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ORIGINAL ARTICLE

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Induction of spermidine/spermine N^1-acetyltransferase in breast cancer tissues treated with the polyamine analogue N^1,N^{11}-diethylnorspermine

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Abstract *Purpose*: The polyamine analogue, N^1,N^{11}-diethylnorspermine (DENSpm), is currently being evaluated in clinical trials for the treatment of solid tumors. The response of solid tumors to this drug has been associated with superinduction of the polyamine catabolic enzyme, spermine/spermidine N^1-acetyltransferase (SSAT). Therefore, to estimate the response of breast cancers to DENSpm, we measured induction of SSAT in breast cancer explants treated in vitro with this polyamine analogue. *Experimental design*: Expression of SSAT protein was evaluated by immunohistochemistry in tissue explants from 38 invasive breast cancer tumors incubated in vitro in the presence (or absence) of DENSpm. In addition, SSAT enzymatic activity was measured in tissue explants from four tumors with high cellularity. *Results*: SSAT expression was significantly increased in 30 of 38 tumor samples treated with DENSpm compared to untreated controls. This induction of SSAT protein expression was found specifically in neoplastic cells of the treated samples, and was seen in all histologic patterns (ductal, lobular, and mucinous) of breast cancer examined. In tumor samples evaluated for changes in SSAT enzymatic activity, these changes correlated closely with changes in protein expression. *Conclusions*: Immunohistochemical staining for induction of SSAT correlates with measures of enzymatic activity in a small sample where measurements were possible and suggests that immunohistochemistry may be used for predicting response of breast cancers to DENSpm. A high proportion of breast cancers induced SSAT in response to DENSpm, supporting the continued consideration of this class of agents for treatment of breast cancer.

Keywords Polyamines · Immunohistochemistry · Catabolism

Abbreviations 5-FU: 5-fluorouracil · DENSpm: N^1, N^{11}-diethylnorspermine · SSAT: spermidine/spermine N^1-acetyltransferase

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Introduction

The polyamine metabolic pathway has been widely investigated as a potential target for cancer treatment since the discovery that polyamine metabolism is frequently dysregulated in neoplastic disease [23, 32, 34, 35]. Early therapeutic strategies, which attempted to inhibit the endogenous synthesis of polyamines, were often ineffective because of the ability of tumor cells to utilize exogenous polyamines and/or compensate metabolically [23]. A more recent therapeutic approach has focussed on increasing catabolism of polyamines in tumor cells [3, 8, 28, 29, 36]. An agent that increases polyamine catabolism is the symmetrically substituted N^1,N^{11}-diethylnorspermine (DENSpm; also known as BENSpm or BE333). The induction of spermidine/spermine N^1-acetyltransferase (SSAT) by this drug has been shown to be associated with a cytotoxic response in non-small-cell lung cancer, melanoma, pancreatic cancer, and some breast cancer cell lines [1, 2, 4, 6, 9, 15, 16, 29]. Recent studies using both conditional and constitutively expressed vectors expressing human SSAT have clearly demonstrated a direct link between SSAT expression and response to specific polyamine analogues [27, 33]. The mechanisms underlying the association between SSAT induction and cytotoxicity appear to be

related to the production of reactive oxygen species (ROS) through the oxidation of the acetylated products by polyamine oxidase [10, 20, 36]. Therefore, the ability to specifically induce high levels of SSAT activity in a tumor-specific manner provides a basis for selective targeting of tumors with ROS-induced apoptotic death.

Since SSAT induction has been directly associated with drug response, and because DENSpm and other similarly acting polyamine analogues are in or are being considered for clinical trial, it is imperative to determine the specificity of the SSAT induction for tumor tissue with respect to normal tissue. This is not only important to demonstrate that high SSAT induction is a tumor-specific drug response, but may also provide an invaluable intermediate marker of such a response. Therefore, it was the aim of the current study to determine whether SSAT induction in response to exposure to the antitumor polyamine analogue, DENSpm, is a tumor cell-specific event, and to determine how commonly SSAT induction occurs in human breast cancers exposed to DENSpm.

Materials and methods

Tissue explants and in vitro exposure to DENSpm. DENSpm was kindly provided by Parks-Davis (Ann Arbor, Mich.). Primary breast cancer tissue samples were obtained aseptically from 38 surgically removed tumors, in compliance with institutional standards for research involving human subjects. All tissues used were in excess of requirements for diagnosis, and no patient identifiers were linked to the tissues. The histologic characteristics of these cancers are summarized in Table 1.

