PERGAMON

European Journal of Cancer 37 (2001) 281–289

European Journal of Cancer

www.ejconline.com

Differential induction of spermidine/spermine N^1-acetyltransferase activity in cisplatin-sensitive and -resistant ovarian cancer cells in response to N^1,N^{12}-bis(ethyl)spermine involves transcriptional and post-transcriptional regulation

G. Marverti *, S. Bettuzzi, S. Astancolle, C. Pinna, M.G. Monti, M.S. Moruzzi

Department of Biomedical Sciences, Section of Biological Chemistry, Via Campi 287, 41100 Modena, Italy

Received 15 June 2000; received in revised form 28 September 2000; accepted 28 September 2000

Abstract

The growth inhibition that occurs in cisplatin-sensitive 2008 human ovarian cancer cells in response to the spermine analogue, N^1,N^{12}-bis(ethyl)spermine (BESpm), is associated with a potent induction of spermidine/spermine N^1-acetyltransferase (SSAT), the rate-limiting enzyme in polyamine catabolism. Conversely, in cisplatin-resistant C13* cells, which are less responsive to BESpm, enzyme induction does not occur at comparable levels after exposure to the bis(ethyl)-derivative. In this study, we investigated the molecular mechanisms underlying the differential induction of SSAT activity in cisplatin-sensitive and -resistant cells. Northern blot analysis revealed a difference in the level of SSAT mRNA expression in the two cell lines; in particular, 2008 cells treated with 10 μM BESpm for progressively increasing periods of time accumulated more heteronuclear (3.5 kb) and mature (1.3/1.5 kb) SSAT mRNAs than its resistant variant. SSAT mRNA accumulation paralleled enzyme activity and both were almost completely prevented in the two lines by co-treatment with 5 μg/ml actinomycin-D (Act-D), suggesting that transcription plays a major role in the analogue-mediated induction of SSAT. Moreover, when Act-D was added 48 h after BESpm exposure, SSAT mRNA and enzyme activity were stabilised in both cell lines. Therefore, the marked difference in the induction of SSAT activity seems to be related to increased enzyme synthesis, particularly in sensitive cells, whose SSAT protein turnover was also greatly reduced (half-life >12 h in 2008 cells versus 5 h in C13* cells) in the presence of BESpm. These findings suggest that cisplatin-resistance modulates the SSAT response to BESpm at transcriptional and post-transcriptional levels.

Keywords: Cisplatin; BESpm; Drug resistance; SSAT; Transcription; mRNA

1. Introduction

The intracellular concentration of polyamines in mammalian cells is controlled by a combination of finely regulated enzymatic steps, including those catalyzed by the biosynthetic enzymes, ornithine decarboxylase (ODC) and S-adenosylmethionine decarboxylase (SAMD), and the catabolic enzyme, spermidine/spermine N^1-acetyltransferase (SSAT) [1,2]. SSAT is a soluble cytosolic enzyme which, in combined action with polyamine oxydase, converts spermine (Spm) to spermidine (Spd) and the latter to putrescine (Put). Through its acetylating activity, SSAT has been implicated both in the regulation of polyamine catabolism via the back-conversion pathway and in the control of polyamine excretion from cells [3,4]. SSAT activity is very low under normal conditions in most mammalian cells, but can be induced by many hormones, drugs and physiological stimuli [5,6].

It has been reported that a number of antitumour polyamine analogues, including the bis(ethyl)-derivatives of Spm, act as potent enhancers of SSAT activity in rodent and human cells [7,8]. The super-induction of SSAT has been associated with a phenotype-specific sensitivity to these compounds, and there are several reports demonstrating an association between super-induction of SSAT and tumour cell toxicity [9–11]. Therefore, there is considerable interest in characterising the potential antitumour activity of these polyamine

* Corresponding author. Tel.: +39-59-205-5394; fax: +39-59-205-5410.

E-mail address: marverti.gaetano@unimo.it (G. Marverti).

0959-8049/01/$ - see front matter
PII: S0959-8049(00)00389-0

analogues and in elucidating the mechanisms that differentiate analogue-sensitive from analogue-insensitive tumour cells.

Previous studies from our group have shown that cisplatin (CDDP)-resistance in human ovarian cancer cells modulates the response to N^1,N^{12}-bis(ethyl)spermine (BESpm), a representative compound of this family of agents [12]. In particular, we have observed that the CDDP-sensitive 2008 cells are more sensitive to the growth inhibitory effect of BESpm than the CDDP-resistant variant C13* cells. Interestingly, the SSAT activity of CDDP- and BESpm-responsive cells was significantly higher than that of their resistant counterparts after exposure to this drug. However, uncertainty remains as to molecular mechanism(s) underlying this phenotype-specific induction of SSAT activity.

Induction of SSAT activity by polyamine analogues is known to involve a number of complex regulatory mechanisms. Among these are accumulation of SSAT mRNA and stabilization of mRNA and enzyme protein [13–15]. In this study, we characterised the molecular basis of the phenotype-specific differential SSAT induction in CDDP-responsive tumour cells in response to N^1,N^{12}-bis(ethyl)spermine as compared to CDDP-resistant cells. The findings indicate that both accumulation of SSAT mRNA and stabilisation of enzyme protein play a role in the different ability of sensitive and insensitive cells to induce SSAT activity in response to BESpm, suggesting that in C13* cells the development of resistance differently modulates SSAT expression at different regulatory levels.

2. Materials and methods

2.1. Drugs and chemicals

BESpm was kindly supplied by the Hoechst Marion Roussel Inc. (Cincinnati, OH, USA). All other chemicals were purchased from Sigma Chemical Co. (St Louis, MO, USA), except where otherwise indicated.

