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Apoptotic effects of the novel histone deacetylase inhibitor BML-210 on HeLa cells

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Histone deacetylase inhibitors (HDACI) have been shown to inhibit cancer cell proliferation, induce cell cycle arrest, and stimulate apoptosis. To date, the key apoptotic proteins and pathways necessary for the anti-tumor activities of HDACI remain ill-defined, and the specific genes regulated by HDACI to mediate these effects have not been fully dissected. We have asked how novel HDACIs induce apoptosis, and how tumour cells can circumvent HDACI-mediated cell death. In the present study, we have investigated the antiproliferative effects of the novel HDACI, BML-210, and its combination with retinoic acid (RA) on human cervical cancer cells (HeLa).

Cell cycle analysis indicated that HeLa cell treatment with BML-210 alone (10 μM) and in its 5 μM combination with 1 μM RA decreased the proportion of cells in G_2/M phase, increased in G_0/G_1 and caused accumulation in subG1 indicating the cells undergoing apoptosis. These changes occurred concomitantly with the increased level of some proteins related to the malignant phenotype. We observed the activation of caspases 3, 8, 9 and an increase of the transcription factor NF-κB in the HeLa cell nucleus at 24 h of treatment with BML-210 or RA alone and in combination. Electrophoretic mobility shift assay (EMSA) experiments revealed an enhanced binding activity of NF-κB and the transcription factor p53 to the p21 promoter immediately after treatment with BML-210.

In conclusion, these results suggest that BML-210 and its combination with RA are effective in inhibiting growth and inducing apoptosis in HeLa cells *in vitro*. The findings imply that BML-210 may prove particularly effective in cancer therapy.

Key words: apoptosis, HeLa cells, retinoic acid, BML-210, histone deacetylase inhibitor

INTRODUCTION

HDAC inhibition was empirically discovered as a novel form of cancer therapy [1]. The biology of various HDAC isoforms and their relationship to tumorigenesis is just beginning to be elucidated and is largely driven by the perceived clinical potential of HDAC inhibitors [1–4]. It remains to be seen if a more detailed understanding of the specific roles played by various HDAC isoforms during human tumorigenesis leads not only to development of isoform-specific inhibitors, but also to more effective or less toxic antitumor therapeutics, as compared to the multiclass HDAC inhibitors which are currently undergoing clinical evaluation. Rationally designed combinations of HDAC inhibitors with various other types of approved or investigational anticancer agents are showing promise in tumour cell culture systems, but must yet be proven in clinical trials [5].

Different HDAC inhibitors are able to change transcription, both positively and negatively, for about 2% of total human genes. While this number seems to be small, common genes affected by these inhibitors are known to exert a critical function in controlling cell cycle and apoptosis or differentiation process [6, 7].

For example, HDACI, such as FK228 (depsipeptide), is a promising new class of antineoplastic agents with the capacity to induce growth arrest and/or apoptosis of cancer cells. FK228 led to a substantial decrease in the expression of Cdc2/Cdk-1, cyclin B1 and phosphorylated pRb and an increase in p21 responsible for cell cycle arrest at G2-M transition and a significant induction of apoptosis. It was demonstrated that another HDACI, suberoylanilide hydroxamic acid (SAHA), and FK228 both regulate a highly overlapping gene set with at least 22% of genes (including Myc, type βTGF, cyclin / cyclin-dependent kinase, TNF, Bcl-2, APAF-1) and the caspase pathway that leads to the induction of apoptosis and decreases cellular proliferation [8–10].

Recently, we have established that BML-210 inhibits cancer cell proliferation (HeLa and NB4) and induces differentiation and apoptosis in leukemia cell lines [11, 12]. In this study, we have investigated the effects of a novel HDACI, BML-210, on HeLa cell apoptosis, and we found that BML-210 alone and in combination with RA caused cell cycle arrest that led to apoptosis in HeLa cells. This was concomitant with overexpression of some proteins related to the malignant phenotype, including a temporal increase of NF-κB and the activation of caspases 3, 8, 9. In EMSA experiments, we observed increased transcription factors, p53 and NF-κB binding activities to p21 specific promotor and consensus sequences, respectively, after treatment with BML-210.

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In conclusion, these results suggest that BML-210 and its combination with RA are effective in inhibiting the growth and activating the apoptosis of HeLa cells *in vitro*. The findings imply that BML-210 may prove particularly effective in cancer treatment.