For incubation of explants, thin slices (about 1 mm in thickness) of tumor were placed in RPMI 1640 medium supplemented with 10% fetal bovine serum and incubated at 37°C in an atmosphere containing 5% CO_2 for 22 h with or without 10 μM DENSpm, a concentration that corresponds to concentrations that have been attained in clinical studies [22].

Table 1 Induction of SSAT in breast cancer explants treated with DENSpm for 24 h

	Total number	SSAT induction by DENSpm	
		Negative (0 to 1+)	Positive (2 to 4+)
Histologic type			
Ductal	29	7	22
Lobular	7	1	6
Mucinous	2	0	2
Estrogen receptor status			
Positive	24	4	20
Negative	14	4	10

Results are tabulated by pathologic diagnosis and by estrogen receptor status. Scoring was based on intensity and distribution of reactivity (3+, strong staining in majority of tumor cells; 2+, moderate reactivity in majority of tumor cells; 1+, weak staining or few cells with moderate staining; 0, no staining).

Immunohistochemical and enzymatic analysis. At the end of this incubation, tissue samples were fixed in 10% buffered formalin for at least 6 h prior to routine histologic processing. When sufficient tissue was available, the samples were snap-frozen in dry ice/methanol and analyzed for measurement of SSAT activity [4].

Sections (5 μm thick) of paraffin-embedded samples were incubated in xylene to remove paraffin, dehydrated, and treated using the high-pH antigen retrieval method (DAKO, Carpinteria, Calif.) as previously described [19]. Immunohistochemical analysis was conducted using a rabbit polyclonal antibody [7] and developed using a DAKO LSAB 2 kit. Samples were scored based on intensity and distribution of reactivity (Table 1).

Results and discussion

Several in vitro and in vivo studies have implicated the high induction of SSAT in the tumor-specific cytotoxic activity of a series of new antitumor polyamine analogues [1, 2, 4, 5, 15, 29]. Recently, the role of SSAT induction in the cytotoxic response of a breast cancer cell line has been unequivocally demonstrated using a conditional SSAT expression system [33] and similar relationships have been demonstrated through the use of other expression vectors to produce cells that express mutant forms of SSAT [25, 27]. Furthermore, in lung cancer tissues, high induction of SSAT activity has now been directly linked to the cytotoxic response of specific polyamine analogues [7, 19].

The current study was undertaken to determine the specificity and frequency of superinduction of SSAT in response to exposure to the polyamine analogue, DENSpm, in breast cancer tissues. Samples from a total of 42 breast cancers were analyzed by immunohistochemistry. Staining these tissue samples with a polyclonal antibody demonstrated increased levels of SSAT in treated samples compared to untreated samples for 36 of these tumors (Fig. 1 and Table 1).

It is important to note that only minimal staining for SSAT was observed in the fibroblastic-type stromal cells of responsive breast tumors, indicating that the high induction of SSAT, when it occurs, is a tumor-specific phenomenon. These findings are consistent with those of previous in vitro studies, which demonstrated SSAT induction in cultured breast cancer cells responding to polyamine analogues, and of studies with lung cancer explants, which showed SSAT induction in cancer cells but not stromal cells [7, 15, 19]. In addition, minimal staining for SSAT was observed in adjacent normal mammary ductal structures of tissues treated with DENSpm (not shown). Collectively, these findings sug-