2.2. Cell lines

The 2008 cell line, established from a patient with serous cystadenocarcinoma of the ovary and the CDDP-resistant C13* subline, generated as previously described [16], were grown as monolayers in RPMI 1640 medium (Whittaker Bioproducts, Walkersville, MD, USA) containing 10% heat-inactivated fetal bovine serum (Gibco/BRL, Gaithersburg, MD, USA) and 50 μg/ml gentamycin sulphate (Sigma). Cultures were equilibrated with humidified 5% CO_2 in air at 37°C. All studies were performed with *Mycoplasma* negative cells, as determined with the *Mycoplasma* T.C. detection kit (Gen-Probe, San Diego, CA, USA).

2.3. Assay of SSAT activity

SSAT was measured essentially as previously described [17]. The cells were harvested, washed twice in phosphate-buffered saline, and suspended in a buffer containing 10 mM Tris(hydroxymethyl)aminomethane (pH 7.2) and 1 mM dithiothreitol. This suspension was frozen and thawed twice, then aliquots of this cytosol were incubated in 100 mM Tris(hydroxymethyl)aminomethane (pH 8.0), 3 mM Spd and 0.5 nmol 1-[^{14}C]acetyl coenzyme A in a final volume of 50 μl for 10 min at 30°C. The reaction was stopped by adding of 10 μl 1 M NH_2–OH HCl and boiling in water for 3 min. The resulting samples were spotted on to P-81 phosphocellulose discs and scintillation counted. The amount of cytosol added to the final reaction mixture was adjusted to maintain the enzyme/substrate concentrations within the linear range. Enzyme activity is expressed as pmol [^{14}C]acetylspermidine formed/min/mg protein. Previous works [18] have shown that SSAT assay also measures other Spd-acetylating enzymes and that, particularly in untreated cells of different cell lines, the authentic SSAT accounts for approximately 27% of the assay-detectable activity versus 96% or more in BESpm-treated cells.

2.4. Northern hybridisation

Total cellular RNA was extracted from cell cultures grown under the indicated conditions using RNAfast (Molecular Systems, San Diego, CA, USA). Ten micrograms aliquots were electrophoresed in a 1% agarose–formaldehyde gel and blotted on to Hybond-N Nylon membranes (Amersham Italia). The RNA was then hybridised alternatively to the specific SSAT or GAPDH cDNA probes, which were purified and labelled by random priming ^{32}[P]-dCTP incorporation. For the detection of SSAT mRNA, a 643 bp cDNA fragment, containing the entire coding region for SSAT, was excised from the plasmid vector pBluescript-II-KS by digestion with Hind III and purified from agarose gel by using the Jetquick gel extraction Kit (Genomed GmbH, Germany), and the specific SSAT mRNA signal was quantified by densitometric scanning, as previously described [19].

2.5. Protein half-life determination

2008 and C13* cells were pretreated for 48 h with 10 μM BESpm, after which 10 μg/ml cycloheximide (CHX) was added to block new protein synthesis. Cells were harvested at 0, 1, 2, 3 and 6 h after CHX administration and SSAT enzyme activity was measured.

2.6. Protein determination

Protein content in the various assays was determined by the method of Lowry and colleagues [20].

3. Results

Previous studies in our laboratory have established that the CDDP-sensitive 2008 cells are more sensitive to the growth-inhibitory effect of BESpm than CDDP-resistant variant C13* cells [12]. In addition, we reported a positive association between the sensitivity to BESpm and the level of induction of the polyamine catabolic enzyme, SSAT, with the consequent more pronounced polyamine depletion [12]. However, our understanding of the precise mechanism(s) responsible for these differences remained unclear.

To characterise further this differential sensitivity, time-course experiments on the effect of BESpm on SSAT activity were undertaken with a concentration of 10 μM BESpm, chosen from the previously reported dose–response studies [12]. In order to verify if extended treatment with the drug might enhance the different increase of SSAT activity between the CDDP-sensitive and -resistant cells, the two cell lines were exposed to 10 μM BESpm for up to 72 h (Fig. 1). A 22-fold induction (220±20 pmol/min/mg versus 10±1 pmol/min/mg in the control (data not shown), $P < 0.01$) was observed at 48 h in the 2008 cells, which continued to increase, reaching a maximum of 435±15 pmol/min/mg (approximately 43-fold induction versus the control value of 10±2 pmol/min/mg (data not shown), $P < 0.01$) at 72 h. By contrast, the C13* cells exibited only a modest (approximately 7-fold) induction up to 95±20 pmol/min/mg versus 11±2 pmol/min/mg of control cells (data not shown) at 48 h, and an approximately 14-fold induction ($P < 0.05$) from 142±20 pmol/min/mg at 72-h exposure to BESpm versus 10±2 pmol/min/mg of control cells (data not shown) over the same period of time.

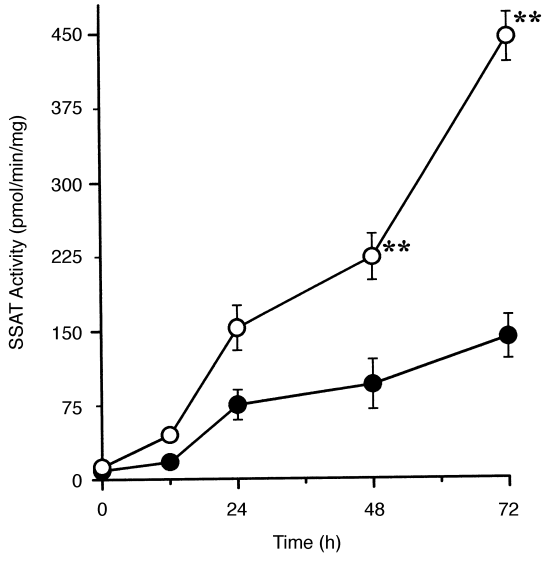

Fig. 1. Time-dependent induction of SSAT activity by 10 μM BESpm in CDDP-sensitive human ovarian carcinoma 2008 cells (○) and its resistant variant C13* cells (●). Cells were treated for the hours indicated with 10 μM BESpm and then harvested and processed for the determination of SSAT activity. All results are expressed as mean values±SE of three separate experiments. $^{**}P < 0.01$ by Student's *t*-test versus resistant cell values.

