Appl Biochem Biotechnol (2013) 170:676–689
DOI 10.1007/s12010-013-0225-z

Chemical Structure and In Vitro Antitumor Activity of Rhamnolipids from *Pseudomonas aeruginosa* BN10

Neli Christova · Boryana Tuleva · Anton Kril · Milka Georgieva · Spiro Konstantinov · Ivan Terziyski · Biliana Nikolova · Ivanka Stoineva

Received: 29 November 2011 / Accepted: 7 April 2013 /
Published online: 19 April 2013

Abstract A newly isolated indigenous strain BN10 identified as *Pseudomonas aeruginosa* was found to produce glycolipid (i.e., rhamnolipid-type) biosurfactants. Two representative rhamnolipidic fractions, RL-1 and RL-2, were separated on silica gel columns and their chemical structure was elucidated by a combination of nuclear magnetic resonance and mass spectroscopy. Subsequently, their cytotoxic effect on cancer cell lines HL-60, BV-173, SKW-3, and JMSU-1 was investigated. RL-1 was superior in terms of potency, causing

N. Christova · B. Tuleva
Institute of Microbiology, Bulgarian Academy of Sciences, Acad. G. Bonchev str., bl 26, 1113 Sofia, Bulgaria

A. Kril
Institute for Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences, Acad. G. Bonchev str., bl 25, 1113 Sofia, Bulgaria

M. Georgieva
Laboratory of Molecular Diagnostics, Department of Pathology, National Oncological Centre, 6 Plovdivsko pole str., 1156 Sofia, Bulgaria

S. Konstantinov
Department of Pharmacology, Pharmacotherapy and Toxicology, Faculty of Pharmacy, Medical University–Sofia, 2 Dunav str., 1000 Sofia, Bulgaria

I. Terziyski
Institute of Physical Chemistry, Bulgarian Academy of Sciences, Acad. G. Bonchev str., bl 11, 1113 Sofia, Bulgaria

B. Nikolova
Institute of Biophysics and Biomedical Engineering, Acad. G. Bonchev str., bl 21, 1113 Sofia, Bulgaria

I. Stoineva (✉)
Institute of Organic Chemistry with Centre of Phytochemistry, Bulgarian Academy of Sciences, Acad. G. Bonchev str., bl 9, 1113 Sofia, Bulgaria
e-mail: ane@orgchm.bas.bg

Ivanka. Stoineva
e-mail: istoineva@yahoo.com

50 % inhibition of cellular viability at lower concentrations, as compared to RL-2. Furthermore, the results from fluorescent staining analysis demonstrated that RL-1 inhibited proliferation of BV-173 pre-B human leukemia cells by induction of apoptotic cell death. These findings suggest that RL-1 could be of potential for application in biomedicine as a new and promising therapeutic agent.

Keywords *Pseudomonas* · Rhamnolipids · NMR · ESI–MS · Antitumor activity

Introduction

Biosurfactants are microbial compounds excreted by a great number of microorganisms such as bacteria, fungi, and yeast. They comprise a diverse group of chemical structures with amphiphilic character. The structures and physico-chemical properties of a large variety of biosurfactants have been studied extensively [1–3]. Generally, the lipophilic parts of their molecules consist of long-chain fatty acids, hydroxyl fatty acids, or α-alkyl-β-hydroxyl fatty acids while the hydrophilic moieties can be carbohydrates, amino acids, cyclic peptides, phosphates, carboxylic acids, or alcohols. Although secondary metabolites, some of them play essential roles for the survival of the producing microorganisms. These include increasing the surface area and bioavailability of hydrophobic substrates, heavy metal binding, bacterial pathogenesis, quorum sensing, and biofilm formation [4].

Microbial surfactants have advantages over their chemical counterparts because they are less toxic and biodegradable, and are effective at extreme temperatures and pH values [5]. These properties explain the fact that biosurfactants have been widely used in bioremediation and waste treatment [6].

Several *Pseudomonas* sp. have been reported to produce rhamnolipids, a glycolipid-type biosurfactant with prominent surface activity. Rhamnolipids can reduce surface tension of water from 72 to 30–32 mN/m at a critical micelle concentration of 5–65 mg/L and can display high emulsifying activity of 10.4–15.5 U/ml filtrate [7]. Regarding their physicochemical properties, they are well suited for application in petrochemical industry and hydrocarbon remediation [8, 9]. Despite their potential uses in environmental applications, little is known about the pharmacological roles of rhamnolipids [7, 10, 11]. The aim of this study was to isolate and structurally characterize rhamnolipid biosurfactants from a novel *Pseudomonas aeruginosa* strain, and to explore new applications of rhamnolipids as antitumor agents by evaluating their in vitro cytotoxic activity in a panel of human cancer cell lines.

Materials and Methods

Microorganism, Media, and Cultivation

The *P. aeruginosa* strain BN10 employed in this study was isolated from soil polluted with hydrocarbons by a standard enrichment technique as described previously [12]. The isolate was selected for its ability to produce surface-active compounds. Strain BN10 was maintained in Nutrient agar slants (Difco Laboratories Inc., Detroit, MI, USA) at 4 °C. Inocula were prepared by transferring bacterial cells from the storage culture to 250-ml flasks containing 50 ml of Nutrient broth and incubated at 30 °C and 150 rpm on a rotary

shaker. Four milliliters of this mid-exponential phase culture was inoculated into 1-l flasks containing 200 ml of mineral medium with the following composition (g l^{-1}): $K_2HPO_4 \cdot 3H_2O$, 7.0; KH_2PO_4, 3.0; $(NH_4)_2SO_4$, 1.0; Mg $SO_4 \cdot 7H_2O$, 0.2; and glycerol, 2.0. The pH of the medium was adjusted to 7.0. Cultures were incubated while shaking (150 rpm) at 30 °C for 7 days.