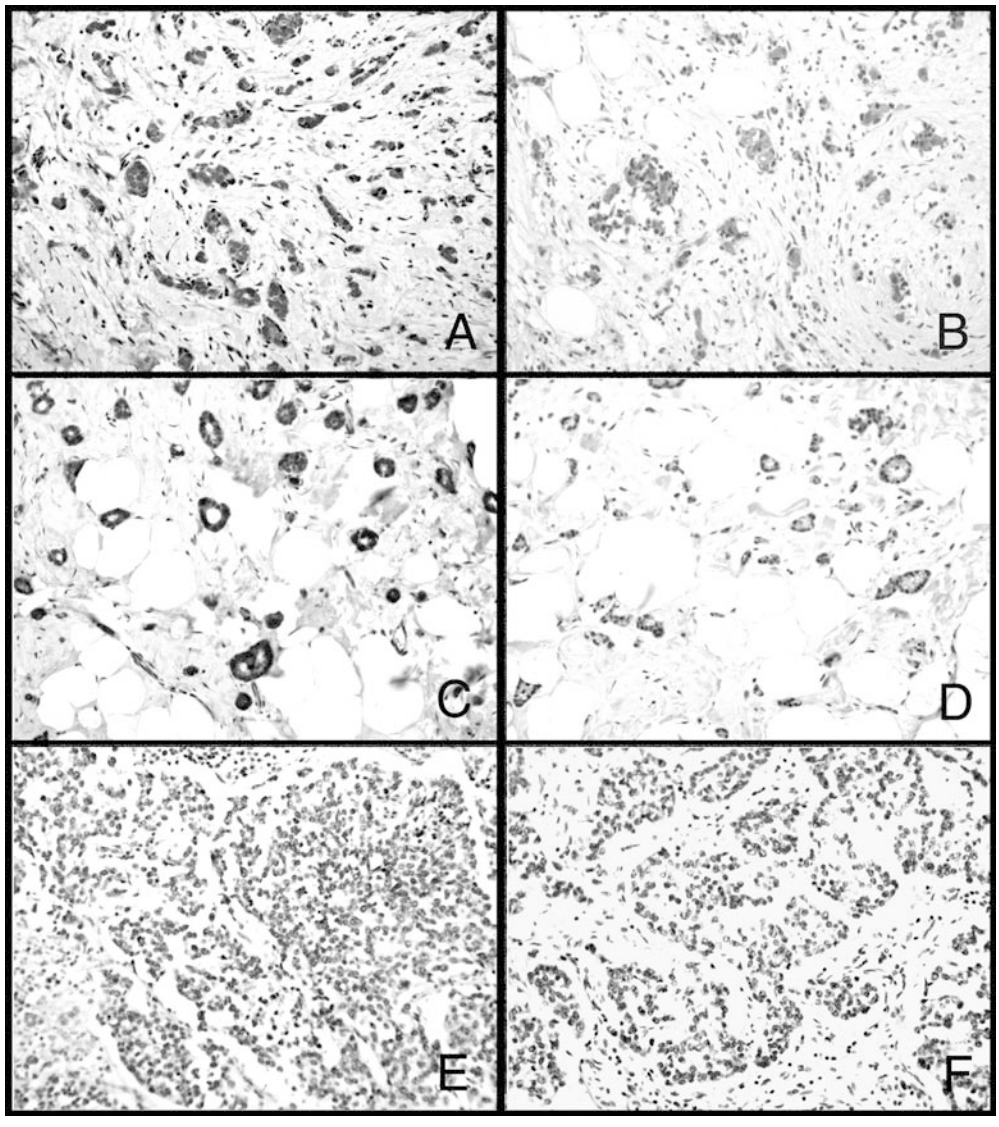

Fig. 1A–F Immunohistochemical staining for SSAT in breast cancer tissue samples. **A**, **C** Samples with strong (3+) induction of SSAT after treatment with DENSpm for 24 h. **B**, **D** Control samples from the same tumors as **A** and **B**, respectively, incubated for 24 h in the absence of DENSpm. **E**, **F** DENSpm-treated and untreated control samples, respectively, representing a tumor that did not show induction of SSAT in response to DENSpm

gest that the high induction of SSAT by DENSpm is restricted to neoplastic cells.

Induction of SSAT protein by DENSpm was seen in samples of ductal carcinoma (22 of 29, 75%), lobular carcinoma (6 of 7, 86%), and mucinous carcinoma (2 of 2, 100%). In addition, induction of SSAT protein was seen in estrogen receptor-negative (10 of 14, 72%) as well as estrogen receptor-positive (20 of 24, 83%) cancers. None of the differences among these various groups reached a level of statistical significance by Chi-squared analysis.

For 4 of the 38 tumors, we had adequate tissue (based on quantity and cellularity) to measure SSAT enzymatic activity in parallel with the immunohistochemical measurements. In three of these tumors, strong induction of SSAT expression (as measured by immunohistochemical staining) following DENSpm treatment was associated with a significant increase in enzymatic activity (Table 2). In the fourth tumor, a poorly differentiated ductal carcinoma, no induction of SSAT could be detected by immunohistochemistry (Fig. 1E, F) or by measurement of enzymatic activity (Table 2, sample no. 080903). Thus, as observed in our previous studies [7, 19], there appears to be a strong correlation between SSAT protein expression and enzymatic activity.

