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Benzamide riboside induces apoptosis independent of Cdc25A expression in human ovarian carcinoma N.1 cells

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Abstract

One of the mechanisms of action of a new oncolytic agent, benzamide riboside (BR) is by inhibiting inosine 5′-monophosphate dehydrogenase (IMPDH) which catalyzes the formation of xanthine 5′-monophosphate from inosine 5′-monophosphate and nicotinamide adenine dinucleotide, thereby restricting the biosynthesis of guanylates. In the present study BR (10–20 μM) induced apoptosis in a human ovarian carcinoma N.1 cell line (a monoclonal derivative of its heterogenous parent line HOC-7). This was ascertained by DNA fragmentation, TUNEL assay, [poly(ADP)ribose polymerase]-cleavage and alteration in cell morphology. Apoptosis was accompanied by sustained c-Myc expression, concurrent down-regulation of cdc25A mRNA and protein, and by inhibition of Cdk2 activity. Both Cdk2 and cdc25A are G_1 phase specific genes and Cdk2 is the target of Cdc25A. These studies demonstrate that BR exhibits dual mechanisms of action, first by inhibiting IMPDH, and second by inducing apoptosis, which is associated with repression of components of the cell cycle that are downstream of constitutive c-Myc expression.

Keywords: IMP dehydrogenase inhibitor; benzamide riboside; apoptosis; cdc25A repression; cyclin D1/prad 1 expression; c-*myc* induction

Abbreviations: ATP, adenosine triphosphate; ATRA, all-trans retinoic acid; BAD, benzamide adenine dinucleotide; BR, 3-(1-deoxy-β-D-ribofuranosyl)benzamide or Benzamide riboside; Cdk2, cyclin dependent kinase 2; dGTP, 2′-deoxyguanosine 5′-triphosphate; DTT, DL-dithiothreitol; EDTA, ethylenediaminetetraacetic acid; FCS, fetal calf serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; gas6, growth arrest specific 6 gene; GTP, guanosine 5′-triphosphate; HEPES, (N-[2-hydroxyethyl]piperazine-N′-[4-butansulfonic acid]); IMP, inosine 5′-monophosphate; IMPDH, inosine 5′-monophosphate dehydrogenase; MEM, alpha minimal essential medium; NAD, nicotinamide adenine dinucleotide; NGF, nerve growth factor; PARP, poly(ADP-ribose)polymerase; PBS, phosphate-buffered saline; PDGF, platelet derived growth factor; PI, propidium iodide, PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; SDS-PAGE, SDS-polyacrylamide gel; TAD, thiazole-4-carboxamide adenine dinucleotide; TNFalpha, tumor necrosis factor alpha; TNFbeta, tumor necrosis factor beta; TR, 2-β-D-ribofuranosylthiazole-4-carboxamide or tiazofurin; TUNEL terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-X nick end labelling; XMP, xanthine 5′-monophosphate; z-VAD-fmk, benzyloxylcarbonyl Val-Ala-DL-Asp-fluoromethylketone

Introduction

Ovarian carcinoma is one of the most lethal gynecological malignancies. To improve the prognosis, there is a need to develop new therapeutic concepts. Since little is known about the molecular mechanisms by which ovarian carcinomas bypass homeostasis, a knowledge of the expression of genes which function in growth-, survival- and death-control would be beneficial.

IMP dehydrogenase (IMPDH) catalyzes the conversion of IMP to XMP and is the rate limiting enzyme in *de novo* guanylate biosynthesis.[1] The activity of this enzyme was shown to be significantly increased in tumor cells and therefore considered to be a potential target for cancer chemotherapy.[2] Tiazofurin (TR) was found to inhibit the growth of human myelogenous leukemia K562[3] and human promyelocytic leukemia HL-60 cells.[4] TR was shown to be an inhibitor of IMPDH[5] and Phase I/II clinical trials conducted with tiazofurin in acute myelogenous leukemia patients, indicated a significant reduction in leukemic cell burden.[6–8]

Benzamide riboside, BR, is a C-nucleoside,[9] which has recently been characterized as having IMPDH inhibitory properties.[10,11] BR exhibited stronger antiproliferative activity in the K562 cells than TR.[12]

The two nucleoside antimetabolites, TR and BR are converted in cancer cells to their active dinucleotide metabolites, TAD (thiazole-4-carboxamide adenine dinucleotide) and BAD (benzamide adenine dinucleotide), which are analogues of NAD, wherein the nicotinamide moiety is

replaced by their respective bases.[11,13] BAD potently inhibits NAD utilization by IMPDH, resulting in the depletion of intracellular guanylate concentrations including GTP and dGTP.

The human ovarian carcinoma N.1 cell line (a monoclonal derivative of the heterogenous parent HOC-7 line) is useful to study the induction of growth arrest, differentiation and apoptosis.[14,15] By analyzing cell morphology, DNA integrity and gene expression, the cytotoxic properties of oncolytic agents can be determined in a first screen.[16]

Therefore, in this report, N.1 cells were utilized to examine the influence of BR on N.1 cell proliferation and to get an insight into the physiological and molecular aspects of antiproliferative mechanisms. Induction of apoptosis by BR was ascertained by distinguishing changes in morphology, DNA fragmentation, TUNEL assay, and [poly (ADP) ribose polymerase] cleavage. The influence of BR on the expression of genes involved in cell cycle progression (c-*myc*, cyclin D1, *cdc25A* expression) and Cdk-2 activity was also examined since conflicting cell cycle signals could activate cell death processes.

