19. **Plant material and isolation of Ludartin:** the plant material of *Artemisia amygdalina* Decne was collected from the high altitude areas of Gurez valley in Kashmir and authenticated by Professor A.R. Naqshi, Department of Botany, University of Kashmir and by comparision with the *A. amygdalina* growing within the institute's gene bank. The shoot and root parts were separately processed, shade dried, chopped and ground to a fine powder. The powdered shoot part was extracted thrice with chloroform. The chloroform extracts so obtained were evaporated under vaccuo on a rotary evaporator to afford a crude extract of 65.0 g. About 50.0 g of the extract was subjected to normal silica-gel column chromatography using successively hexane (100%) and hexane–EtOAc (95:5, 90:10, 85:15, 80:20, 75:25, 70:30) as eluent (unpublished work). All the 90:10 (hexane/EtOAc) fractions were pooled up and concentrated to furnish pure white crystals of Ludartin (2.0 g) whose structure was elucidated on the basis of spectral data analysis and by comparision with that of literature data.[13,14] **Synthesis of Amino analogs of Ludartin (2–18):** A solution of **1** (30 mg, 0.121 mmol) in acetonitrile (2 ml) and amine (0.121 mmol) was heated under reflux for 8–24 h either in presence of base (DBU) or more often without the base. After cooling the reaction mixture was evaporated under vaccuo on a rotary evaporator and the residue obtained was subjected to normal silica-gel column chromatography using Hexane-EtOAc as eluent to furnish the pure product. **Spectral data of reference compound (9)**: Yield 90%; Rf = 0.5 (5% MeOH-DCM); IR (KBr cm^{-1}) 3013, 2960, 1758, 1205, 780; ^1H NMR (400 MHz, $CDCl_3$); δ 3.64 (t, J = 10.8, 10.8 Hz, 1H), 3.39 (s, 1H), 3.04 (d, J = 10.7, 1H), 2.83 (d, J = 5.7 Hz, 2H), 2.71–2.68 (m, 1H), 2.52–2.43 (m, 5H), 2.36–2.31 (m, 1H), 2.18–2.15 (m, 3H), 2.02–1.98 (m, 1H), 1.75 (s, 4H), 1.68 (s, 3H), 1.62 (s, 3H), 1.30–1.18 (m, 1H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 175.60, 133.99, 131.79, 78.84, 66.03, 62.59, 53.96, 53.21, 52.74, 50.53, 44.39, 33.04, 32.28, 26.43, 22.44, 21.22, 17.96. ESI-MS (m/z): 318 $(M+H)^+$. Similarly other compounds were synthesized and characterized using spectral data analysis.
20. Matsuda, H.; Kageura, T.; Inoue, Y.; Morikawa, T.; Yoshikawa, M. *Tetrahedron* **2000**, *56*, 7763.
21. Cytotoxicity assay: RPMI-1640 medium, streptomycin, fetal bovine serum, sodium bicarbonate, phosphate buffer saline, sulphorhodamine, trypsin, 5-flurouracil, gentamycin sulphate were purchased from Sigma Chemicals Co. Glacial acetic acid from Fischer scientific and trichloroactetic acid (TCA) from Merck specialties private limited. All the human cancer cell lines (A-549, THP-1, PC-3, HCT-116) were obtained from ATCC Sigma. All the cells used were grown in RPMI-1640 medium containing 10% FBS, 100 unit penicillin/100 μg streptomycin per ml medium. Cells were allowed to grow in carbon dioxide incubator (Thermoscientific USA) at 37 °C with 98% humidity and 5% CO_2 gas environment. In the present study the cytotoxic effect of the different analogs was evaluated using sulpharhodamine B (SRB) assay. The SRB dye binds to the basic protein of cells that have been fixed to tissue-culture plates by trichloroacetic acid (TCA). As the binding of SRB is stoichiometric, the amount of dye extracted from stained cells is directly proportional to the cell number. In the present case, all cells lines seeded in flat-bottomed 96-well plates were allowed to adhere overnight, and then media containing different analogs (varying concentrations) were added. The plates were assayed for 48 h. The cells were fixed by adding 50 μl of ice-cold 50% TCA to each well for 60 min. The plates were washed five times in running tap water and stained with 100 μl per well SRB reagent (0.4% w/v SRB) in 1% acetic acid for 30 min. The plates were washed five times in 1% acetic acid to remove unbound SRB and allowed to dry overnight. SRB was solubilised with 100 μl per well, 10 mM tris–buffer, shaken for 5 min and the optical density was measured at 570 nm.[22]
22. Kaur, S.; Kamboj, S.; Singh, J.; Saxena, A. K.; Dhuna, V. A. *Biotechnol. Lett.* **2005**, *27*, 1815.
23. Csuk, R.; Heinold, A.; Siewert, B.; Schwarz, S.; Barthel, A.; Kluge, R.; Strohl, D. *Arch. Pharm. Chem. Life Sci.* **2012**, *345*, 215.
24. Shaikenov, T. E.; Adekenov, S. M.; Williams, R. M.; Prashad, N.; Baker, F. L.; Madden, T. L.; Newman, R. *Oncol. Rep.* **2001**, *8*, 173.
25. Adekenov, S. M. U.S. Patent 5,902,809 A, 1999.