The recent confirmation of the involvement of SSAT induction and tumor response underscores the significance of the current findings. The results presented here demonstrate that in human breast cancers, the induction of SSAT in response to a clinically tested polyamine analogue is primarily a tumor-specific event. Thus, this approach may have the potential to be used as both a predictive indicator and an intermediate marker of drug response in accessible tumors.

Recently completed phase II clinical trials with DENSpm in breast cancer have demonstrated it to be a well-tolerated drug. However, the objective responses observed in this limited trial were minimal [37]. The possible reasons for the limited success with DENSpm

Table 2 SSAT enzyme activity in treated tissues corresponding to immunohistochemical staining. Treated samples were exposed to 10 μM DENSpm for 22 h as detailed in Methods. SSAT enzyme activity values are the means ± SD of three replicates. The scale used to score immunohistochemical staining is detailed in Materials and methods

Sample number	Treatment	SSAT activity (pmol/mg protein/min)	Protein by immunohistochemistry
050101	Without DENSpm	22 ± 5.9	0 (Fig. 1A)
	With DENSpm	552 ± 4	2+ (Fig. 1B)
092502	Without DENSpm	51.5 ± 29	0
	With DENSpm	887 ± 1	2+
062603	Without DENSpm	5.7 ± 31	0
	With DENSpm	1388 ± 37	3+
080903	Without DENSpm	23 ± 24	0 (Fig. 1E)
	With DENSpm	72.9 ± 4.5	0 (Fig. 1F)

to date may relate to the dosing schedule used in the trials (once daily/5 days) and/or the amount of drug reaching the desired target. With the current data demonstrating that the induction of SSAT in human breast cancers is a common response to DENSpm exposure, it should now be possible to determine if sufficient drug is reaching the target tumor tissue with the current dosing schedule or with modified dosing schedules. Additionally, recent studies have provided a rationale for the use of SSAT-inducing polyamine analogues in combination with some standard chemotherapeutic agents [21, 24]. For example, Maxwell et al. have demonstrated that treatment of MCF-7 breast cancer cells with 5-FU leads to an increase in SSAT steady-state message as determined by array analysis [24]. In addition, Hahm et al. have demonstrated a synergistic effect both in growth inhibition and in polyamine pool depletion when MCF-7 cells are pretreated with 5-FU or other standard antineoplastic agents including 4-hydroxycyclophosphamide, fluorodeoxyuridine, and *cis*-diaminechloroplatinum(II), followed by the SSAT-inducing analogue CPENSpm, a close structural relative to DENSpm [21]. However, it should be noted that none of the standard antineoplastic agents affected polyamine pools when used alone. These results suggest that treatment with 5-FU alone produces an increase in SSAT mRNA without increasing SSAT protein or activity, but pretreating cells with 5-FU before treatment with a polyamine analogue results in greater increases in SSAT activity and polyamine depletion than treatment with polyamine analogue alone. These results are consistent with those from several laboratories that have demonstrated extensive post-transcriptional regulation of SSAT [11–14, 17, 18, 26, 30, 31, 38].

Such findings suggest great potential for the use of polyamine analogues in combination with current antineoplastic agents for cancer treatment. However, to effectively validate the synergy of standard chemotherapeutic agents in combination with polyamine analogues, it will be critical to demonstrate that the synergistic effects resulting from such a combination remain tumor-specific. The methodologies presented here provide a facile means to determine the selectivity of such a combination in actual human tumor tissue.

In summary, the present study validates the use of immunohistochemistry to assess induction of SSAT in breast cancers after in vitro treatment with polyamine analogues, such as DENSpm. Furthermore, our results demonstrate that a high proportion of breast cancers have a significant polyamine catabolic response after treatment with DENSpm. The clinical potential of alternative dosing using DENSpm or combination therapy with other chemotherapeutic agents are exciting alternatives that will benefit from the ability to measure tumor-selective effects as presented here.