Taxonomic Identification

Preliminary identification was carried out by both microscopic observations and biochemical experiments based on Bergey's Manual of Determinative Bacteriology [13]. Confirmation was obtained through determining the 16S rRNA gene sequence. DNA was extracted from a pure culture by standard salting out procedure [14]. The extracted DNA was amplified by polymerase chain reaction (PCR) using the designed by us primers for 16S rRNA of *P. aeruginosa* (GenBank accession number PACS2). We used the forward primers 752 F_1 (5′ ccctggtagtccacgccgt 3′) and 1168 R_1 (5′ ccggtactactgaactgcagta 3′). The PCR was performed in the following mixture: about 100 ng bacterial DNA, 10 pmol of each primer, 0.2 mmol l^{-1} of each dNTPs, 1× supplied PCR buffer [including 1.25 mmol l^{-1} $MgCl_2$, 0.5 U ExPrime Taq (Genet Bio, Chungnam, Korea)], and ddH_2O up to final volume of 25 μl. The cycling conditions were as follows: initial denaturation at 95 °C for 5 min; 30 cycles of denaturation at 95 °C for 45 s, annealing 58 °C for 45 s, and synthesis 72 °C for 90 s; and final synthesis at 72 °C for 5 min.

The obtained amplification products were controlled in 2 % agarose gel electrophoresis and then sequenced by the same primers used in the PCR. The sequencing reaction was performed by BigDye terminator sequencing kit (Applied Biosystems, Foster City, CA, USA) and analyzed by automated sequencer ABI 310 Genetic Analyzer (Applied Biosystems).

Detection of Biosurfactant Production

Two simple preliminary methods were used for detection of biosurfactant production:

1. Surface tension (ST) measurement of the supernatant fluid was carried out after centrifugation at 8,000×*g* for 20 min. The surface tension measurements were made by the du Noüy ring method using a Krüss tensiometer (Hamburg, Germany). Before each measurement, the instrument was calibrated against triple distilled water.
2. Emulsifying activity of the culture supernatant was estimated by adding 0.5 ml of sample fluid and 0.5 ml of kerosene to 4.0 ml of distilled water. The tube was vortexed for 10 s, held stationary for 1 min, and then visually examined for turbidity of a stable emulsion.

The orcinol assay [15] was used for direct assess of the amount of glycolipids in the sample. The rhamnolipid concentrations were calculated from standard curves prepared with L-rhamnose and expressed as rhamnose equivalents (RE) (mg/ml).

Isolation and Purification of Biosurfactants

To remove the bacterial cells, the culture broth was centrifuged at 8,000×*g* for 20 min. Then the supernatant was acidified with 6 M HCl and allowed to stay at 4 °C for one night. The precipitate was collected after centrifugation at 12,500×*g* for 20 min and extracted with equal volume of ethyl acetate. The organic phase was removed under reduced pressure at

40 °C and the resulting light brown colored viscous liquid was considered as the crude biosurfactant.

The biosurfactant-containing extract was dissolved in 3 ml of chloroform/methanol/water (65:15:2 v/v/v) and afterwards purified by using normal pressure chromatography on silica gel 60 (particle size 0.063–0.200 mm, mesh size 70–230; Merck, Darmstadt, Germany). The surface-active compounds were eluted with dichloromethane/methanol/water ($CH_2Cl_2/CH_3OH/H_2O$) 65:15:2, and fractions (1.5 ml) were collected. The purification of the rhamnolipids was monitored off-line by thin layer chromatography (TLC). TLC was carried out on Si gel 60 plates as stationary phase with the same solvent system as used for normal pressure chromatography and spraying with orcinol/sulfuric acid reagent for detection of rhamnolipid compounds. The purified fractions were compared with reference substances of rhamnolipids from Jeneil Biosurfactant Company, USA.

Structure Elucidation

All nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Advance II+600 spectrometer, operating at 600.13 MHz for hydrogen, equipped with a pulse gradient unit. The NMR experiments were carried out in $CD_3OD/CDCl_3$ at 293 K. Chemical shifts were reported as parts per million (ppm) referenced to internal TSP (trimethylsilyl-2,2,3,3-tetradeuteropropionic acid) as standard. The two-dimensional COSY and HSQC experiments were acquired in phase-sensitive mode using the standard Bruker pulse sequences.

Two-dimensional $^1H/^1H$ correlation spectra (COSY) and gradient-selected $^1H/^{13}C$ heteronuclear single quantum coherence (HSQC) spectra were acquired using the standard Bruker software.

Ion electrospray mass spectra (ESI–MS) of the same were recorded on a Finnigan $QTOF^2$ mass spectrometer (Thermo Quest LC and LC/MS Division, CA, USA). Stock solution of the surface-active compound was prepared by dissolving of 2 mg substance in 1 ml chloroform/methanol (1:1, v/v). Aliquots of 0.1 ml were diluted in 1.9 acetonitrile/water (7:3, v/v) and infused at a flow rate of 10 μl min^{-1} with a syringe pump (Hamilton syringe, 500 μl) directly connected to the electrospray ionization (ESI).

ESI mode was as follows: sheath gas—nitrogen (6 l min^{-1}); negative mode $[M–H]^-$ at −4 kV of ionization; the temperature and the voltage of the heated capillary were 300 °C and 25 V, respectively; tube lens offset 5 V. Helium was used as a collision gas at the collision-induced dissociation.

Cell Lines and Culture Conditions

The following cell lines were used in the experiments: Hl-60 (DSMZ no. ACC 3, established from the peripheral blood of a 35-year-old woman with acute myeloid leukemia), BV-173 (DSMZ no. ACC 20 from the peripheral blood of a 45-year-old man with chronic myeloid leukemia in blast crisis), SKW-3 (DSMZ no. ACC 53, established from the peripheral blood of a 61-year-old man with T-cell lymphocytic leukemia), and JMSU-1 (DSMZ no. ACC 505, derived from malignant ascitic fluid of a 75-year old man with urinary bladder carcinoma).

The cells were passaged and kept in log phase as previously described [16]. Briefly, cells were grown as suspension cultures [RPMI-1640 medium supplemented with 10 % fetal bovine serum (Sigma Chemical Co., Poole, UK)] at 37 °C in an incubator with humidified atmosphere and 5 % CO_2. Cells were passaged two or three times a week.