MATERIALS AND METHODS

Cell cultures

The human epithelial cervical cancer cell line HeLa was cultured in DMEM medium (Gibco) containing 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin (Gibco) at 37 °C in humidified air and 5% CO_2.

Chemicals and antibodies

RA were purchased from Sigma Chem. Co (St. Louis, MO) and added to the medium from stock solution of 500 μM (in ethanol) to a final concentration of 1 μM. BML-210 (Biomol, Plymouth Meeting, PA) was dissolved in DMSO (stock solution 10 mM) and added to the medium to the final concentrations of 5 and 10 μM. The final DMSO concentration in the medium did not exceed 0.001%.

Antibodies against NF-κB (p65), pro-caspase-3, pro-caspase-8, pro-caspase-9 and FasL were purchased from Santa Cruz Biotechnology, Inc. (California).

Oligonucleotides were synthesized by MWG-Biotech AG (Denmark).

Preparation of cytosolic and nuclear proteins

The cells (5×10^6 to 10^7) were harvested by centrifugation at 500× g for 6 min, washed twice in ice-cold phosphate-buffered saline (PBS) and suspended in Nuclei EZ lysis buffer (Sigma, St. Louis, MO) for 5 min on ice. The cell homogenates were then centrifuged at 1500× g for 5 min. The supernatant, corresponding to the cytosolic fraction, was clarified by centrifuging at 15,000× g for 15 min, frozen at –76 °C or immediately used for sodium dodecyl sulfate / polyacrylamide gel electrophoresis (SDS/PAGE). Nuclei were washed in the same cold Nuclei EZ buffer, vortexed briefly and set on ice for 5 min. The nuclei were pelleted at 500× g for 5 min, completely resuspended in Nuclei EZ storage buffer (Sigma, St. Louis, MO) and frozen at –76 °C.

For SDS-PAGE analysis of total nuclear proteins, the nuclei were resuspended in two volumes (v/v) of 2× lysis solution (100 mM Tris, pH 7.4, 5 mM $MgCl_2$, 200 mM DTT and 4% SDS), and thereafter three volumes of 1× lysis solution and benzonase (Pure Grade, Merck, Germany) were added to give a final concentration of 2.5 units/ml. The lysates were incubated for 1 h at 0 °C and then centrifuged at 15,000× g for 30 min. The supernatants were immediately subjected to electrophoresis or frozen at –76 °C.

Cell cycle analysis

Untreated and chemical-agent-treated cells were collected by centrifugation, suspended in PBS and fixed in ice-cold 70% ethanol (ratio 1 : 10) for 24 h at –20 °C. After centrifugation at 500× g for 5 min, the cells were suspended in PBS containing propidium iodide (PI) (50 μg/ml) and RNase (0.2 mg/ml) and incubated at room temperature for 30 min. The tubes were then kept at 4 °C in the dark until analysis by flow cytometry (Becton–Dickinson FACSCalibur, USA). The percentage of cells in G0/G1, S and G2/M was evaluated with CellQuest software. Apoptotic cells were quantified on a PI histogram as a hypodiploid peak, and the data were registered on a logarithmic scale.

Electrophoretic mobility shift assay (EMSA)

The probes used were synthetic oligonucleotides representing binding sites: (5'-AGTTGAGGGGACTTTCCCAGGC-3') consensus NFκB; (5'-ATCAGGAACATGTCCCAA CATGTTGAGCTCT-3') p53 from the p21 promoter.

Standard DNA reactions were performed with 10 μg nuclear extracts in 20 μl of the reaction buffer (10 mM HEPES pH 7.9, 3 mM $MgCl_2$, 0.1 mM EDTA, 40 mM NaCl, 10% glycerol) containing 2 μg BSA, 1 μg poly(dI-dC), 1 pM labeled oligonucleotide for 30 min at room temperature. When desired, the unlabeled competitor oligonucleotide was added to protein extracts at a 50- or 100-fold molar excess for a 15 min-preincubation. DNA–protein complexes were resolved on 5% polyacrylamide gel containing 1× Tris-borate buffer. After electrophoresis, the gels were dried and then exposed to X-ray films.

RESULTS AND DISCUSSION

The present study was designed to investigate the novel HDACI, BML-210 and their combination with RA on HeLa cell growth and apoptosis. BML-210 (N-(2-Aminophenyl)-N'-phenyloctanediamide) is a novel compound synthesized by Biomol, and it has a similar structure as other HDACIs including SAHA. BML-210 can easily fit into a HDAC catalytic pocket and inhibit HDAC activity.