Although comparable amounts of BESpm were accumulated in both cell lines, BESpm-induced SSAT activity in 2008 cells was significantly enhanced at 48 and 72 h ($P < 0.01$) with respect to C13* cells, suggesting that the differential SSAT induction was not due to different levels of accumulation of the analogue. However, the polyamine content of both cell lines indicated that the natural polyamine pool of the two cell lines was differentially affected by the analogue, with a higher depletion of sensitive cells [12].

Since it has been clearly shown that SSAT induction by BESpm occurs by both transcriptional and post-transcriptional mechanisms [14], we sought to determine at what molecular level the observed differential increase in SSAT between sensitive and resistant cells was mediated.

Assessment of mRNA levels under conditions in which SSAT activity is induced was used to determine the contribution of possible increased transcription in response to the polyamine analogue. Fig. 2 shows that the time-dependent increase in steady-state SSAT-specific RNAs, as determined by northern blot analysis of the cell extracts from the two cell lines, paralleled with time-dependent induction of the SSAT activity by BESpm treatment. However, the increase in mRNA in response to BESpm treatment did not occur to the same extent as SSAT activity in both lines (approximately 11-fold in 2008 cells and 2.5-fold in C13* cells after 72 h, compared, respectively, with the control values at 0 h that did not change up to 12 h of treatment). As shown, 2008 cells significantly accumulated more SSAT mRNAs than C13* cells from 24 to 72 h, indicating that BESpm treatment enhanced steady-state SSAT mRNA level particularly in sensitive cells. This was also accompanied by the evident and progressive increase in the 3.5-kb band of SSAT mRNA, which has been shown to represent preprocessed (heteronuclear) SSAT RNA [13]. In contrast, in the C13* cell line the increase in the level of the mature 1.3/1.5 Kb mRNA form was not associated with a parallel increase in the heteronuclear 3.5 Kb SSAT RNA form. Interestingly, in the resistant cells, the mature 1.3/1.5 Kb SSAT mRNA form accumulated after 24, 48 and 72 h treatment (with respect to controls), while the level of the heteronuclear 3.5 Kb SSAT RNA was almost undetectable. This different pattern of accumulation of the two transcripts suggests that different regulation of SSAT mRNA splicing activation could be an early event as a response to analogue exposure in C13* cells.

As an initial approach in determining the mechanism of RNAs accumulation, cells were treated simultaneously with BESpm and actinomycin-D (Act-D), an inhibitor of RNA synthesis. After 12, 18 and 24 h of

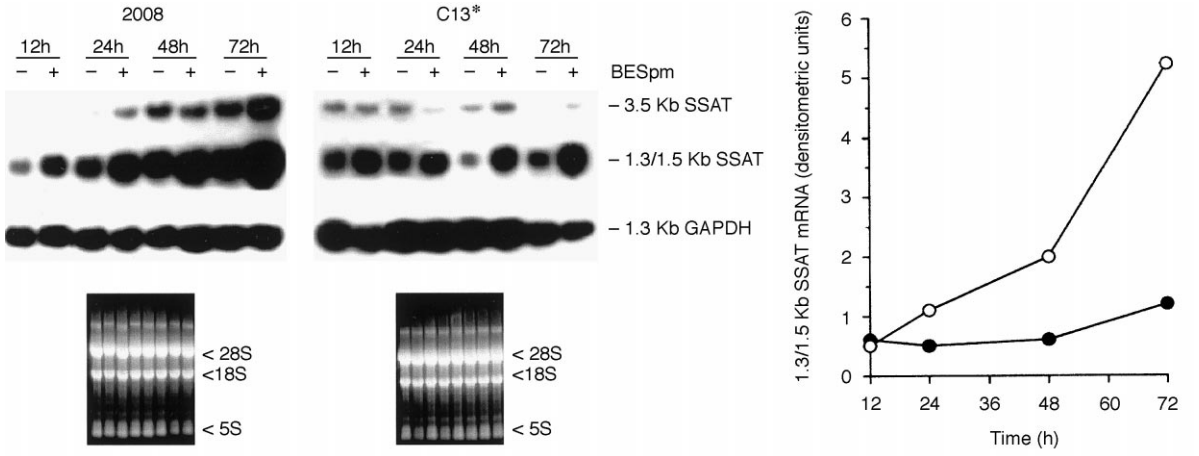

Fig. 2. Time-dependent responses of SSAT mRNA induction as detected by northern blot hybridisation in sensitive (2008) and resistant (C13*) cells treated with 10 μM BESpm for the times indicated. Heteronuclear (3.5 Kb) and mature (1.3/1.5 Kb) forms of SSAT mRNA are shown. GAPDH signal is also shown for comparison. The specific mRNA radioactive signals were quantified by densitometric scanning of the autoradiograms and the data, normalised by substracting the control values, were also graphed the better to compare the kinetics of SSAT mRNA induction in 2008 cells (○) and in C13* cells (●). *Y*-axis: relative optical density. The ethidium bromide staining of the same RNA specimens, separated on agarose gel, is also shown. Ten micrograms of total RNA was loaded on each lane. The data shown are representative of two determinations obtained in separate sets of experiments.

treatment, cells were harvested and SSAT mRNA content was analysed by northern blot hybridisation in parallel with the determination of SSAT enzyme activity. The cells were exposed to Act-D for no longer than 24 h to avoid toxic effects; in fact, longer exposure to this concentration of Act-D caused cell toxicity, as revealed by cell rounding and detachment. As shown in Fig. 3, under these conditions 12 h Act-D treatment reduced SSAT mRNA accumulation by 60% in both cell lines treated with BESpm; at 24 h, Act-D inhibition of the increase of SSAT transcript by BESpm was greater than 90–95%, both in sensitive and resistant cells. In both cell lines, heteronuclear SSAT RNA completely disappeared after Act-D exposure, as expected as a result of inhibition of transcription. This suggested that the increase in steady-state SSAT mRNA observed with BESpm alone is primarily the result of increased transcription. As a consequence, the induction of SSAT enzyme activity was progressively prevented by 60–90% in both cell lines. As shown in Fig. 4, acetylase activity was blocked to the level of control values in both cell lines by Act-D treatment, representing a difference at $P < 0.01$ in 2008 cells and at $P < 0.05$ in C13* cells after 18 and 24 h of exposure.