Cytotoxicity Assessment

Cytotoxicity of the biosurfactants was assessed using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] dye reduction assay as described by Mosmann [17]. Exponentially growing cells were seeded in 96-well microplates (100 μl/well at a density of 3.5×10^5 cells/ml for the adherent and 1×10^5 cells/ml for the suspension cell lines) and allowed to grow for 24 h prior to the exposure to the studied compounds. Stock solutions of the biosurfactants were freshly prepared in DMSO and then diluted with the corresponding growth medium. At the final dilutions, the solvent concentration never exceeded 0.5 %. Cells were exposed to the tested agents for 72 h whereby for each concentration a set of eight separate wells was used. Every test was run in triplicate. After incubation with the tested compounds, MTT solution (10 mg/ml in PBS) aliquots were added to each well. The plates were further incubated for 4 h at 37 °C and the formazan crystals formed were dissolved by adding 110 μl of 5 % HCOOH in 2-propanol. Absorption of the samples was measured by an ELISA reader (Uniscan Titertec) at 580 nm. Survival fraction was calculated as percentage of the untreated control. The experimental data were processed using GraphPad Prism software and were fitted to sigmoidal concentration/response curves.

In Vitro Safety Testing

The in vitro safety testing was performed as described by Borenfreund and Puerner [18] and the latest modification of the validated Balb/c 3T3 (clone 31) Neutral Red Uptake Assay (3T3 NRU test). Balb/c 3T3 mouse embryo cells were grown as monolayers in 75-cm^2 tissue culture flasks in low-glucose (1‰) DMEM (Sigma), supplemented with 5 % fetal calf serum and antibiotics. Cultures were maintained at 37.5 °C in a humidified atmosphere and 5 % CO_2. Cells were plated at a density of 1×10^4 cells in 100 μl culture medium in each well of 96-well flat-bottomed microplates and allowed to adhere for 24 h before treatment with test compounds diluted in culture medium. A concentration range from 1 mM to 0.008 mM was applied by serial twofold dilutions. After 24-h treatment, Neutral Red containing medium was added, cultures were incubated for 3 h, and the cells were washed several times with phosphate-buffered saline (PBS). The cell monolayers were treated with ethanol/acetic acid desorbing solution and the optical density was measured by a microplate reader at 540 nm. Cell viability was calculated as percentage of the untreated (negative control) cell cultures. The statistical analysis included application of one-way ANOVA followed by Bonferroni's post hoc test, and $p < 0.05$ was accepted as the lowest level of statistical significance. Microsoft Excel was used for the elaboration of dose–response curves and estimation of IC_{50} values.

Fluorescence Studies

BV-173 cells (1×10^5/ml) were seeded in 24-well plates (Costar), allowed to grow overnight at 37.5 °C in a humidified atmosphere under 5 % CO_2, and treated with two concentrations (25 μM and 50 μM) of the monorhamnolipid RL-1, dissolved in phosphate-buffered saline (pH 7.2) for 24 h. At the end of the period for treatment, the cells were incubated for 1 h with 0.1 μg/ml *bis*-benzimide DNA-binding dye Hoechst 33258 and harvested by centrifugation at 1,500 rpm. The cell pellet was resuspended and smears were prepared on diagnostic glass slides (Menzel Glaeser). The slides were air-dried, fixed in cold (−20 °C) acetone for 20 min, covered, and examined with a Leica DM 5000 B (Wetzlar, Germany) fluorescent microscope equipped with the appropriate filter set, a digital camera, and the appropriate Leica Software.

At least 250 cells on each slide were counted and the numbers of apoptotic and necrotic cells were determined according to the accepted criteria [19].

All experiments were performed in duplicate. The statistical analysis included application of one-way ANOVA, followed by Bonferroni's post hoc test, and $p < 0.05$ was accepted as the lowest level of statistical significance.

Lymphocytes from Healthy Donors

Fresh human peripheral blood from healthy donors, obtained from the National Centre of Blood Transfusion in Sofia, Bulgaria, was used for isolation of lymphocytes using Ficoll Paque gradient centrifugation. The lymphocytes were resuspended in RPMI-1640 cell culture medium (without phenol red), supplemented with 10 % FCS and Pen Strep (1 %) at a concentration of 10^7 cells/ml, and activated by adding 3 μg/ml phytochemagglutinin, dissolved in the culture medium.

The lymphocyte cultures were treated at the same conditions as the cell lines.

Results

Characterization of Strain BN10

The isolate was aerobic, Gram (−), motile (with one flagellum), oxidase-positive, catalase-positive, rod-shaped, and non-spore forming bacterium. The optimal growth temperature for strain BN10 was 29–30 °C in a minimal medium containing 2 % glycerol. Morphological observations and biochemical data for the metabolic pattern of the strain showed high similarity to a type strain of *P. aeruginosa*.

Comparative sequence analysis of the 16S rRNA (1,080 bp) in the GenBank was performed by BLAST search and manually reading through the GenBank accession number PACS2. Since the gene sequence comparison gave 99 % similarity to *P. aeruginosa* PACS2, the identity of the strain was confirmed.

Surfactants Recovery and Characterization

Acid precipitation and extraction with ethyl acetate, separation, and purification by column chromatography on silica gel gave two tensio-active fractions named RL-1 and RL-2. TLC results suggested that the biosurfactants RL-1 and RL-2 excreted by strain BN10 were glycolipids since yellowish green spots appeared when using anisaldehyde as color developing agent. Their structures were further analyzed as described below.

NMR spectral analyses of the purified compounds RL-1 and RL-2 were assigned to a typical rhamnolipid-type structure. The appearance of the characteristic for lipids resonance signals at 13.7 ppm (CH_3), 22.5–32.7 ppm (CH_2), 171.5 ppm (C=O), and 173.6 ppm (C=O) were observed in the ^{13}C NMR spectra of both fractions. On the ^{13}C spectrum, the corresponding signals of the anomeric carbons of rhamnose ring were at 96.7 ppm and 102.4 ppm. The ^{13}C NMR signals of C-β and C-β′ appeared at 71.4 and 71.1 ppm as it was determined from the analysis of the $^{1}H/^{13}C$ HSQC spectra, and that was evidence for the presence of O-substitution in these positions in both fatty acid chains (Fig. 1).