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References

1. Bernacki RJ, Bergeron RJ, Porter CW (1992) Antitumor activity of *N*,*N*′-bis(ethyl)spermine homologues against human MALME-3 melanoma xenografts. Cancer Res 52:2424
2. Bernacki RJ, Oberman EJ, Seweryniak KE, Atwood A, Bergeron RJ, Porter CW (1995) Preclinical antitumor efficacy of the polyamine analogue N^1,N^{11}-diethylnorspermine administered by multiple injection or continuous infusion. Clin Cancer Res 1:847
3. Casero RA Jr, Pegg AE (1993) Spermidine/spermine N^1-acetyltransferase—the turning point in polyamine metabolism. FASEB J 7:653
4. Casero RA Jr, Celano P, Ervin SJ, Porter CW, Bergeron RJ, Libby PR (1989) Differential induction of spermidine/spermine N^1-acetyltransferase in human lung cancer cells by the bis(ethyl)polyamine analogues. Cancer Res 49:3829
5. Casero RA Jr, Celano P, Ervin SJ, Wiest L, Pegg AE (1990) High specific induction of spermidine/spermine N^1-acetyltransferase in a human large cell lung carcinoma. Biochem J 270:615
6. Casero RA Jr, Mank AR, Xiao L, Smith J, Bergeron RJ, Celano P (1992) Steady-state messenger RNA and activity correlates with sensitivity to N^1,Nc^{12}-bis(ethyl)spermine in human cell lines representing the major forms of lung cancer. Cancer Res 52:5359
7. Casero RA Jr, Gabrielson EW, Pegg AE (1994) Immunohistochemical staining of human spermidine/spermine N^1-acetyltransferase superinduced in response to treatment with antitumor polyamine analogues. Cancer Res 54:3955
8. Casero RA, Wang Y, Stewart TM, Devereux W, Hacker A, Smith R, Woster PM (2003) The role of polyamine catabolism in anti-tumour drug response. Biochem Soc Trans 31:361
9. Chang BK, Bergeron RJ, Porter CW, Vinson JR, Liang Y, Libby PR (1992) Regulatory and antiproliferative effects of N-alkylated polyamine analogues in human and hamster pancreatic adenocarcinoma cell lines. Cancer Chemother Pharmacol 30:183
10. Chen Y, Kramer DL, Diegelman P, Vujcic S, Porter CW (2001) Apoptotic signaling in polyamine analogue-treated SK-MEL-28 human melanoma cells. Cancer Res 61:6437