Results

Effect of BR on the proliferation of human ovarian adenocarcinoma N.1 cells *in vitro*

Cells (2.4×10^4) were seeded into 12-well plates and cultured for 3 days. Then saline, 10 or 20 μM BR was added, adherent cells were harvested every day for 6 days and the cell number was determined. Incubation of N.1 cells with 10 μM BR resulted in a significant reduction in proliferation rate compared to the logarithmic growth rate of control cells. Incubation of N.1 cells with 20 μM BR resulted in a highly significant cytocidal effect ($P<0.0001$) (Figure 1; Table 1). Elucidation of cytocidal effect of BR (20 μM) was based on the fact that the number of cells present at 72 h was less than that observed at 48 h after treatment. Thus, it was of interest to determine cytotoxicity by a specific assay.

Cytotoxicity of BR to human ovarian adenocarcinoma N.1 cells

Cells (2.4×10^4) were seeded into 12-well plates and cultured for 3 days. Then saline, 10 or 20 μM BR was added, cells were trypsinized, pooled with the cells floating in the culture medium and stained with PI to determine the percentage of dead cells.[17–19] In control cells the rate of spontaneous dead cells was between 4.6–7.6% which increased to 14.1–20.5% upon treatment with 10 μM BR. The highest percentage of dead cells was observed after treatment with 20 μM BR for 72 h (35.7%; Figure 2e) which is consistent with the decrease of cell number found in the cell proliferation assay (Figure 1; Table 1). Simultaneous treatment with the pan-caspase and apoptosis inhibitor z-VAD-fmk together with 20 μM BR repressed approximately 50% of the overall cytotoxic effect. Thus, it was of interest to further determine the type of cell death.

BR induced apoptosis of N.1 cells

The nature of BR-induced cell death was determined first by examining DNA-integrity. Apoptotic DNA degradation characterized by oligonucleosomal fragmentation resulted after 72 h of BR treatment (Figure 2b) but was not observed when cells were treated with 10 μM BR (data not shown). In addition, PARP was cleaved to the apoptosis specific 85 kDa signature type fragment following treatment with 20 μM BR for 72 h (Figure 2c and d) but not after exposure to 10 μM BR. Apoptosis induced by 20 μM BR treatment was dependent on caspase activity because the pan caspase inhibitor z-VAD-fmk abrogated PARP cleavage (Figure 2d), apoptosis (Figure 2f) and overall cytotoxicity (Figure 2e).

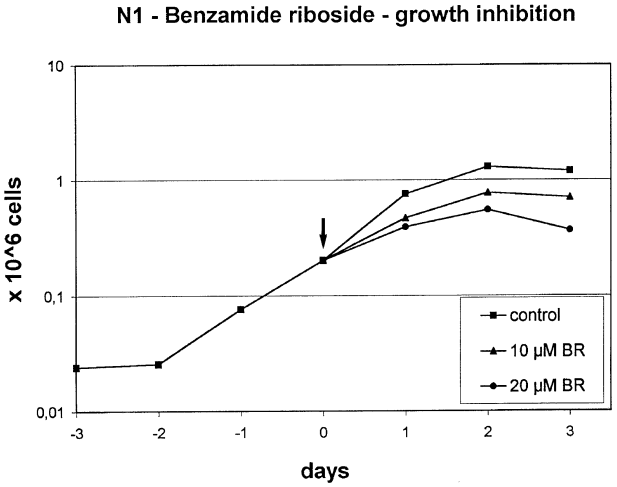

Figure 1 Inhibition of proliferation of N.1 cells after treatment with benzamide riboside (BR). For each time point, 12-well plates were seeded with 2.4×10^4 cells and allowed to grow for 3 days and then 10 or 20 μM BR (indicated by an arrow, day 0) was added. One set of N.1 cells, treated with saline, served as control. Cells were detached from the wells with trypsin on days -2, -1, 0, 1, 2, 3 after saline or BR treatment and the cell number was determined. The abscissa shows the time in days of incubation and the ordinate shows the amount of cells per well. The results were from triplicate experiments

Table 1 Human ovarian carcinoma N.1 cell number before and after BR treatment

Day	−3	−2	−1	0	1	2	3
Control	24300	25800	76400	201900	750000	1292000	1192200
10 μM BR	24300	25800	76400	201900	465600	768000	697700
20 μM BR	24300	25800	76400	201900	388500	546000	364200

Seeding of N.1 cells into 12 well plates was performed on day -3. Benzamide riboside (BR) (10 or 20 μM) was added on day 0

Dose-dependent BR mediated apoptosis

To further elucidate the type of N.1 cell death, cells were incubated with 10 or 20 μM BR and the cell morphology was analyzed by phase contrast microscopy (Figure 2a). A typical apoptotic phenotype was exhibited by N.1 cells at 72 h following treatment with 10 or 20 μM BR (Figure 2a lower panel; arrows (→) point at cells undergoing apoptosis).