For cell cycle analysis, HeLa cells were treated with 10 μM BML-210 or 1 μM RA alone and in combination (1 μM RA and 5 μM BML-210) for 48 h, the cells were harvested and stained with propidium iodide. Cell populations in cell cycle phases were determined by flow cytometry. As is shown in Fig. 1, the subG1 population, which indicates apoptotic cells, increased in response to treatment with BML-210 alone up to 13% and after combined treatment, to 15%, while treatment with RA alone did not induce apoptosis.

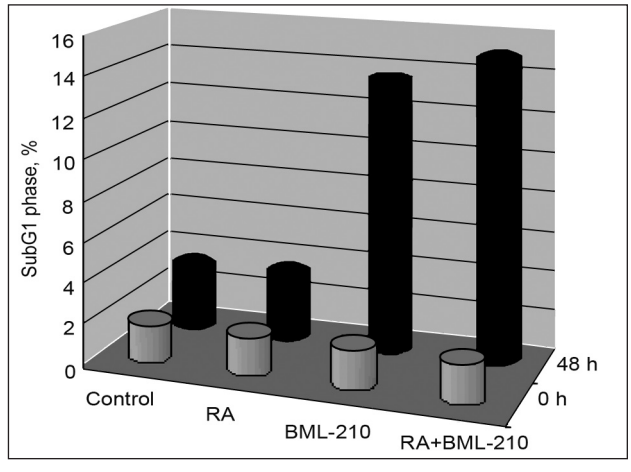

Fig. 1. Effect of RA and BML-210 on HeLa cell death. HeLa cells were treated with 1 μM RA, 10 μM BML-210 or in combination with 1 μM RA and 5μM BML-210 for 48 h. Apoptosis of control and treated HeLa cells was determined as a hypodiploid peak (subG1) from flow cytometric analysis of PI stained cells. Apoptosis is presented as a percentage of the total events collected. Results are from three separate experiments where standard deviations (not shown) were less than ±10%

We quantitated the distribution of HeLa cells in cell cycle phases after treatment with 1 μM RA, 10 μM BML-210 or combination of 1 μM RA and 5 μM BML-210 for 48 h. Cell cycle analysis revealed that BML-210 alone and its combination with RA caused an increase of the proportion of HeLa cells in G1 phase up to 73% and 68%, respectively, and an decrease in G2/M phase to 4.7% and 2%, respectively (Fig. 2). Thus, BML-210 alone or in combination with RA increased the growth inhibitory effect which could be attributed to the arrest of transition through the cell cycle. It is now well established that inhibition of cyclin / CDK complexes by p21 can result in G1 and G2/M cell cycle arrest [13–15]. p53 is a transcriptional activator of several genes regulating the events such as transformation and DNA synthesis. An important target of p53 is p21/WAF1 [16].

In the present study, we have investigated p53 protein binding activity to the specific sequences of the p21 promoter after treatment with BML-210. By EMSA, the transcription factor p53 binding to the specific sequence of the p21 promoter increased at hours 8 and 24 of treatment (Fig. 4, B). This usually leads to an increase in p21 expression (data not shown) and cell cycle arrest (Fig. 2).

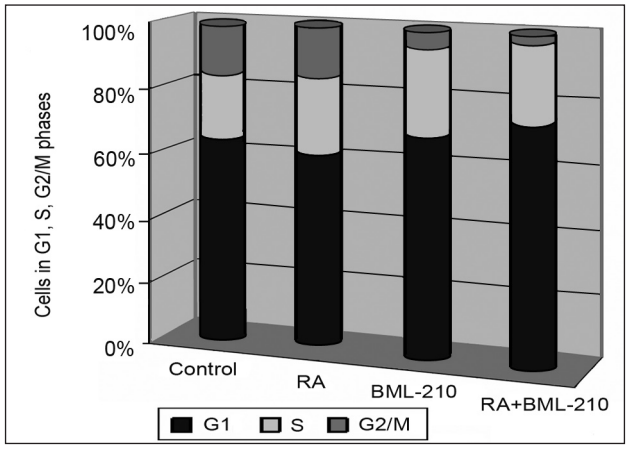

Fig. 2. Effects of RA and BML-210 on cell cycle progression of HeLa cell line. HeLa cells were treated with 1 μM RA, 10 μM BML-210 or in combination with 1 μM RA and 5 μM BML-210 for 48 h. The cell cycle phase distribution (%) was determined from DNA frequency distribution histograms of PI-stained cells. Results are from one representative experiment of three where standard deviations (not shown) were less than ±10%