Mass spectrometry analysis revealed the presence of one major component for RL-1 and RL-2, respectively, detected as the anions of *m*/*z* 503 (ca. 71 %) and *m*/*z* 649 (ca. 80 %). They correspond to the deprotonated molecules of Rha-C_{10}-C_{10} (mono-rhamnolipid) and

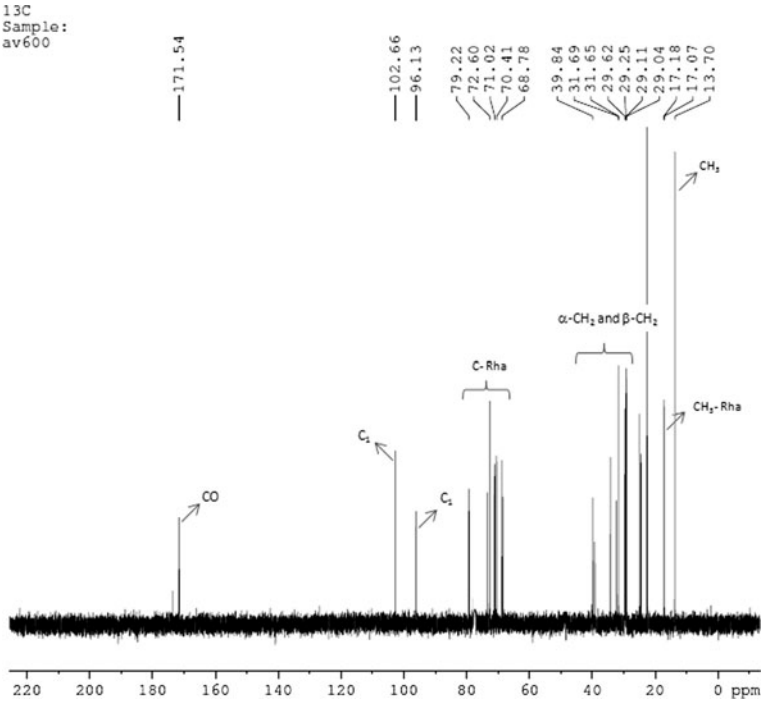

Fig. 1 ^{13}C NMR spectrum of di-rhamnolipid from *Pseudomonas aeruginosa* BN10

Rha_2-C_{10}-C_{10} (di-rhamnolipid). In order to confirm these structural assignments, the main ions of fractions RL-1 and RL-2 were subjected to tandem-MS mode. Thus, the pseudomolecular ion of *m*/*z* 503 (Fig. 2) gave two major fragments of *m*/*z* 333 and *m*/*z* 339 corresponding to the rupture of the ester links, along with *m*/*z* 169 corresponding to the released fatty acid and of *m*/*z* 163 corresponding to the rupture of the link in the rhamnose–alkylic chain. The fragmentation of the main pseudomolecular ion of *m*/*z* 649 (Fig. 3) gave daughter ion of *m*/*z* 479 representing the rupture of the ester bond between the two alkylic chains in the di-rhamnolipid. Other characteristic ion was that of *m*/*z* 339 representing the cleavage of the link between the rhamnose and alkylic moiety. Daughter ion at *m*/*z* 163 representing single rhamnose was also detected.

In Vitro Antitumor Activity

A comparative evaluation of the antitumor effects of the two studied compounds, RL-1 and RL-2, was performed on a panel of four human tumor cell lines, using the standard MTT-dye reduction assay for cell viability. Evident from the cytotoxicity data summarized in Fig. 4, RL-1 exerted high cytotoxic activity to cells from human pre-B leukemia line BV-173 (IC_{50}=50 μM) and the cell line SKW-3 (T-cell chronic lymphocytic leukemia) with IC_{50}= 54 μM. Slightly weaker cytotoxic effect of the tested compound (IC_{50}=60 μM) was found to cells derived from poorly differentiated transitional cell carcinoma of the urinary bladder (line JMSU1) and to cells (IC_{50}=67 μM) from the cell line HL-60 (human promyelocytic leukemia). In contrast, the di-rhamnolipid RL-2 showed in vitro antitumor activity at higher concentrations (Fig. 4) when compared to the mono-rhamnolipid RL-1. Medium in vitro

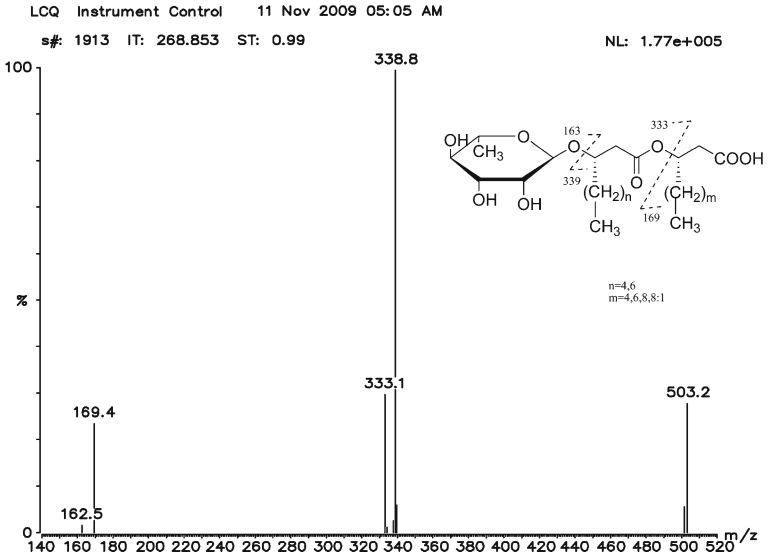

Fig. 2 **a** Mass spectrum of RL-1 and **b** fragmentation of molecular $[M–H]^-$ ion of *m*/*z* 503

cytotoxic effect of RL-2 was found to HL-60 leukemia cells (IC_{50}=77 μM) and approximately twice higher IC_{50} values, as compared to the effect of RL-1, were observed after testing on cell lines SKW-3 and JMSU1 (108 μM and 140 μM, respectively).