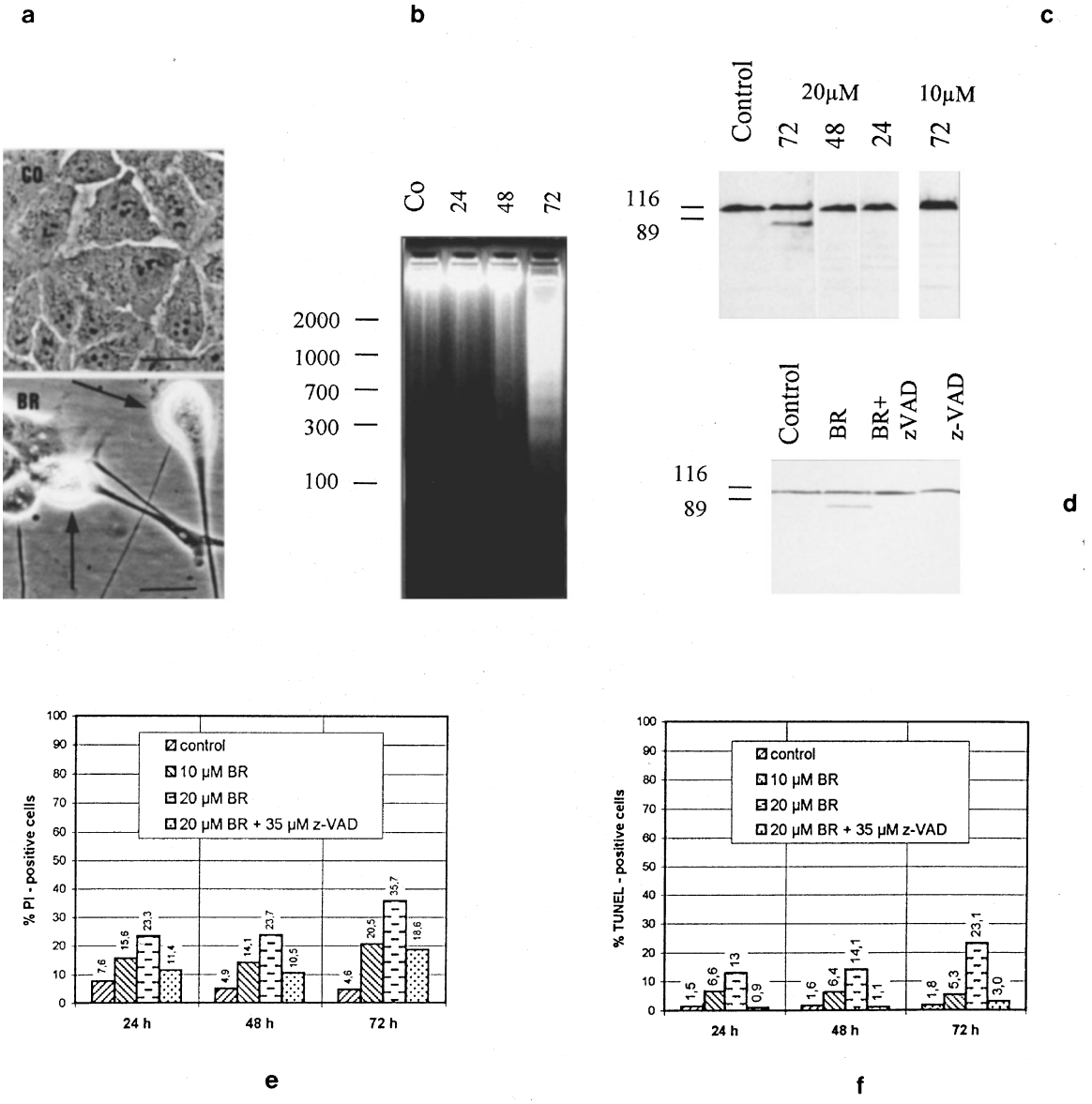

Figure 2 (**a**) Phase contrast light microscopy of N.1 cells following treatment. Cells were incubated with saline (panel A) or 20 μM benzamide riboside (panel B) at 37°C. Photographs were taken at 400-fold magnification after 72 h of incubation. Arrows point at cells that show an apoptotic morphology typical for N.1 cells. The scale bars at lower right corners: 25 μm. (**b**) Effect of benzamide riboside (BR) on DNA degradation. N.1 cells were incubated with saline (control) or 20 μM BR for increasing periods of time (hours) and processed as detailed in Materials and Methods. Numbers on the left refer to base pairs determined by a DNA marker. (**c**) Degradation of PARP during benzamide riboside (BR) treatment. N.1 cells were treated with 20 μM BR for 24, 48 and 72 h or with 10 μM BR for 72 h, protein was extracted and separated on SDS–PAGE, transferred to nitrocellulose and immunoblotted with monoclonal anti-PARP antibody C-2-10 (right lane). The lane to the left shows an untreated control. The numbers to the left indicate kilo-Daltons. (**d**) Degradation of PARP during benzamide riboside (BR) treatment. N.1 cells were treated with 20 μM BR, 20 μM BR+35 μM z-VAD-fmk, or 35 μM z-VAD-fmk for 72 h. Protein was extracted and separated on SDS–PAGE, transferred to nitrocellulose and immunoblotted with monoclonal anti-PARP antibody C-2-10 (right lane). The lane to the left shows an untreated control. The numbers to the left indicate kilo-Daltons. (**e**) Cytotoxicity of benzamide riboside (BR) to N.1 cells. For each time point, 12-well plates were seeded with 2.4×10^4 cells and allowed to grow for 3 days and then 10 μM BR, 20 μM BR or 20 μM BR+35 μM z-VAD-fmk (indicated by an arrow) was added. One set of N.1 cells, treated with saline, served as control. Cells were detached from the flasks with trypsin and pooled with the cells floating in the cell culture supernatant on days 2, 3, 4, 5 and 6 following BR treatment: Cells were incubated with PI and examined under a microscope for nuclear PI-staining. The percentage of PI-positive cells (dead cells) was calculated. (**f**) Effect of benzamide riboside (BR) on the induction of apoptosis in N.1 cells. Cells were incubated with saline (control), 10 μM, 20 μM BR or 20 μM BR+35 μM z-VAD-fmk for 24, 48 and 72 h and apoptosis was determined by TUNEL assay. Numbers in the ordinate indicate the percentage of apoptotic cells obtained from the mean values of triplicate counts