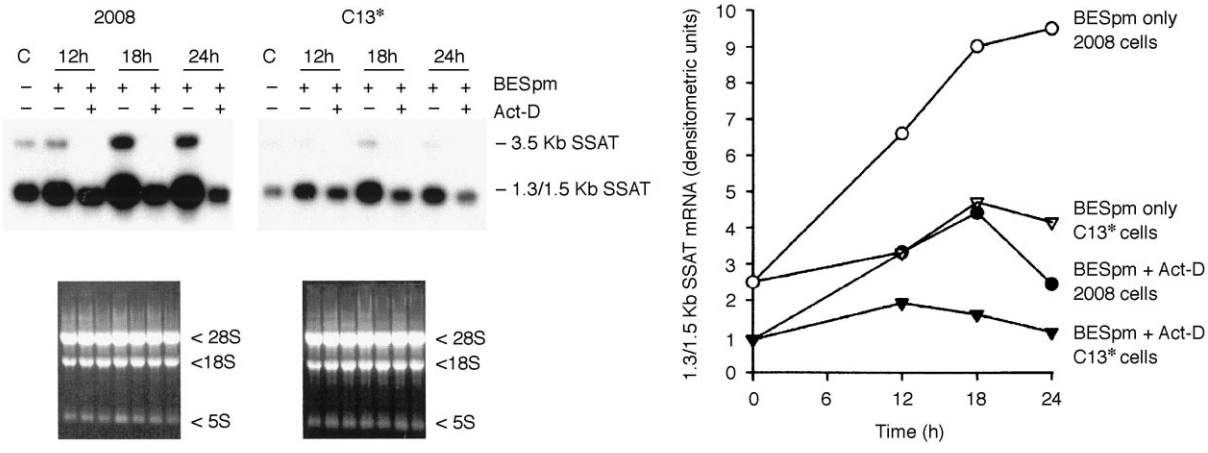

Fig. 3. Northern blot detection of SSAT mRNA induced by BESpm alone and by BESpm/Act-D co-exposure in sensitive and resistant cells. At 80–90% confluency, cells were treated with 10 μM BESpm alone or in combination with 5 μg/ml Act-D for the times indicated prior to isolation of RNA and determination of SSAT mRNA. Heteronuclear (3.5 Kb) and mature (1.3/1.5 Kb) forms of SSAT mRNA are shown. GAPDH mRNA did not change significantly and it was not shown. The specific mRNA radioactive signals were quantified by densitometric scanning of the autoradiograms and the values, normalised by subtracting the control values, were graphed. *Y*-axis: relative optical density. The ethidium bromide staining of the same RNA specimens, separated on agarose gel, is also shown. Ten micrograms of total RNA was loaded on each lane. The data shown are representative of two determinations obtained in separate sets of experiments.

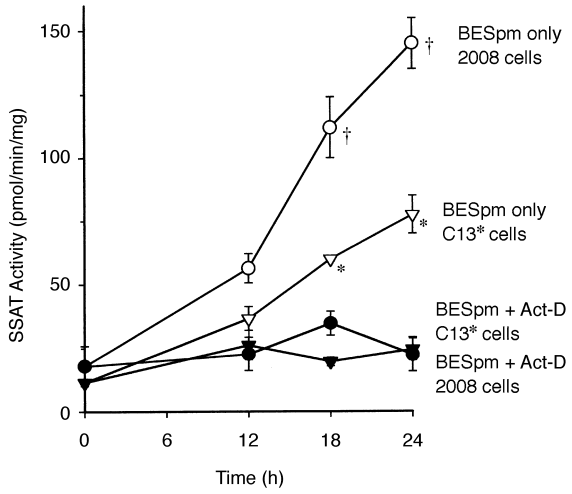

Fig. 4. SSAT activity induced by BESpm alone (open symbols) and by BESpm/Act-D co-exposure (filled symbols) in 2008 cells (circle) and C13* cells (triangle down). At 80–90% confluency, cells were treated with 10 μM BESpm alone or in combination with 5 μg/ml Act-D for the times indicated prior to determination of enzyme activity. Results are expressed as means of at least three determinations. Error bars denote standard error. $*P<0.05$; $\dagger P<0.01$ by Student's *t*-test versus both controls and Act-D treated samples.

Although BESpm-mediated accumulation of SSAT mRNA occurred at least in part through changes in the transcriptional process, the possibility that changes at the post-transcriptional level in response to BESpm might also occur was investigated. Thus, to study SSAT mRNA turnover, Act-D was used to block transcription, and the decay of SSAT mRNA in cells stimulated with BESpm was determined at 6 h time intervals. In control cells, treated with Act-D, the SSAT mRNA decreased progressively with a half-life of about 20 h in both sensitive and resistant lines (data not shown). However, when cells were pretreated with BESpm and then exposed to Act-D, as observed for many enzymes, including ODC, in a number of different cell systems [21,22], this inhibitor of RNA synthesis caused a slight increase in endogenous SSAT mRNA concentrations with resultant complications in estimating SSAT mRNA half-life. Fig. 5 depicts this effect after 24 h exposure to Act-D; there was a 1.2-fold increase in the accumulation of mature SSAT mRNA with respect to BESpm-treated sensitive 2008 cells, and a 1.8-fold increase with respect to BESpm-treated C13* resistant cells (Fig. 5b). The induction of the 1.3/1.5 Kb mRNA form coincided with a strong reduction (2008 cells) and a complete disappearance (C13* cells) of the heteronuclear SSAT RNA form (Fig. 5a). This phenomenon, which continued until the appearance of cytotoxic effects of Act-D, strengthens the hypothesis of a splicing activation of SSAT mRNA, particularly in C13* cells. Nevertheless, these results confirm, as previously reported [9,13], that BESpm treatment causes significant stabilisation of mature SSAT mRNA. As shown, BESpm-induced SSAT activity paralleled the changes in the transcript levels (Fig. 5c).