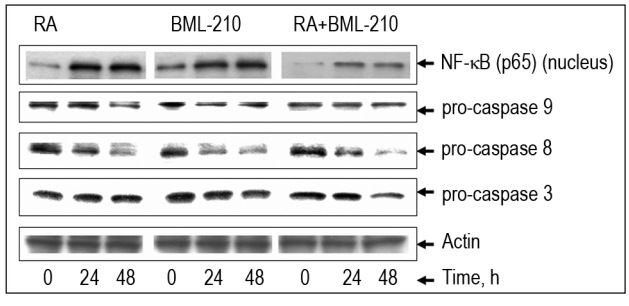

Fig. 3. Expression of cytoplasmic and nuclear proteins in response to RA and BML-210 treatment. HeLa cells were treated with 1 μM RA, 10 μM BML-210 or in combination with 1 μM RA and 5 μM BML-210 for indicated time-points. Cytosolic and nuclear proteins isolated from control and the treated HeLa cells were fractionated on 6–18% SDS-PAGE and analysed by Western blotting with antibodies against NF-κB, pro-caspase-3, pro-caspase-8 and pro-caspase-9

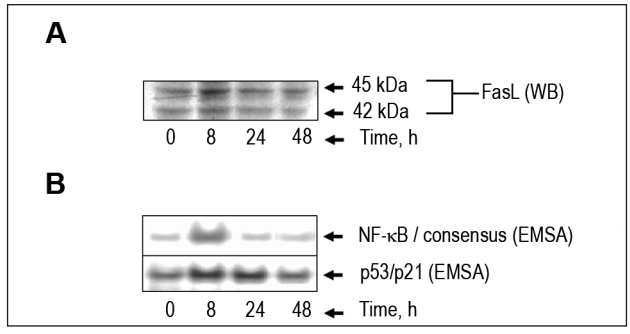

Fig. 4. FasL protein expression and p53 and NFκ-B binding activities to specific DNA sequences in response to BML-210. A – HeLa cells were treated with 10 μM BML-210 for indicated time-points. Cytosolic proteins were fractionated on 6–18% SDS-PAGE and analysed by Western blotting with antibodies against FasL. B – EMSA was performed using nuclear extracts from control (0 h) and BML-210-treated 8, 24, 48 h HeLa cells and oligonucleotides containing binding sites of NF-κB consensus sequences and p53 to the p21 promoter. Arrows indicate DNA complexes with NF-κB and p53 proteins

The NF-κB family is also important for many processes within the cell. NF-zB controls the expression of many genes that participate in cell cycle, apoptosis and other key cellular processes [17, 18]. In a canonical pathway, NF-κB activation depends on the IKK complex activity, which is formed by three subunits (IKKα and IKKβ and IKKγ/NEMO). There is an alternative NF-κB activation pathway that does not require IKKβ or IKKγ/NEMO, where RelB is the major player [17–19].

We have examined the NF-κB protein expression level after different treatments and also NF-κB binding activity to consensus DNA sequences in HeLa cells. By EMSA, we found an upregulation of NF-κB binding activity to the consensus sequence at 8 h of BML-210 treatment (Fig. 4, B). By Western blotting, the NF-κB protein expression level revealed an increase after treatment with RA and BML-210 alone or in its combination at 24 and 48 h (Fig. 3). One of the target genes of NF-κB is p53 involved in the regulation of p21 [20]. Our observation allows to suggest that NF-κB may be involved in the inhibition of proliferation and apoptosis induction in HeLa cells in response to treatment with BML-210 and its combination with RA (Fig. 5).