Similar morphological changes were noticeable when N.1 cells were exposed to unrelated apoptosis inducing agents such as ATRA, TNF-alpha and TNF-beta.[15,16] Incubation of N.1 cells with either lower doses of benzamide riboside (1–5 μM) or with tiazofurin (40 μM), a well characterized IMPDH inhibitor, did not induce programmed cell death (data not shown). It has been demonstrated that in human myelogenous leukemia K562 cells, TR is converted into its active metabolite, TAD, at a rate that is 50% less than that obtained with BR.[12] Therefore, N.1 cells were incubated with 40 μM TR in order to compare it to the effect observed with 20 μM BR. Incubation with TR did not induce apoptosis in N.1 cells. Apoptosis exhibited by N.1 cells after 24, 48 and 72 h exposure to BR was further confirmed by TUNEL-assay.

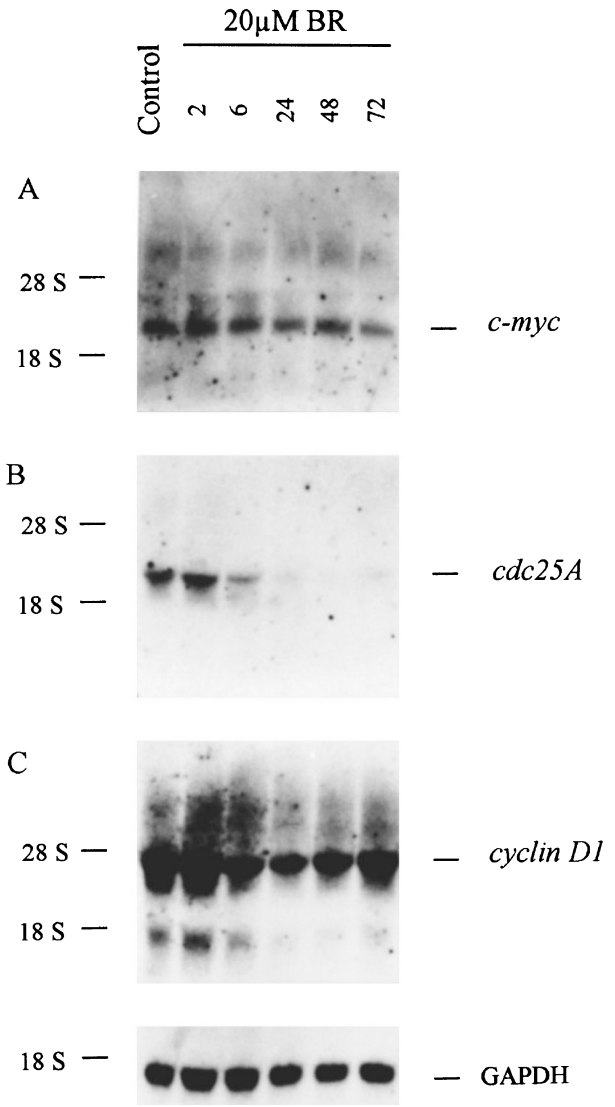

Figure 3 Kinetics of mRNA expression in cells treated with benzamide riboside (BR). N.1 cells were incubated with 20 μM BR for the time periods indicated in the lanes. Control lane refers to cells treated with saline. Filters were hybridized against *cdc25A* (panel B); stripped and rehybridized against c-*myc* (panel A); stripped and rehybridized against cyclin D1 (panel C); and stripped and rehybridized against GAPDH (bottom panel)

Control N.1 cells exhibited a spontaneous apoptosis rate of 1.5–1.8%, and exposure of cells to 5 μM BR did not significantly increase this rate (2.9%, data not shown). Treatment with 10 μM BR enhanced the apoptotic response to 5.3–6.6% and treatment with 20 μM to 23.1% at 72 h (Figure 2f). These values are statistically highly significant ($P<0.001$). Combination of z-VAD-fmk (35 μM) with BR (20 μM) reduced the percentage of TUNEL-positive cells to control levels at all times investigated.