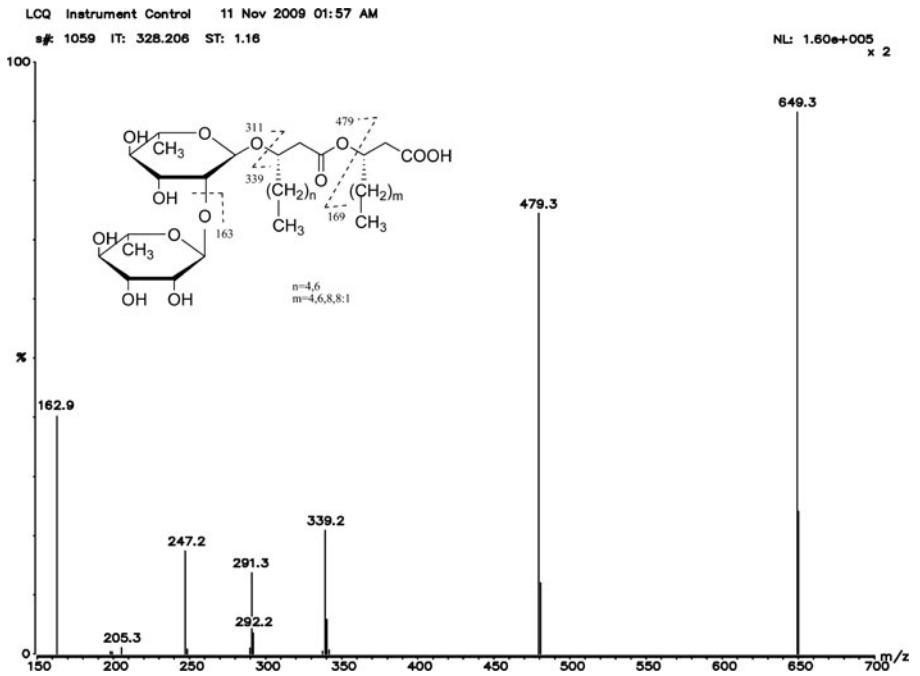

Fig. 3 **a** Mass spectrum of RL-2 and **b** fragmentation of molecular $[M–H]^-$ ion of *m*/*z* 649

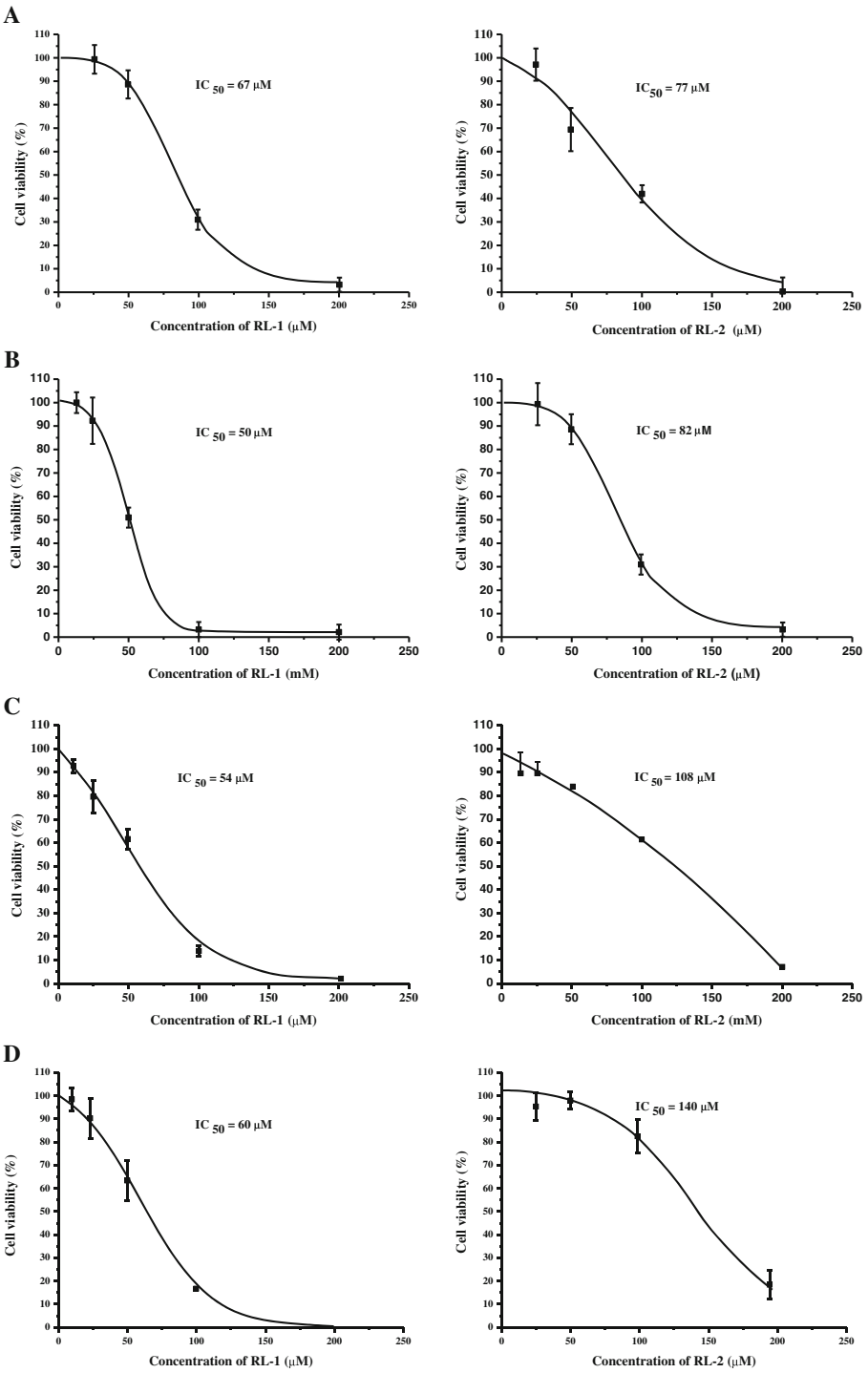

Fig. 4 Effect of RL-1 and RL-2 on the HL-60 (**a**), BV-173 (**b**), SKW-3 (**c**), and JMSU-1 (**d**) cell viability. The cells were incubated for 72 h. Values are means ± SD from three experiments

In Vitro Safety Testing

The results from the validated Balb/c 3T3 (clone 31) Neutral Red Uptake Assay (3T3 NRU test) revealed a dose-dependent cytotoxic activity of rhamnolipids from *P. aeruginosa*, strain BN10. RL-1 and RL-2 induced pronounced reduction of cell viability of non-tumorigenic Balb/c 3T3 (clone 31) only at highest test concentrations (Fig. 5). No significant cytotoxicity was observed after treatment of Balb/c 3T3 cell cultures with concentrations from 0.125 mM to 0.08 mM of both substances. However, RL-1 (IC_{50}=0.233 mM) induced approximately twofold higher reduction of cell viability compared to RL-2 (IC_{50}=0.447 mM).