Another possible mechanism by which SSAT activity might be increased is via stabilisation of the protein, since SSAT normally turns over very rapidly [14,15]. To test this possibility, we compared the stability of SSAT

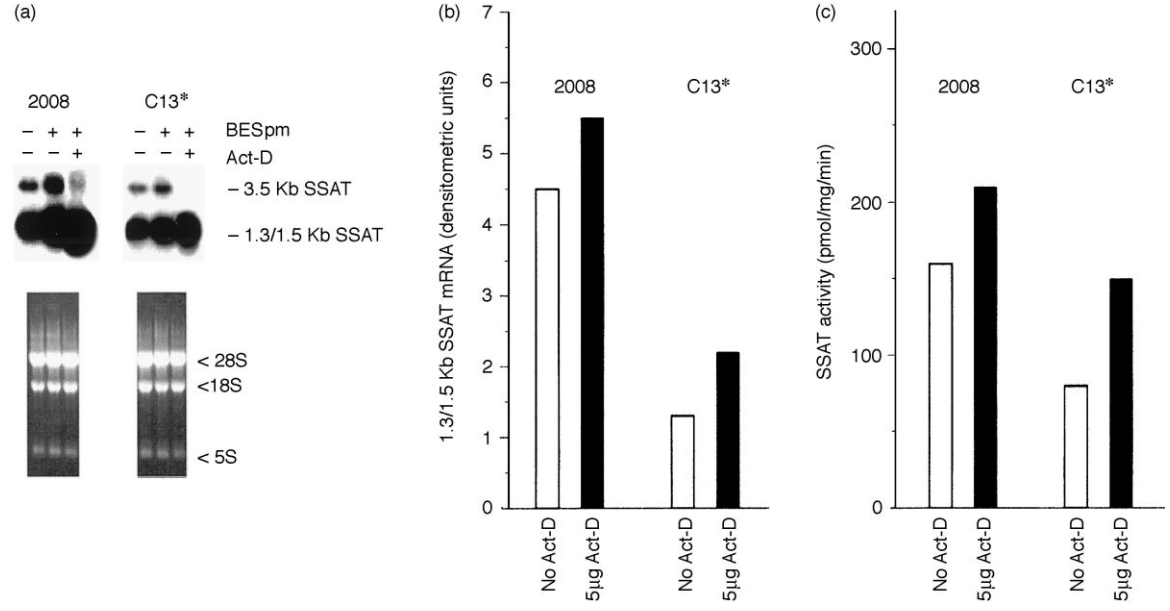

Fig. 5. Northern blot (a) and bar graph of SSAT mRNA (b) and SSAT enzyme activity (c) from 2008 and C13* cells. Sample monolayers were pretreated for 48 h with 10 μM BESpm, washed and then postincubated for an additional 24 h in the absence or presence of 5 μg/ml Act-D. Northern blots are representative of two identical experiments; enzyme activity is based on data from three experiments in which the standard error was within ±10% for each data point. Panel (a) also shows the ethidium bromide staining of the same RNA specimens, separated on agarose gel. Ten micrograms of total RNA was loaded on each lane. GAPDH mRNA did not change significantly and it was not shown.

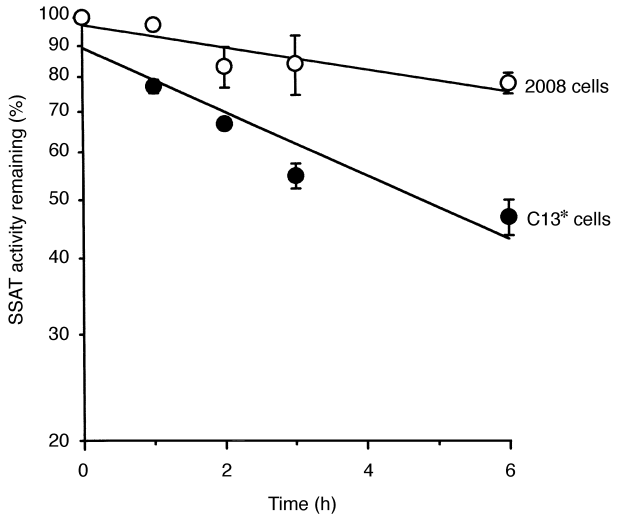

Fig. 6. Half-life determination of SSAT activity in DDP-sensitive 2008 cells and in its resistant variant C13* cells. Both lines were treated for 48 h with 10 μM BESpm and then incubated with 10 μg/ml cycloheximide. Cell samples were then removed at the times indicated and were analysed for SSAT activity. The points represent three separate determinations and are expressed as percentages of induced SSAT activity using the value at the time of cycloheximide addition as 100% (212±31 pmol/min/mg of protein in 2008 cells and 95±16 pmol/min/mg of protein in C13* cells).

in DDP-sensitive and -resistant cells after 48 h exposure to BESpm. The decay of SSAT activity was monitored at different time intervals after inhibition of protein synthesis by cycloheximide (10 μg/ml). As shown in Fig. 6, the activity of SSAT in C13* cells declined with a half-life of approximately 5 h, whereas the acetylase activity in the 2008 cells was more stable, and declined with a half-life of approximately 10–12 h.