Regulation of Cdc25A during apoptosis

The four different methods of analysis shown above demonstrated that BR induced apoptosis in N.1 cells. Earlier investigations revealed that N.1 cells undergo c-*myc*-dependent apoptosis.[15,20,21] Therefore, it was of interest to examine the influence of BR on the expression of: (i) the proto-oncogene, c-*myc* which induces cell-cycle progression from G_0 to G_i,[22] (ii) cdc25A, a G_1 specific cell cycle gene which also plays a role in apoptosis,[23,24] and (iii) cyclin D1, another G_1 cell cycle specific gene, which together with cdc25A is required for successful passage of cells from G_1 to S-phase.[25,26] Incubation of N.1 cells with BR (20 μM) did not modulate either c-*myc* mRNA expression (Figure 3; densitometric values are provided in Table 2) or the expression of c-Myc protein as illustrated in Figure 4a. However, there was a transient down-regulation of cyclin D1 transcripts at 24 h to 45% of control levels (Figure 3; densitometric values are provided in Table 2) as well as of gas6 (data not shown). Cyclin D1 protein levels were expressed in accordance with the mRNA levels observed following 20 μM BR treatment which underscores that Cdc25A down-regulation is specific to the induction of apoptosis (Figure 4a).

Incubation of N.1 cells with BR (20 μM) resulted concurrently in down-regulation of cdc25A transcripts reaching a nadir at 48 h to 21% of control (a 79% reduction) in a time-dependent manner (Figure 3; densitometric values are provided in Table 2). Down-regulation of cdc25A transcripts was also reflected at the protein level. Treatment of N.1 cells with 20 μM BR resulted in a decrease in cdc25A protein at 48 h (Figure 4a). The effect of treatment with 20 μM BR on the activity of Cdk2, the target of cdc25A, was next examined. As anticipated, Cdk2 activity was inhibited when analyzed in an *in vitro* kinase assay in which histone H1 served as a substrate for Cdk2

Table 2 Densitometer evaluation of c-*myc*-mRNA, *cdc25A*-mRNA, and cyclinD1-mRNA expressions after treatment with benzamide riboside

mRNA	Control	2 h	6 h	24 h	48 h	72 h
c-*myc*	100	107	103	92	99	73
cdc25A	100	91	64	32	21	27
cyclin D1	100	99	59	45	81	90

The intensities of the bands shown in Figure 3a–c (Northern blot) were quantitated. The values are expressed as per cent of control following treatment of cells with 20 μM benzamide riboside for 2, 6, 24, 48 and 72 h. The densitometer readings of c-*myc*-mRNA, *cdc25A*-mRNA, and cyclinD1-mRNA expressions were normalized utilizing the densitometer readings of GAPDH-mRNA expression which served as an internal control to indicate equal loading of the samples

(Figure 4b). Therefore, BR treatment resulted in down-regulation of cdc25A with concurrent inhibition of Cdk2-activity, in spite of unchanged c-Myc expression.

As indicated earlier, incubation of N.1 cells with 40 μM TR which did not induce apoptosis in these cells and only transiently suppressed cdc25A transcript after 6 and 24 h exposure (Figure 5) without affecting Cdc25A protein level (data not shown).

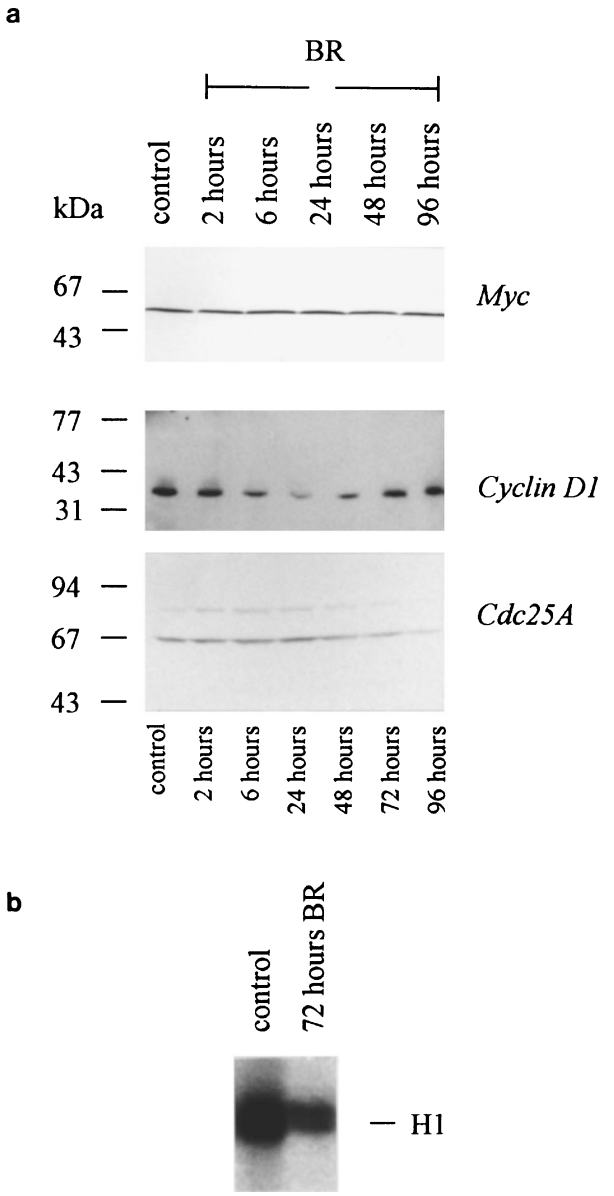

Figure 4 (**a**) Kinetic of c-Myc, cyclin D1 and Cdc25A protein expression in cells treated with benzamide riboside (BR). N.1 cells were incubated with 20 μM BR for the time periods indicated in the lanes. Control lane refers to cells treated with saline. (**b**) Effect of benzamide riboside on Cdk2 immunocomplex formation. N.1 cells were exposed to 20 μM benzamide riboside for 72 h (lane to the right). Lane 1 shows a control treated with saline. Protein was extracted as described in Materials and Methods. Cdk2 was immuno-precipitated with monoclonal anti-Cdk2 antibody. For the kinase reaction and in presence of $^{32}P\gamma$ATP histone H1 was used as a target. The reaction was terminated by boiling and samples were subjected to SDS–PAGE. Gels were dried and exposed to X-ray films at -80°C