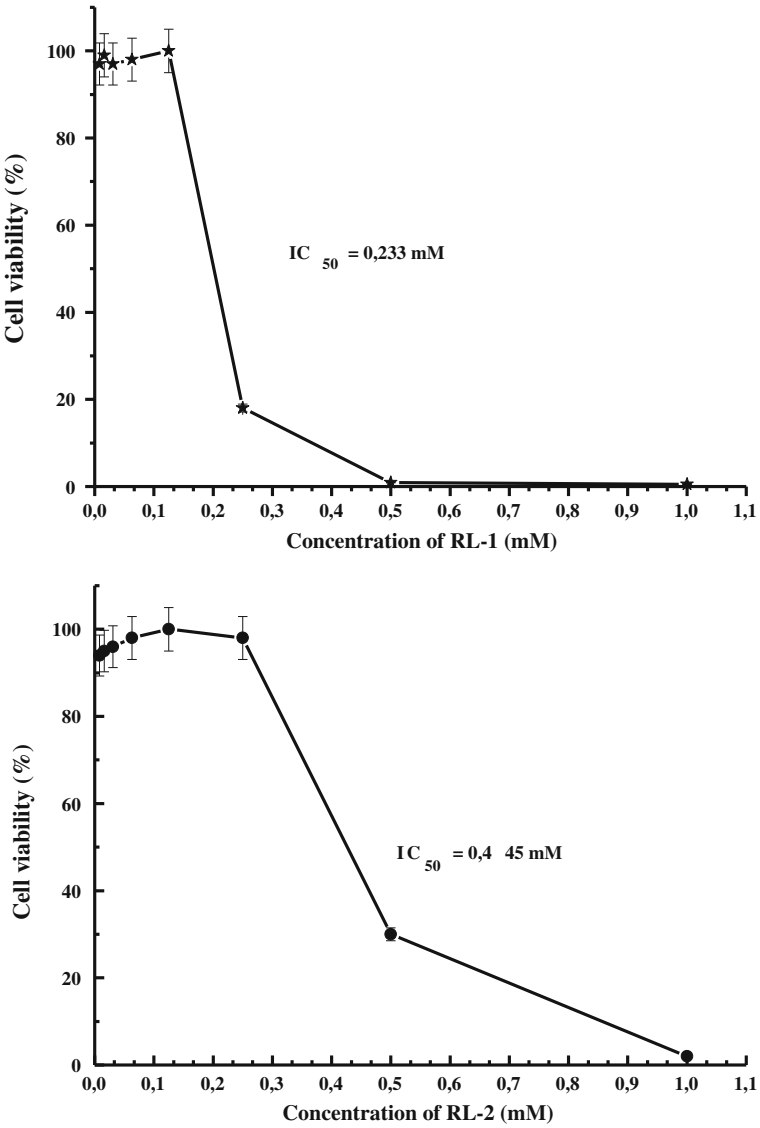

Fig. 5 In vitro cytotoxicity of rhamnolipids RL-1 and RL-2 from *P. aeruginosa* strain BN10 on cultures from cell line Balb/c 3T3, clone 31 (3T3 NRU test)

Proapoptotic Effect of RL-1

The specific blue/cyan fluorescence allowed easy detection of apoptotic and necrotic BV-173 leukemic cells. The results presented in Fig. 5 show that the studied compound in a concentration of 25 μM induced specific apoptotic alterations in BV-173 cells after 24 h of exposure. The value was significantly higher ($p<0.05$) when compared to the number of apoptotic cells observed in the control tumor cell cultures (Fig. 7). In addition, the number of RL-1-treated (25 μM; 24 h) BV-173 apoptotic cells was also higher ($p<0.01$) than values of necrotic cells after treatment with 25 μM and 50 μM of the tested substance for 24 h, which indicates that the RL-1-induced apoptosis is the leading event during the first 24 h of treatment.

Surprisingly, incubation of BV-173 cells with a higher concentration (50 μM) of the tested mono-rhamnolipid did not result in a significant elevation of the numbers of both apoptotic and dead leukemic cells in comparison with the control (Fig. 6).

Cytotoxic Effect of RL-1 on Human Lymphocytes from a Healthy Donor

The viability of lymphocytes from a healthy donor was used as a control for the cytotoxic effect of the studied compound. Figure 7 shows the percentage of lymphocytes viability after treatment with 25 μM and 50 μM RL-1. A weak but statistically significant ($p<0.05$) reduction of cell viability to 72 % was observed after 72 h of treatment with the higher concentration of 50 μM RL-1. In contrast, the same concentration is IC_{50} for the cell line BV-173 (Fig. 8).

Discussion

Morphological and physiological characterization combined with a comparative sequence analysis of the 16S rRNA of the newly isolated strain BN10 confirmed its identity as *P. aeruginosa*. Regarding previous studies, the active ingredient of *Pseudomonas* biosurfactants are rhamnolipids [7, 20]. This was proved in this study by using TLC, NMR, and ESI–MS analysis. The chemical characterization of the main components was confirmed to be L-rhamnopyranosyl-β-hydroxydecanoyl-β-hydroxydecanoate (Rha-C_{10}-C_{10}) for RL-1 and L-rhamnopyranosyl-L-rhamnopyranosyl-β-hydroxydecanoyl-β-hydroxydecanoate (Rha_2-C_{10}-C_{10}) for RL-2.