4. Discussion

Acquired cisplatin-resistance has been shown to lead to some morphological and functional modifications developed during prolonged drug exposure [23]. The repair of the DNA damage caused by DDP adducts is often enhanced in all cell lines where resistance is observed, while the induction of other mechanisms of resistance such as reduced drug uptake, increased glutathione or metallothionein levels are not always observed [24,25]. However, in C13* cells all these alterations are present along with mitochondrial defects that play an important role in the DDP-resistance of C13* cells [12,16]. It is well known that cells with acquired anticancer drug resistance frequently exhibit broad cross-resistance to other anticancer agents due to some of the intracellular modifications which occurred [26]. In this regard, our previous results showed that ovarian cancer DDP-resistant cells also exhibit cross-resistance to the polyamine analogue BESpm. The cross-resistance to DDP and BESpm does not seem to be a phenomenon restricted to these cell lines, because we also found that the 2-fold DDP-resistant human breast-cancer cell line MCF7/CH were 1.5-fold resistant to BESpm when compared with its parental sensitive MCF7 line [27]. A correlation between BESpm sensitivity and the level of SSAT induction during analogue treatment has been observed both in ovarian and breast cancer cell lines [12,27]. Since SSAT appears directly or indirectly involved in the response of specific tumour phenotypes to treatment with the polyamine analogue [28], here we studied the underlying mechanisms by which SSAT is differently regulated in response to drug treatment in DDP-sensitive and -resistant ovarian cancer cells. Studies on the mechanistic basis for SSAT accumulation in response to the spermine analogue have demonstrated, in human melanoma cells, that BESpm induces accumulation of SSAT mRNA by combined effects on increased gene transcription and mRNA stabilisation [13]. In addition, a significant portion of the SSAT response to such analogues has been ascribed to posttranscriptional mechanisms, including enhanced mRNA translation and stabilisation of the SSAT enzyme protein [14]. In accordance with these and other reports [9,28], our results provide further evidence that increased transcription is at least partially responsible for the accumulation of SSAT mRNA in response to BESpm. Indeed we show here that, after treatment with Act-D, BESpm stimulation of SSAT mRNA accumulation in the two cell lines can be almost completely prevented. In addition, we provide the first evidence of a differential accumulation of SSAT transcripts caused by the treatment with the analogue in cisplatin-sensitive and -resistant human ovarian cancer cell lines. The quantitative difference in transcript content between sensitive and resistant lines was also supported by the increasing abundance of unprocessed heteronuclear SSAT RNA in sensitive cells as compared with their resistant counterparts. Along with increased transcription, BESpm action also involves the stabilisation of SSAT mRNA with little or no difference in either cell line, as appears from analogue-pretreatment experiments. Furthermore, these studies indicate that the increased activation of specific SSAT RNA splicing could contribute to the enhanced level of mature mRNA form, particularly in C13* cells. Assuming that SSAT induction during analogue treatment is a compulsory step for the cell, it is conceivable that in the resistant line the less efficient transcription may be partly compensated by an augmented conversion of the heteronuclear SSAT RNA into the mature 1.3/1.5 Kb SSAT mRNA forms, at least at the times observed.

There are several potential mechanisms whereby the intracellular changes responsible for DDP-resistance could contribute to the differential regulation of SSAT expression in DDP-sensitive and -resistant ovarian

cancer cells. Recently, a *cis*-element in the SSAT promoter has been identified, the polyamine-responsive element (PRE), which mediates the transcriptional induction of SSAT by polyamine analogue [29] in association with the nuclear protein, Nrf-2, which is expressed in the analogue-responsive cell types along with a partner protein more recently identified as polyamine-modulated factor-1 (PMF-1) [30]. Due to the importance of these elements in playing a critical role in the polyamine analogue-induced expression of SSAT in responsive cell types, we could hypothesize that C13* cells may present changes in these factors with respect to the parental line. In particular, it may be suggested that different amounts of PMF-1 are present in sensitive and resistant lines. In fact, the human PMF-1 gene is located on chromosome 1 near the 1q12/1q21 border, close to chromosomal regions 1q21–q22, in which gains were found to be related to the cisplatin-resistant phenotype in ovarian cancer patients [31]

These differences may be the expression of the accumulation of multiple genetic changes, such as gains in chromosomes 2q, 4, 6q, and 8q or losses in chromosomes 2p, 7p, 11p, 13 and X, which have been recognised as being responsible for cisplatin resistance in ovarian cancer cell lines [32]. In particular, the losses in the chromosome regions Xp22.2 and Xp22.1 observed in the 2008 cell line selected for acquired resistance to DDP (C13* cells) may have an important role in the transcription activity of the SSAT gene, which is located at the Xp22.1 locus [28]. Thus, it is conceivable that the reduced level of transcription of SSAT gene that C13* resistant cells exibit, could derive from altered regulation of this process, due to loss of chromosomal material in regions spanning the SSAT gene or its promoter. This alteration may explain the fact that resistant cells, even under basal conditions, do not increase the level of SSAT message with time as much as sensitive cells (Fig. 2). The increase in SSAT mRNA level occurring in control cells could be related to a progressive accumulation of cultured cells in G_2/M phases of the cell cycle, a condition that has been shown to induce SSAT expression in normal human fibroblast [19]. The phenomenon is particularly evident at 72 h after seeding, when both 2008 and C13* cell cultures are also approaching confluency. The fact that this accumulation in SSAT RNAs is particularly evident in untreated 2008 cells, but not in C13* cells, suggests once more that altered SSAT gene expression may be among the intracellular changes caused by DDP selection.

Further, it has been proposed [33] that the mechanisms by which polyamine analogues induce mRNA transcription could be the consequence of a direct interaction of the analogue at RNA sites or with secondary regulatory protein(s), which in turn act at those sites. The kinetics of SSAT mRNA turnover we observed in BESpm pretreated cells, i.e. the stabilisation of the mRNA level in 2008 cells and the slight induction in C13* cells, might suggest that Act-D could inhibit the transcription of a gene that is required for SSAT mRNA degradation. Fogel-Petrovic and colleagues [34] demonstrated in human melanoma cells the involvement of labile regulatory protein(s) controlling SSAT gene transcription and/or mRNA stabilisation, suggesting that gene expression is repressed under basal conditions and released by inhibitors of protein synthesis. Further studies are in progress to elucidate the precise nature of this regulation of SSAT expression in DDP-sensitive and-resistant cells.