Discussion

Four different methods were employed to demonstrate second mechanism of BR action, which has previously been shown to be an inhibitor of IMP dehydrogenase, and was now shown to induce apoptosis in human ovarian adenocarcinoma N.1 cells. Although treatment of N.1 cells with 10 μM BR rendered about 6% of TUNEL-positive cells, no PARP-cleavage or oligosomal DNA fragmentation was detected under these conditions which suggests that these methods of analysis are less sensitive than TUNEL assay or that other caspases, perhaps different from caspase 3, play a major role in BR-induced apoptosis of N.1 cells. The overall cytotoxic effect of 20 μM BR after 72 h (35.7%; determined by PI-staining) could only be in part ascribed to apoptosis (23.1%; determined by TUNEL assay), therefore, 12.6% (calculated) of the remaining cells succumbed to non-apoptotic cell death.

Strikingly, BR triggered apoptosis in N.1 cells despite the presence of 10% FCS (fetal calf serum). All other agents examined so far (ATRA, TNF-alpha, TNF-beta and trimidox) induced apoptosis only in the presence of low serum concentration.[15,20] This action suggests that BR induced apoptosis in spite of the presence of factors in the serum that support cell survival.

Another analogous agent, TR, which was shown to inhibit IMPDH activity similar to that of BR,[11] was included for comparison in the examination of induction of apoptosis in N.1 cells. However, no apoptosis of N.1 cells was observed following TR treatment. Therefore, BR may exert a unique apoptosis inducing activity on N.1 cells.

It was shown that apoptosis induction by serum withdrawal in IL-3 in rodent fibroblasts,[27,28] and by TNF in HeLa cells depended on c-Myc expression.[29] It was

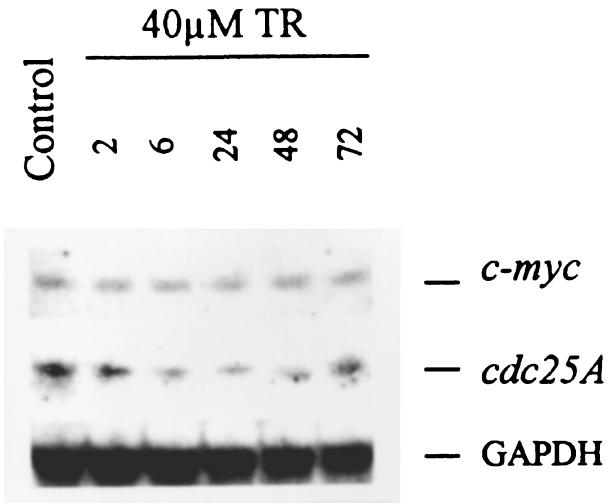

Figure 5 Kinetics of mRNA expression in cells treated with tiazofurin (TR). N1 cells were incubated with 40 μM TR for the time periods indicated in the lanes. Control lane refers to cells treated with saline. Filters were hybridized against *cdc25A*; stripped and rehybridized against c-*myc*; and stripped and rehybridized against GAPDH

recently shown that N.1 cells underwent c-*myc*-dependent cell death.[15,20,21] Apoptosis-inducing concentrations of BR, however, did not interfere with the constitutive expression of c-Myc in this fast growing human ovarian carcinoma N.1 cell line.

It has been shown that the transcription factor c-Myc directly regulated cdc25A expression and that apoptosis induced by conditional ectopic Myc activation depended entirely on Cdc25A expression.[24] Our results with N.1 cells demonstrate that during BR-induced apoptosis, constitutive c-Myc expression was not altered, but cdc25A transcript was down-regulated, thereby resulting in the reduction in Cdc25A protein levels. In concurrence with the present results, apoptosis in N.1 cells was induced by TNF in spite of constitutive c-Myc expression, with a concurrent decrease of cdc25A transcript and reduced Cdc25A protein levels.[20] Therefore, in this instance apoptosis was independent of Cdc25A in N.1 cells. In concurrence with our findings, recent studies have demonstrated that c-Myc induction did not regulate cdc25A expression in rat fibroblasts.[30–32]

In a preliminary study with rat fibroblast cells, apoptosis was triggered by serum removal that was accompanied by down-regulation of cdc25A. Supplementation with PDGF re-established cdc25A expression and prevented apoptosis (unpublished observations). These studies reiterate that cdc25A may have an interesting role to play in apoptosis and survival.

The analysis of c-*myc* and cdc25A expression during treatment with TR (another related IMPDH inhibitor) that did not induce apoptosis revealed a transient down-regulation of cdc25A transcript levels that recovered after 48 h and that the expression of Cdc25A protein was unaffected. Therefore, BR exhibits dual mechanisms of action, one by inhibiting IMPDH and the other by inducing apoptosis, in addition having a selective action on cdc25A expression.