11. Coleman CS, Pegg AE (1997) Proteasomal degradation of spermidine/spermine N^1-acetyltransferase requires the carboxyl-terminal glutamic acid residues. J Biol Chem 272:12164
12. Coleman CS, Pegg AE (2001) Polyamine analogues inhibit the ubiquitination of spermidine/spermine N^1-acetyltransferase and prevent its targeting to the proteasome for degradation. Biochem J 358:137
13. Coleman CS, Huang H, Pegg AE (1995) 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 34:13423
14. Coleman CS, Huang H, Pegg AE (1996) Structure and critical residues at the active site of spermidine/spermine-N1-acetyltransferase. Biochem J 316:697
15. Davidson NE, Mank AR, Prestigiacomo LJ, Bergeron RJ, Casero RA Jr (1993) Growth inhibition of hormone-responsive and -resistant human breast cancer cells in culture by N^1,N^{12}-bis(ethyl)spermine. Cancer Res 53:2071
16. Davidson NE, Hahm HA, McCloskey DE, Woster PM, Casero RA Jr (1999) Clinical aspects of cell death in breast cancer: the polyamine pathway as a new target for treatment. Endocr Relat Cancer 6:69
17. Fogel-Petrovic M, Vujcic S, Brown PJ, Haddox MK, Porter CW (1996) Effects of polyamines, polyamine analogs, and inhibitors of protein synthesis on spermidine-spermine N^1-acetyltransferase gene expression. Biochemistry 35:14436
18. Fogel-Petrovic M, Vujcic S, Miller J, Porter CW (1996) Differential post-transcriptional control of ornithine decarboxylase and spermidine-spermine N^1-acetyltransferase by polyamines. FEBS Lett 391:89
19. Gabrielson EW, Pegg AE, Casero RA Jr (1999) The induction of spermidine/spermine N^1-acetyltransferase (SSAT) is a common event in the response of human primary non-small cell lung carcinomas to exposure to the new antitumor polyamine analogue N^1,N^{11}-bis(ethyl)norspermine. Clin Cancer Res 5:1638
20. Ha HC, Woster PM, Yager JD, Casero RA Jr (1997) The role of polyamine catabolism in polyamine analogue-induced programmed cell death. Proc Natl Acad Sci U S A 94:11557
21. Hahm HA, Dunn VR, Butash KA, Deveraux WL, Woster PM, Casero RA Jr, Davidson NE (2001) Combination of standard cytotoxic agents with polyamine analogues in the treatment of breast cancer cell lines. Clin Cancer Res 7:391
22. Hahm HA, Ettinger DS, Bowling K, Hoker B, Chen TL, Zabelina Y, Casero RA Jr (2002) Phase I study of N^1,N^{11}-diethylnorspermine in patients with non-small cell lung cancer. Clin Cancer Res 8:684
23. Marton LJ, Pegg AE (1995) Polyamines as targets for therapeutic intervention. Annu Rev Pharmacol Toxicol 35:55
24. Maxwell PJ, Longley DB, Latif T, Boyer J, Allen W, Lynch M, McDermott U, Harkin DP, Allegra CJ, Johnston PG (2003) Identification of 5-fluorouracil-inducible target genes using cDNA microarray profiling. Cancer Res 63:4602
25. McCloskey DE, Pegg AE (2000) Altered spermidine/spermine N^1-acetyltransferase activity as a mechanism of cellular resistance to bis(ethyl)polyamine analogues. J Biol Chem 275:28708
26. McCloskey DE, Coleman CS, Pegg AE (1999) Properties and regulation of human spermidine/spermine N^1-acetyltransferase stably expressed in Chinese hamster ovary cells. J Biol Chem 274:6175
27. Murray-Stewart T, Applegren NB, Devereux W, Hacker A, Smith R, Wang Y, Casero RA Jr (2003) Spermidine/spermine N^1-acetyltransferase (SSAT) activity in human small-cell lung carcinoma cells following transfection with a genomic SSAT construct. Biochem J 373:629
28. Porter CW, Bergeron RJ (1988) Enzyme regulation as an approach to interference with polyamine biosynthesis—an alternative to enzyme inhibition. Adv Enzyme Regul 27:57
29. Porter CW, Ganis B, Libby PR, Bergeron RJ (1991) Correlations between polyamine analogue-induced increases in spermidine/spermine N^1-acetyltransferase activity, polyamine pool depletion, and growth inhibition in human melanoma cell lines. Cancer Res 51:3715
30. Shappell NW, Miller JT, Bergeron RJ, Porter CW (1992) 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 12:1083
31. Shappell NW, Fogel-Petrovic MF, Porter CW (1993) Regulation of spermidine/spermine N^1-acetyltransferase by intracellular polyamine pools. Evidence for a functional role in polyamine homeostasis. FEBS Lett 321:179
32. Thomas T, Thomas TJ (2001) Polyamines in cell growth and cell death: molecular mechanisms and therapeutic applications. Cell Mol Life Sci 58:244
33. Vujcic S, Halmekyto M, Diegelman P, Gan G, Kramer DL, Janne J, Porter CW (2000) Effects of conditional overexpression of spermidine/spermine N^1-acetyltransferase on polyamine pool dynamics, cell growth, and sensitivity to polyamine analogs. J Biol Chem 275:38319
34. Wallace HM (2003) Polyamines and their role in human disease—an introduction. Biochem Soc Trans 31:354
35. Wallace HM, Caslake R (2001) Polyamines and colon cancer. Eur J Gastroenterol Hepatol 13:1033
36. Wallace HM, Duthie J, Evans DM, Lamond S, Nicoll KM, Heys SD (2000) Alterations in polyamine catabolic enzymes in human breast cancer tissue. Clin Cancer Res 6:3657
37. Wolff A, Armstrong DK, Fetting JH, Carducci MC, Riley CD, Bender JF, Casero RA, Davidson NE (2003) A phase II study of the polyamine analogue N^1,N^{11}-diethylnorspermine (DENSpm) daily of five days every 21 days in patients with previously treated metastatic breast cancer. Clin Cancer Res 9:5922
38. Xiao L, Casero RA Jr (1996) Differential transcription of the human spermidine/spermine N^1-acetyltransferase (SSAT) gene in human lung carcinoma cells. Biochem J 313:691