In addition, BESpm availability at important sites could be limited to a different extent in the two cell lines by resistance-associated intracellular modifications. For instance, the double intracellular concentration of negatively charged molecules (glutathione, metallothioneins, etc.) in resistant cells, that can bind and neutralise the cationic analogue, and/or the higher number of mitochondria with their hyperpolarised membranes in C13* cells can divert much more analogue from macromolecules such as DNA, proteins, etc. than in the parental line.

Our studies indicate that the differential analogue-induced accumulation of SSAT-specific mRNA alone does not account entirely for the different increase in SSAT activity resulting from analogue exposure in the two cell lines. Thus, the addition of BESpm stimulates the transcription of SSAT mRNA and probably enhances its stability, as previously reported [13], but it is only the combination of these effects, together with the delay in SSAT protein degradation, that lead to a several-fold increase in SSAT enzyme activity. It is well known that SSAT has a very short half-life [18,35,36]. However, polyamine analogues have been shown to increase protein stability several times [34], prolonging its turnover in many cell types. In our case, the BESpm-extended enzyme half-life appeared to be a factor accounting both for enzyme activation and, in particular, for the differential induction of SSAT activity as demonstrated by the comparison of the half-life in the two cell lines. In BESpm-treated 2008 cells, the SSAT half-life was approximately 2.4-fold greater than in the treated C13* cells. Stabilisation of enzyme activity might be caused by a direct analogue interaction with the enzyme. Coleman and colleagues showed that BESpm protects the protein from protease digestion [15]. In a very recent work, McCloskey and Pegg [11] have identified a point mutation in the SSAT gene of CHO cells selected for BESpm-resistance, C55.7Res and suggest that SSAT mutated proteins may have altered interaction with the polyamine analogue. Therefore, the decreased enzyme activity of resistant cells may derive from change in protein/analogue interaction that compromises the ability of the analogue to protect SSAT protein from degradation. Thus, it is possible that rapidly turning

over SSAT protein might be stabilised more effectively by BESpm in sensitive cells by binding the enzyme with higher affinity, thus allowing the enzyme to accumulate to levels higher than in C13* cells.

In conclusion, these results demonstrate that the more pronounced increase in SSAT activity induced by BESpm in the sensitive line is primarily the consequence of an increase in steady-state SSAT mRNA level and then of a more prolonged stability of the enzyme protein, indicating that multiple events involved in the control of SSAT gene expression are differently affected in sensitive and resistant cell lines and thus contribute in a different cumulative manner to final enzyme activities. Further studies attempting to demonstrate a functional correlation between cellular alterations caused by DDP selection and the altered SSAT expression that results in the polyamine analogue-resistance of C13* cells are currently in progress.

Acknowledgements

This work was supported by a grant from MURST 60%, 40% and from Associazione Angela Serra per la Ricerca sul Cancro, Azienda Ospedaliera Policlinico di Modena, Modena, Italy.