Rhamnolipids are produced as a natural mixture of mono- and di-rhamnolipids and are known to exhibit a great variety of biological activities. They have been shown to have antimicrobial [11, 21], antiphytoviral [22], and zoosporic activity [23]. There is a growing

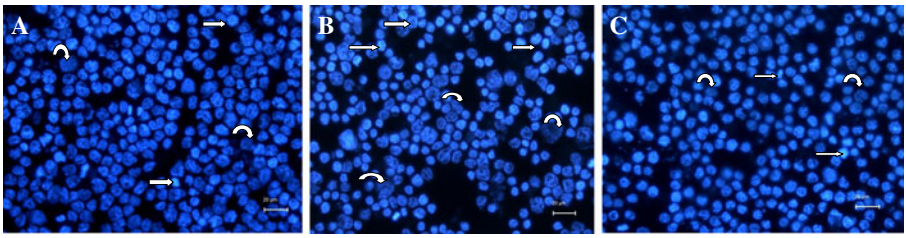

Fig. 6 Nuclear morphology of BV-173 cells. Untreated control cells (**A**), cells treated with 25 μM RL-1 for 24 h (**B**), and cells treated with 50 μM RL-1 for 24 h (**C**)

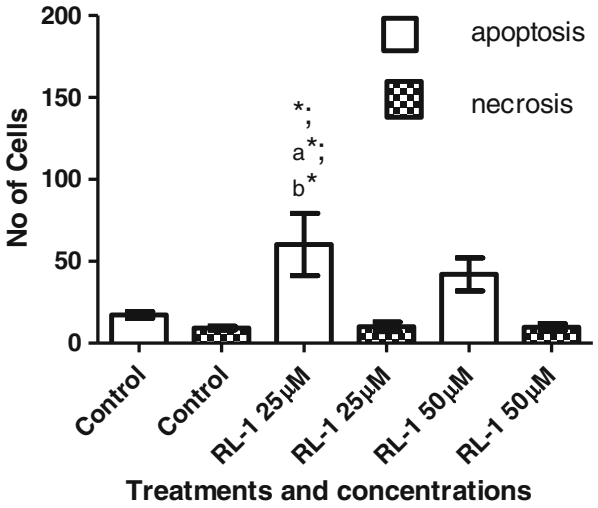

Fig. 7 In vitro induction of apoptosis and necrosis in BV-173 cells after 24 h of treatment with the mono-rhamnolipid RL-1. Statistics: one-way ANOVA, followed by Bonferroni's post hoc test; *$p<0.05$, compared to the respective control; *a* compared to number of necrotic cells after treatment with 25 μM; *b* compared to number of necrotic cells after treatment with 50 μM

interest concerning the effect of rhamnolipids on human and animal cells and cell tissues. In several investigations, the di-rhamnolipid fraction has been used to obtain most of the published data. Di-rhamnolipids were demonstrated to induce morphological alterations in phagocytic and non-phagocytic cells by reorganizing the cytoskeleton [24], to induce differentiation in keratinocyte and fibroblast cell cultures [25], and to affect cellular immunosupression [26]. Di-rhamnolipids have been tested in the treatment of psoriasis [27] and wound healing [28]. Thanomsub et al. [10] found that di-rhamnolipid a (Rha_2-C_{10}-C_{10}) and di-rhamnolipid b (Rha_2-C_{10}-C_{12}) from *P. aeruginosa* strain B189 significantly inhibited the growth of human breast cancer cell line MCF-7 and the insect cell line C6/36.

To the best of our knowledge, this is the first report demonstrating a mono-rhamnolipid-mediated cytotoxic activity to human cancer cell lines. The mono-rhamnolipid RL-1 was superior in terms of potency, causing 50 % inhibition of cell viability at lower concentrations than the di-rhamnolipid RL-2 in all tested leukemic cell lines and in the epithelial bladder carcinoma cell line. Further studies are needed for elucidation of the mechanisms of tumor cell death, which could include suppression of cellular oncogenes c-myc and N-src [29]

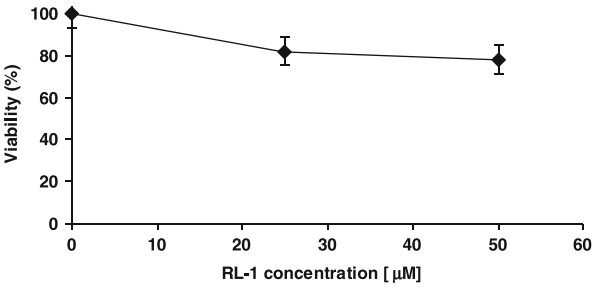

Fig. 8 Viability of lymphocytes after 72 h of treatment with 25 μM and 50 μM RL-1

and/or activation of tumor suppressor program like retinoblastoma pathway [30]. The results of our studies, however, indicate that the process of rhamnolipid-induced tumor cell death requires prolonged exposure, which suggests that multiple mechanisms leading to a cascade of events are involved in the destruction of leukemic and epithelial human cancer cells.

Furthermore, the results from our initial experiments indicate that RL-1 from *P. aeruginosa* strain BN10 induced a rapid process of rapidly triggered apoptosis after 24-h treatment of human leukemia cells from cell line BV-173, demonstrated by the presence of specific morphological alterations. Chromatin condensation and margination, nuclear fragmentation, plasma membrane blebbing, and presence of apoptotic bodies [31, 32] were readily observed in the treated tumor cell cultures.

The apoptotic process, however, appeared to be dose independent, which could be due to the presence and function of efflux pumps, activated after incubation with the higher concentration of the tested substance, or to a saturation of the cell membrane receptors, which does not allow penetration of more active molecules within the tumor cells. In addition, it is also possible that higher doses of RL-1 could influence the overexpression of Bcl-2 and c-myc genes, present in BV-173 pre-B leukemia cells, which leads to antiapoptotic effects and higher proliferation rates. Another important point is that BV-173 cells possess the Philadelphia chromosome, which encodes the Bcr-Abl oncoprotein, and the activated Bcr-Abl tyrosine-kinase stimulates several signal transduction pathways [33]. Nevertheless, the continuous (72 h) treatment of BV-173 tumor cells resulted in a dose-dependent cytotoxic effect, indicating that the antiapoptotic processes have been overcome after prolonged incubation with the mono-rhamnolipid RL-1.