Raf1 was shown to phosphorylate Cdc25A to activate it.[33] This interaction seems to be mediated by members of the 14-3-3 family of proteins.[34] Activated Raf was shown to be important for the survival of IL-3 dependent hematopoietic cells;[35,36] increased the survival response in cells exposed to ionizing radiation;[37,38] mediated insulin-, NGF- and PDGF-dependent growth and survival signals;[39] and protected cells from c-Myc-induced apoptosis in v-*abl* transformed cells.[40]

Moreover, an anti-apoptotic effect mediated by Cdc25A in Rat1MycER cells was demonstrated, wherein serum induced cell growth was not sufficient to generate full expression of Cdk2 activity because cdc25A phosphatase activity was rate limiting and was incompletely expressed in the absence of external growth factors.[41] Since Myc-dependent apoptosis in Rat1MycER cells was triggered by serum deprivation,[42] Cdc25A activity seems to promote cell survival.

Activation of c-Myc can lead to either proliferation or apoptosis based on the presence of survival signals.[27,28] Therefore, extracellular factors which promote growth and/or survival might be mediated across Raf and cdc25A to the cell cycle machinery.[33]

It was suggested that consistent expression of c-Myc in N.1 cells promotes cell cycle initiation from G_0 to G_1 phase,[22,27,28,30,31,43] whereas concurrent down-regulation of cdc25A (such as with BR) might interrupt further cell cycle progression.[25,44,45] In our studies, cdc25A expression correlated with survival, and down-regulation of cdc25A preceded apoptosis. An anticipated, cdc25A down-regulation paralleled with the inactivation of Cdk2.

BR induced active cell death in spite of the presence of serum. This activity suggests that BR might have interrupted anti-apoptotic signals from serum factors. This is because a functional relation between cell survival, Raf1 and the cdc25A proto-oncogene has been demonstrated.[23,24,35–40] Tilting a balance between factors, which contribute to cell cycle start signals and those which contribute to cell cycle progression, might trigger apoptosis.

During BR treatment, gas6 (a gene specifically up-regulated during growth arrest)[45] and c-*myc* was expressed at unchanged levels, whereas cyclin D1 mRNA and protein levels became transiently inhibited after 24 h. Therefore cdc25A repression was specific to the induction of apoptosis and not due to a cell cycle arrest in G_0 phase.[22,43,46–48]

This is the first report demonstrating the induction of apoptosis in cancer cells by BR, a new C-nucleoside, with a concurrent down-regulation of cdc25A expression. The exact role of cdc25A in apoptosis and survival will be addressed in future studies.

Materials and Methods

Materials

BR was synthesized as described.[9] Monoclonal anti-Myc antibodies were purchased from Genosys (UK), and polyclonal anti-Cdc25A antibodies and monoclonal anti-cyclin D1 antibodies were from Santa Cruz (CA, USA). All other chemicals of highest quality were purchased from Sigma Chemical Co., St. Louis (MO, USA). Z-VAD-fmk was from Alexis Biochemicals, San Diego (CA, USA). The cDNAs of cdc25A were kindly provided by Dr. David Beach, Cold Spring Harbor (NY, USA); cyclin D1 by Dr. Hannes Hofmann, IMP, Vienna, Austria; c-*myc* by Dr. Rainer deMartin, University of Vienna, Austria; and GAPDH by Dr. Paul Amstad, University of Maryland (College Park, MD, USA).

Cell culture

The monoclonal human ovarian adenocarcinoma N.1 cell line is a derivative of the heterogenous HOC-7 cell line which was cultured in MEM supplemented with 10% FCS (GIBCO, Paisley, UK) at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

Benzamide riboside treatment

Human ovarian adenocarcinoma N.1 cells were cultured in 12-well plates or T-25 plates (Falcon) and after reaching 70% confluence, cells were treated with either saline or BR (1–20 μM) for periods up to 72 h.

Determination of cell proliferation

Human ovarian adenocarcinoma N.1 cells were seeded at a density of 2.4×10^4 cells into 12-well plates and allowed to grow into a

logarithmic phase before the addition of BR (day 3 after seeding). To determine the cell number the culture medium was aspirated, cells were trypsinized and counted under a Zeiss Axiovert 35 inverse microscope.

Determination of cytotoxicity

Human ovarian adenocarcinoma N.1 cells were seeded at a density of 2.4×10^4 cells into 12-well plates and allowed to grow into a logarithmic phase before the addition of BR (day 3 after seeding). To determine the cytotoxic activity of BR, cells were trypsinized, pooled with the cells floating in the cell culture supernatant, stained with PI, counted under a microscope and the percentage of PI-positive cells was calculated.[17–19]

Northern blot analysis

Human ovarian adenocarcinoma N.1 cells were incubated with BR as described above. Incubations were terminated by removing drug containing medium. The cells were washed twice with ice cold PBS, and subsequently lysed with RNAzol (BioTex, Houston, TX, USA). Total RNA (30 μg/lane) was separated on 1% agarose gels containing formaldehyde and transferred to Millipore S membranes (Millipore, Bedford, MA, USA) by the capillary method. Biotinylated probes were allowed to hybridize to filter-bound RNA at 67°C overnight. Biotinylation procedures and filter processing were conducted as described.[49] Filters were then exposed to Kodak X-ray films (Rochester, NY, USA).