References

1. Tabor C, Tabor H. Polyamines. *Annu Rev Biochem* 1984, **53**, 749–790.
2. Pegg AE. Recent advances in the biochemistry of polyamines in eukaryotes. *Biochem J* 1986, **234**, 249–262.
3. Seiler N. Functions of polyamine acetylation. *Can J Physiol Pharmacol* 1987, **65**, 2024–2035.
4. Shappell NW, Miller JT, Bergeron RJ, Porter CW. Differential effects of the spermine analog, N^1,N^{12}-bis(ethyl)spermine, on polyamine metabolism and cell growth in human melanoma cell lines and melanocytes. *Anticancer Res* 1992, **12**, 1083–1089.
5. Della Ragione F, Pegg AE. Role of spermidine/spermine N^1-acetyltransferase in polyamine metabolism. In Caldarera CM, Bachrach U, eds. *Advances in Polyamines in Biomedical Research*. Bologna, CLUEB, 1984, 97–104.
6. Casero RA Jr, Pegg AE. Spermidine/spermine N^1-acetyltransferase — the turning point in polyamine metabolism. *FASEB J* 1993, **7**, 653–661.
7. Casero RA Jr, Celano P, Ervin SJ, Porter CW, Bergeron RJ, Libby PR. Differential induction of spermidine/spermine N^1-acetyltransferase in human lung cancer cells by the bis(ethyl)polyamine analogues. *Cancer Res* 1989, **49**, 3829–3833.
8. Pegg AE, Wechter R, Pakala R, Bergeron RJ. Effect of N^1,N^{12}-bis(ethyl)spermine and related compounds on growth and polyamine acetylation, content and excretion in human colon tumour cells. *J Biol Chem* 1989, **264**, 11744–11749.
9. Casero Jr RA, Mank AR, Xiao L, Smith J, Bergeron RJ, Celano P. Steady-state messenger RNA and activity correlates with sensitivity to N^1,N^{12}-bis(ethyl)spermine in human cell lines representing the major forms of lung cancer. *Cancer Res* 1992, **52**, 5359–5363.
10. Shappell NW, Fogel-Petrovic M, Porter CW. Regulation of spermidine/spermine N^1-acetyltransferase by intracellular polyamine pools. *FEBS Lett* 1993, **321**, 179–183.
11. McCloskey DE, Pegg AE. Altered spermidine/spermine N^1-acetyltransferase activity as a mechanism of cellular resistance to bis-(ethyl)polyamine analogues. *J Biol Chem* 2000, **275**, 28708–28714.
12. Marverti G, Piccinini G, Ghiaroni S, Barbieri D, Quaglino D, Moruzzi MS. N^1,N^{12}-bis(ethyl)spermine effect on growth of *cis*-diamminedichloroplatinum(II)-sensitive and-resistant human ovarian-carcinoma cell lines. *Int J Cancer* 1998, **78**, 33–40.
13. Fogel-Petrovic M, Shappell NW, Bergeron RJ, Porter CW. Polyamine and polyamine analog regulation of spermidine/spermine N^1-acetyltransferase in MALME-3M human melanoma cells. *J Biol Chem* 1993, **268**, 19118–19125.
14. Parry L, Balana-Fouce R, Pegg AE. Post-transcriptional regulation of the content of spermidine/spermine N^1-acetyltransferase by N^1,N^{12}-bis(ethyl)spermine. *Biochem J* 1995, **305**, 451–458.
15. Coleman CS, Huang H, Pegg AE. Role of the carboxyl terminal MATEE sequence of spermidine/spermine N^1-acetyltransferase in the activity and stabilization by the polyamine analog N^1,N^{12}-bis(ethyl)spermine. *Biochemistry* 1995, **34**, 13423–13430.
16. Andrews PA, Albright KD. Mitochondrial defects in *cis*-diaminedichloroplatinum(II)-resistant human ovarian carcinoma cells. *Cancer Res* 1992, **52**, 1895–1901.
17. Casero RA Jr, Celano P, Ervin SJ, Porter CW, Wiest L, Pegg AE. High specific induction of spermidine/spermine N^1-acetyltransferase in human large cell lung carcinoma. *Biochem J* 1990, **270**, 615–620.
18. Persson L, Pegg AE. Studies of the induction of spermidine/spermine N^1-acetyltransferase using specific antiserum. *J Biol Chem* 1984, **259**, 12364–12367.
19. Bettuzzi S, Davalli P, Astancolle S, *et al*. Coordinate changes of polyamine metabolism regulatory proteins during the cell cycle of normal human dermal fibroblasts. *FEBS Lett* 1999, **446**, 18–22.
20. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951, **193**, 265–275.
21. Wallon MU, Persson L, Heby O. Superinduction of ornithine decarboxylase (ODC) by actinomycin D is due to stimulation of ODC mRNA translation. *FEBS Lett* 1990, **268**, 161–164.
22. Hltta K, Sistonen L, Alitalo K. The mechanisms of ornithine decarboxylase deregulation in c-Ha-ras oncogene-transformed NIH 3T3 cells. *J Biol Chem* 1988, **263**, 4500–4507.
23. Andrews PA. Mechanisms of acquired resistance to cisplatin. In Goldstein LJ, Ozols RF, eds. *Anticancer Drug Resistance, Advances in Molecular and Clinical Research*. Boston, Kluwer Academic, 1994, 217–246.
24. Chu G. Cellular responses to cisplatin. *J Biol Chem* 1994, **269**, 787–790.
25. Andrews PA, Howell SB. Cellular pharmacology of cisplatin: perspectives on mechanisms of acquired resistance. *Cancer Cells* 1990, **2**, 35–43.
26. Yang YY, Robbins PD, Lazo JS. Differential transactivation of human metallothionein-IIa in cisplatin-resistant and-sensitive cells. *Oncol Res* 1998, **10**, 85–98.
27. Marverti G, Monti MG, Piccinini G, Moruzzi MS. Differential induction of spermidine/spermine N^1-acetyltransferase in cisplatin-sensitive and-resistant human breast cancer cells by N^1,N^{12}-bis(ethyl)spermine. In Bardocz S, Koninkx J, Grillo MA, White A, eds. *Biologically Active Amines in Food Processing and Amines Produced by Bacteria, and Polyamines and Tumour Growth*. vol. II. Luxembourg, COST 917, 1999, 94–98.
28. Xiao L, Casero Jr RA. Differential transcription of the human spermidine/spermine N^1- acetyltransferase (SSAT) gene in human lung carcinoma cells. *Biochem J* 1996, **313**, 691–696.
29. Wang Y, Xiao L, Thiagalingam A, Nelkin BD, Casero Jr RA. The identification of a cis-element and a trans-acting factor involved in the response to polyamines and polyamine analogues in the regulation of the human spermidine/spermine N^1-acetyltransferase gene transcription. *J Biol Chem* 1998, **273**, 34623–34630.

30. Wang Y, Devereux W, Murray Steward T, Casero Jr RA. Cloning and characterization of human polyamine-modulated factor-1, a transcriptional cofactor that regulates the transcription of the spermidine/spermine N^1-acetyltransferase gene. *J Biol Chem* 1999, **274**, 22095–22101.
31. Kudoh K, Takano M, Koshikawa T, *et al*. Gains of 1q21–q22 and 13q12–q14 are potential indicators for resistance to cisplatin-based chemotherapy in ovarian cancer patients. *Clin Cancer Res* 1999, **5**, 2526–2531.
32. Wasenius VM, Jekunen A, Monni O, *et al*. Comparative genomic hybridization analysis of chromosomal changes occurring during development of acquired resistance to cisplatin in human ovarian carcinoma cells. *Genes Chromosomes Cancer* 1997, **18**, 286–291.
33. Fogel-Petrovic M, Kramer DL, Vujcic S, *et al*. Structural basis for differential induction of spermidine/spermine N^1-acetyltransferase activity by novel spermine analogs. *Mol Pharmacol* 1997, **52**, 69–74.
34. Fogel-Petrovic M, Vujcic S, Brown PJ, Haddox MK, Porter CW. Effects of polyamines, polyamine analogs, and inhibitors of protein synthesis on spermidine/spermine N^1-acetyltransferase gene expression. *Biochemistry* 1996, **35**, 14436–14444.
35. Pegg AE, Pakala R, Bergeron RJ. Induction of spermidine/spermine N^1-acetyltransferase activity in Chinese-hamster ovary cells by N^1,N^{11}-bis(ethyl)norspermine and related compounds. *Biochem J* 1990, **267**, 331–338.
36. Matsui J, Pegg AE. Effects of inhibitors of protein synthesis on rat liver spermidine N^1-acetyltransferase. *Biochim Biophys Acta* 1981, **675**, 373–378.