The analysis of the results from the 3T3 NRU test indicates very low toxicity to non-tumorigenic Balb/c 3T3 (clone 31) mouse fibroblasts. In fact, the obtained IC_{50} values are much higher than values for the in vitro antitumor activity of the tested rhamnolipids. However, in both cases, RL-1 appeared as a more potent cytotoxic agent than RL-2. It appears highly likely that the structure of the molecule of the mono-rhamnolipid is responsible for higher levels of penetration in both tumor and normal cells, thus exerting higher reduction of cell viability.

It should be also pointed out that the observed weak cytotoxicity after prolonged incubation with RL-1 to normal human lymphocytes indicates lower adverse effect to normal cells. In fact, the treatment with a concentration of 50 μM of compound RL-1 induced 50 % reduction of cell viability of BV-173 human pre-B leukemic cells (Fig. 4). The results obtained strongly support the notion that RL-1 could be used for the development of drugs for treatment of pre-B human leukemias.

Conclusions

In order to evaluate the individual contribution of rhamnolipids RL-1 and RL-2, isolated from *P. aeruginosa* strain BN10, we studied their cytotoxic effect on four human tumor cell lines. Our study showed for the first time that the mono-rhamnolipid RL-1 had significant inhibitory activity on the growth of the human cancer cell lines compared to the di-rhamnolipid RL-2. Apparently, the in vitro antitumor activity of RL-1 has been mediated by induction of apoptosis. Therefore, the mono-rhamnolipid RL-1, produced by *P. aeruginosa* strain BN10, could be considered as a novel naturally derived compound, promising for a future work on the development of antitumor agents.

Acknowledgment This work was financially supported by the Ministry of Education, Youth and Science of Bulgaria, Project BG051PO001-3.3-05/0001 "Science & Business", Operational Programme "Human Resources Development".

References

1. Desai, J. D., & Banat, I. M. (1997). *Microbiology and Molecular Biology Reviews, 61*, 47–64.
2. Mulligan, C. N., & Gibbs, B. F. (2004). *Proceedings of the Indian National Science Academy, B70*, 31–55.
3. Muthusamy, K. S., Gopalakrishman, S., Ravi, T. K., & Sivachidambaram, P. (2008). *Current Science, 94*, 736–747.
4. Cameotra, S. S., & Makkar, R. S. (1998). *Applied Microbiology and Biotechnology, 50*, 51–59.
5. Ron, E. Z., & Rosenberg, E. (2001). *Environmental Microbiology, 3*, 229–236.
6. Banat, I. M., Makkar, R., & Cameotra, S. (2000). *Applied Microbiology and Biotechnology, 53*, 495–508.
7. Abalos, A., Pinaso, A., Infante, M. R., Casals, M., Garcia, F., & Manresa, A. (2001). *Langmuir, 17*, 1367–1371.
8. Abalos, A., Vinas, M., Sabate, J., Manresa, A., & Solanas, A. M. (2004). *Biodegradation, 15*, 249–260.
9. Urum, K., Grigson, S., Pekdemir, T., & McMenamy, S. (2006). *Chemosphere, 62*, 1403–1410.
10. Thanomsub, B., Pumeechockchai, W., Limtrakul, A., Arunrattiyakorn, P., Petchleelaha, W., Nitoda, T., et al. (2006). *Bioresource Technology, 9*, 2457–2461.
11. Benincasa, M., Abalos, A., Oliveira, I., & Manresa, A. (2004). *Antonie Van Leeuwenhoek, 85*, 1–8.
12. Tuleva, B., Christova, N., Cohen, R., Stoev, G., & Stoineva, I. (2008). *Journal of Applied Microbiology, 104*, 1703–1710.
13. Bergey, D.H. & Holt, J.G. (1994). *Bergey's manual of determinative bacteriology* (9th ed.) (pp. 93–94). Baltimore: Williams & Wilkins
14. Miller, L., Dykes, L., & Polesky, H. (1988). *Nucleic Acids Research, 16*, 1215.
15. Chandrasekaran, E.V., & Bemiller, J.N. (1980). In Whistler R.L. (Ed.), *Methods in carbohydrate chemistry* (pp. 89–96). New York: Academic
16. Konstantinov, S. M., Eibl, H., & Berger, M. R. (1998). *Cancer Chemotherapy and Pharmacology, 41*, 210–216.
17. Mosmann, T. (1983). *Journal of Immunological Methods, 65*, 55–63.
18. Borenfreund, E., & Puerner, J. A. (1985). *Toxicology Letters, 24*, 119.
19. Kroemer, G., Galluzzi, L., Vandenabeele, P., Abrams, J., Alnemri, E. S., Baehrecke, E. H., et al. (2009). *Cell Death and Differentiation, 16*, 3–11.
20. Bodour, A. A., Drees, K. P., & Maier, R. M. (2003). *Applied and Environmental Microbiology, 69*, 3280–3287.
21. Itoch, A., Tomato, F., & Suzuki, T. (1971). *The Journal of Antibiotics, 24*, 855–859.
22. Haferburg, D., Hommel, R., Kleber, H. P., Kluge, S., Schuster, G., & Zshiegner, H. J. (1987). *Acta Biotechnologica, 7*, 353–356.
23. Stanghellini, M. E., & Miller, R. M. (1997). *Plant Disease, 19*, 4–12.
24. Häußbler, S., von Neuhoff, N. R., Nimtz, M., & Steinmetz, I. (2003). *Infection and Immunity, 10*, 2970–2975.
25. Stipcevic, T., Piljac, A., & Piljac, G. (2005). *Journal of Dermatological Science, 40*, 141–143.
26. Piljac, G., & Piljac, V. (1995). *US Patent, 5*, 466–675.
27. Piljac, G., & Piljac, V. (1995). *US Patent, 5*, 455–232.
28. Stipcevic, T., Piljac, A., & Piljac, G. (2006). *Burns, 32*, 24–34.
29. Collins, S. J. (1987). *Blood, 70*, 1233–1244.
30. Hurst, C. D., Tomlinson, D. C., Williams, S. V., Platt, F. M., & Knowles, M. A. (2008). *Oncogene, 27*, 2716–2727.
31. Kaufmann, S. H., & Hengarther, M. O. (2001). *Trends in Cell Biology, 262*, 526–534.
32. Reed, J. (2003). *Cancer Cells, 3*, 17–25.
33. Arlinghaus, R.B., invited guest editor. (2002), Oncogene (Review), 21, 8560–8567.