DNA analysis

Human ovarian adenocarcinoma N.1 cells were treated with saline or BR as described above. Supernatant cells were centrifuged and lysed in 0.4 ml buffer containing 50 mM Tris-HCl, pH 8.0; 10 mM EDTA; and 0.5% sodium lauryl sarcosine. The adherent cells (100% in controls) were lysed in the same buffer (1.2 ml). Aliquots of 0.4 ml lysates from adherent and detached cells were treated with 2 μl RNAse A (11 U/l, USB, Cleveland, OH, USA) for 1 h at 37°C, followed by the addition of 10 μl of proteinase K (15 mg/ml; Boehringer Mannheim, Germany) and further incubation at 50°C for 3 h. An equal amount of phenol : chloroform : isoamyl alcohol (25 : 24 : 1) was added and DNA was extracted by gentle treatment (wide-bore pipettes were used without vortexing). After two washes with chloroform isoamyl alcohol (24 : 1), DNA was precipitated with ethanol, and resuspended in 30 μl TE buffer (10 mM Tris-HCl, pH 7.5 with 1 mM EDTA) containing 2 μl RNAse (2 U/l). The lysates derived from adherent and detached cells were pooled, the DNA content was quantitated and equal amounts of DNA were subjected to separation on 2% agarose gels.[15]

TUNEL assay

Cells were exposed to saline, 10 or 20 μM BR for 72 h. Floating cells were collected and pooled with the trypsinized monolayer cells. Trypsin activity was stopped by the addition of serum. Aliquots of pooled samples were subjected to cytospin onto siliconised glass plates (2 min at $70 \times g$), air dried, fixed with 4% paraformaldehyde and further processed as described in the instructions of the *In Situ* Cell Death detection Kit manual (Boehringer, Mannheim, Germany). After the reaction with terminal deoxynucleotidyl transferase, total cell number was first determined by phase contrast microscopy. Then fluorescing cells were counted within the same frame by utilizing fluorescence microscopy. From these counts the percentage of apoptotic cells was calculated.

Western blot analysis

Human ovarian adenocarcinoma N.1 cells were treated with BR or saline, washed twice with ice cold PBS, and then lysed with SDS sample buffer (3% SDS, 10% glycerol, 36 mM DTT, 18.5 mM EDTA, and 25 mM Tris-HCl pH 6.8). Cells were sonicated and protein concentrations were quantitated by dot-metric analysis (Novus Molecular, San Diego, CA, USA). Equal amounts of protein (40–100 μg/lane) were loaded onto SDS-polyacrylamide gels (10% acrylamide/bisacrylamide) separated at 80 V (constant) and electrophoretically transferred to 0.45 nitrocellulose membranes (Schleicher Schuell, Dassel, Germany). Filters were blocked with 5% skimmed milk, 0.5% Tween 20, followed by incubation overnight at 4°C with the first antibody (monoclonal anti-Myc antibody at a 1 : 200 dilution; monoclonal anti cyclin D1 antibody at a 1 : 1000 dilution; monoclonal anti-PARP C-2-10 antibody at a 1 : 5000 dilution; and polyclonal anti Cdc25A antibody at a 1 : 20000 dilution). Secondary antibody reaction was carried out by incubating filters with horseradish peroxidase conjugated with sheep anti-mouse antibody (Amersham International, UK) at a 1 : 5000 dilution for 2 h at room temperature. Signal was developed using the ECL kit according to the instructions of the manufacturer (Amersham International, UK).

Laser scanning analysis

The images of Northern blots were developed on X-ray films and scanned by a laser dot beam (1 mm diameter) across the length axis of the individual mRNA bands. The peak areas underneath the plotted curves were excised and weighed on an analytical balance. The corresponding peak values of the GAPDH mRNA bands (internal loading standard) were utilized for factor-correction of the peak values of the c-*myc*-, cdc25A- and cyclin D1 mRNA bands.

Kinase assay

Protein extracts were prepared in buffer containing 20 mM HEPES pH 7.9, 0.4 M NaCl, 2.5% glycerol, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 0.5 mM NaF, 0.5 mM Na_3VO_4, 0.02 μg/ml leupeptin, 0.02 μg/ml aprotinin, 0.003 μg/ml benzamidine hydrochloride, 0.1 μg/ml trypsin inhibitor and 0.5 mM DTT. After a freeze-thaw cycle in liquid nitrogen, cellular debris was precipitated by centrifugation and supernatants were stored at -70°C.[32] Protein concentrations were determined using the BioRad protein reagent with bovine serum albumin, Fraction V, as standard. Cdk immunoprecipitation and Cdk-analysis were performed as cited.[50] Anti-Cdk2 antibody (St. Cruz) was coupled to protein G-Sepharose beads and incubated with 30 μg protein extract for several hours at 4°C. For protein kinase assay these beads were resolved in a mixture containing 50 mM Tris pH 7.4, 10 mM $MgCl_2$, 1 mM DTT, 0.2 mM PMSF, 1 mM NaF, 80 mM radioactive ATP and 1 μg histone H1. After incubation at 37°C for 30 min, proteins were separated by electrophoresis on a 12% SDS-polyacrylamide gel. The gel was dried and exposed to X-ray film at -80°C.

Statistical analyses

Statistical differences were computed with the SPSS software. Results are given as mean $\pm$ standard deviation (S.D.). Means were compared by using paired samples *t*-test or by one way analysis of variance using Bonferron's *post hoc* range test for multiple comparisons, as appropriate. A P value of <0.05 was considered significant.

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