Since caspase plays the major role in the apoptotic process, the involvement of the caspase cascade has been examined in BML-210-induced HeLa cell apoptosis. There are at least two

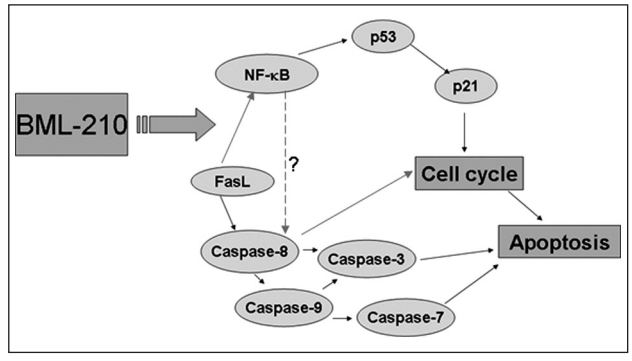

Fig. 5. Possible signaling ways of apoptosis in BML-210-treated HeLa cells, based on our results

major pathways that initiate caspase cascades. One pathway is mediated by cell-surface death receptors, including Fas/CD95/Apo-1 and TRAIL, and is characterized by a critical requirement for caspase-8 [21]. A second signalling pathway centers on the disruption of the mitochondrial function which is characterized by a release of the proapoptotic mediator, cytochrome *c*, and caspase-9 activation [22, 23]. Once activated, these caspases in turn activate executioner caspases (caspase-3, -6, -7). The active executioners promote apoptosis by cleaving the cellular substrates that induce the morphological and biochemical features of apoptosis [22, 23].

We have examined the role of caspase 3, 8 and 9 in BML-210/ RA/ RA + BML-210-treated HeLa cell apoptosis. Western blot analysis showed an upregulated expression of FasL at an 8 h time-point (Fig. 4, A) and a downregulated expression of pro-caspase-8 (Fig. 3), suggesting that the Fas / FasL pathway was activated in BML-210-induced cell apoptosis. We have also demonstrated that the downregulation of pro-caspase-9 may lead to the activation of the mitochondrial apoptotic pathway. The data correlated with the downregulation of pro-caspase-3. Pro-caspase-8 downregulation was observed after treatment of HeLa cells with RA alone and in combination with BML-210, while downregulation of pro-caspase-9 was more pronounced in HeLa cells treated with RA alone. Based on our results, we concluded that two pathways of apoptosis were initiated in HeLa cells in response to HDACI and RA (Fig. 5).

In general, our data suggest that BML-210 and its combination with RA can be promising as a novel combination in cancer cell treatment, acting as a growth inhibition agent and apoptosis inducer via caspase pathway activation.

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NAUJO HISTONŲ DEACETILAZIŲ INHIBITORIAUS BML-210 APOPTOTINIS POVEIKIS HeLa LĄSTELĖMS

Santrauka

Histonų deacetilazių inhibitoriai (HDACI) – tai nauja cheminių medžiagų grupė, galinti sulėtinti vėžinių ląstelių augimą bei sukelti užprogramuotą ląstelių mirtį. Kai kurie HDACI gana efektyviai naudojami klinikinėje praktikoje, tačiau šių medžiagų veikimo molekuliniai mechanizmai iki šiol nėra visiškai ištirti. Tikimasi, kad vėžinių ląstelių mirties dėl HDACI poveikio mechanizmo atskleidimas pagerintų vėžio gydymo perspektyvą.

Šiame darbe vertinamas naujo HDAC inhibitoriaus BML-210 poveikis epitelinių gimdos kaklelio vėžio (HeLa) ląstelių augimui. Bandymuose naudoti šie reagentai: 1 μM retinoinė rūgštis (RA), 10 μM BML-210 ir 5 μM BML-210 kartu su 1 μM RA. Ląstelių ciklo tyrimai rodo, kad BML-210 kombinacija su RA lėmė ląstelių susikaupimą G0/G1 fazėje ir ląstelių skaičiaus sumažėjimą G2/M fazėje. Šiuos ląstelės ciklo pokyčius lydėjo padidėjęs kaspazių aktyvumas. Po BML-210 kombinacinio poveikio buvo pastebėtas padidėjęs HeLa ląstelių skaičius subG1 fazėje ir tolimesnė ląstelių mirtis.Mes nustatėme NF-κB baltymo raiškos padidėjimą praėjus 8 val. nuo visų reagentų poveikio. Šis baltymo raiškos padidėjimas sutapo su NF-κB transkripcijos faktoriaus ryšio su DNA promotorinėse sekose aktyvumo padidėjimu paveikus HeLa ląsteles BML-210. Taip pat buvo nustatytas FasL baltymo raiškos padidėjimas BML-210 paveiktose ląstelėse.

Taigi naujas HDACI BML-210 bei jo kombinacija su RA gali būti taikoma siekiant sulėtinti vėžio ląstelių augimą bei sukelti jų mirtį.