Journal of Cellular Biochemistry, Supplement 23:87-95 (1995)

Polyamines as Biomarkers of Cervical Intraepithelial Neoplasia

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Abstract Polyamines (putrescine, spermidine and spermine) play critical roles in cell growth and transformation. Ornithine decarboxylase (ODC), a key enzyme in polyamine biosynthesis, is considered a putative protooncogene crucial to the regulation of cell growth and transformation. Cancer patients have elevated levels of polyamines in their physiological fluids compared to normal counterparts. α-Difluoromethylornithine (DFMO), a specific suicide inhibitor of ODC, exhibits antitumor and anti-metastasis activities, and displays effectiveness in many carcinogen-induced animal chemoprevention models. Therefore, we are using DFMO in a chemoprevention trial for cervical intraepithelial neoplasia grade III (CIN III), and evaluating patients for changes in polyamine metabolism as an intermediate marker of DFMO effect. A preliminary study showed that several milligrams of abnormal cervical biopsy tissue contained detectable levels of ODC activity and polyamines. Additionally, the presence of cadaverine suggested bacterial contamination of these tissues. For this reason, normal and abnormal biopsies collected during colposcopy were rinsed prior to frozen storage. In most patients, abnormal tissues showed greater ODC activities and lower spermidine/spermine ratios than normal tissues. Patients are now being treated with de-escalating doses of DFMO (1–0.06 g/m^2/day) for one month. To study the effects of DFMO in patients with CIN III, we are collecting blood and cervical tissue specimens to measure the following parameters: plasma DFMO, ornithine and arginine levels; plasma N^1-acetylspermidine levels; erythrocyte (blood polyamine carrier) free polyamine levels; cervical tissue free polyamine levels; cervical tissue N^1-acetylspermidine levels; and cervical tissue ODC activities. N^1-acetylspermidine will be examined as this compound is known to exist primarily in tumor tissues, not in normal tissues. We therefore established a high-performance liquid chromatography method for N^1-acetylspermidine. We expect to find that polyamines are effective markers in analyzing DFMO effects in this chemoprevention trial, thus functioning as pharmacodynamic parameters as well as biomarkers for transformation.

Key words: Biomarkers, cervical intraepithelial neoplasia, DFMO, pharmacodynamic parameters, polyamines

Polyamines are organic polycations known to play important roles in many biological functions [1,2]. The structures of relevant polyamines are depicted in Table I. Because spermidine is an asymmetrical molecule, there exist two forms of monoacetylspermidines, N^1- and N^8-acetylspermidine. Polyamines are now known to be involved in proliferation, differentiation, neoplastic

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TABLE I. Structures of Polyamines

Polyamine	Chemical Structure
Putrescine	$H_2N(CH_2)_4NH_2$
Cadaverine	$H_2N(CH_2)_5NH_2$
Spermidine	$H_2N(CH_2)_3NH(CH_2)_4NH_2$
N^1-Acetylspermidine	$CH_3CONH(CH_2)_3NH(CH_2)_4NH_2$
N^8-Acetylspermidine	$NH_2(CH_2)_3NH(CH_2)_4NHCOCH_3$
Spermine	$H_2N(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$
N^1-Acetylspermine	$CH_3CONH(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$

transformation, cellular maintenance, apoptosis, as well as angiogenesis, which is essential for tumor cell metastasis [3–6]. Although the mechanisms of action of natural, ubiquitous polyamines are not entirely clear, it is known that polyamines interact with nucleic acids, proteins, membranes, and other intracellular organelles. On interaction with DNA, polyamines can induce conformational changes in DNA structure [7]. As illustrated in Figure 1, polyamine metabolism in mammalian cells starts at arginine through the action of arginase. The resultant ornithine is converted by one of the key enzymes in polyamine metabolism, ornithine decarboxylase (ODC), to putrescine. The enzymatic activity of ODC is uniquely regulated by multiple mechanisms. Putrescine is then converted to spermidine and spermine by *S*-adenosylmethionine decarboxylase (SAMDC) and each respective polyamine synthase (aminopropyltransferase). The required aminopropyl moiety at this step derives originally from methionine. Spermidine/spermine N^1-acetyltransferase (SSAT) can acetylate spermidine and spermine, following which these polyamines can be excreted. In addition, N^1-acetylspermidine and N^1-acetylspermine can be converted to putrescine and spermidine, respectively, by the action of polyamine oxidase. This is called the back-conversion pathway. In this polyamine metabolism scheme, one of the most effective ways to inhibit polyamine biosynthesis is to specifically inhibit ODC. This can be accomplished by α-difluoromethylornithine (DFMO), an analog of ornithine and a specific enzyme-activated irreversible inhibitor of ODC. It covalently binds to ODC. The chemical structure of DFMO is depicted in Figure 2.

Cancer patients are known to have elevated levels of polyamines in their physiological fluids compared to their normal counterparts, indicating changes in polyamine metabolism in cancer patients due to the occurrence of tumors [8]. Expression of ODC is transiently increased upon stimulation by growth factors, but becomes constitutively activated during cellular transformation induced by carcinogens, oncogenic viruses, or oncogenes. Biochemical and molecular aspects of polyamine metabolism specifically associated with tumorigenesis have been extensively studied in recent years. Bachrach *et al.* [9] originally showed that chick embryo fibroblasts transformed by Rous sarcoma virus contained putrescine levels 3–7 times higher than normal chick embryo fibroblasts, although both cell lines propagated at the same rate. ODC induction has been shown to be critical to the expression of the c-*myc* protooncogene in a human colonic carcinoma cell line [10]. NIH/3T3 cells transformed with human c-Ha-*ras* oncogene displayed markedly enhanced ODC activity, showing ODC deregulation at multiple levels [11]. Decreased expression of the protooncogenes, c-*fos*, c-*myc* and c-*jun*, has recently been observed following polyamine depletion with DFMO in IEC-6 (rat intestinal crypt) cells [12]. *myc* has been shown to be a potent transactivator of ODC transcription [13]. These results indicate the basic importance of polyamines in oncogene expression. Furthermore, cell transformation is induced in NIH/3T3 cells transfected with human ODC cDNAs [14,

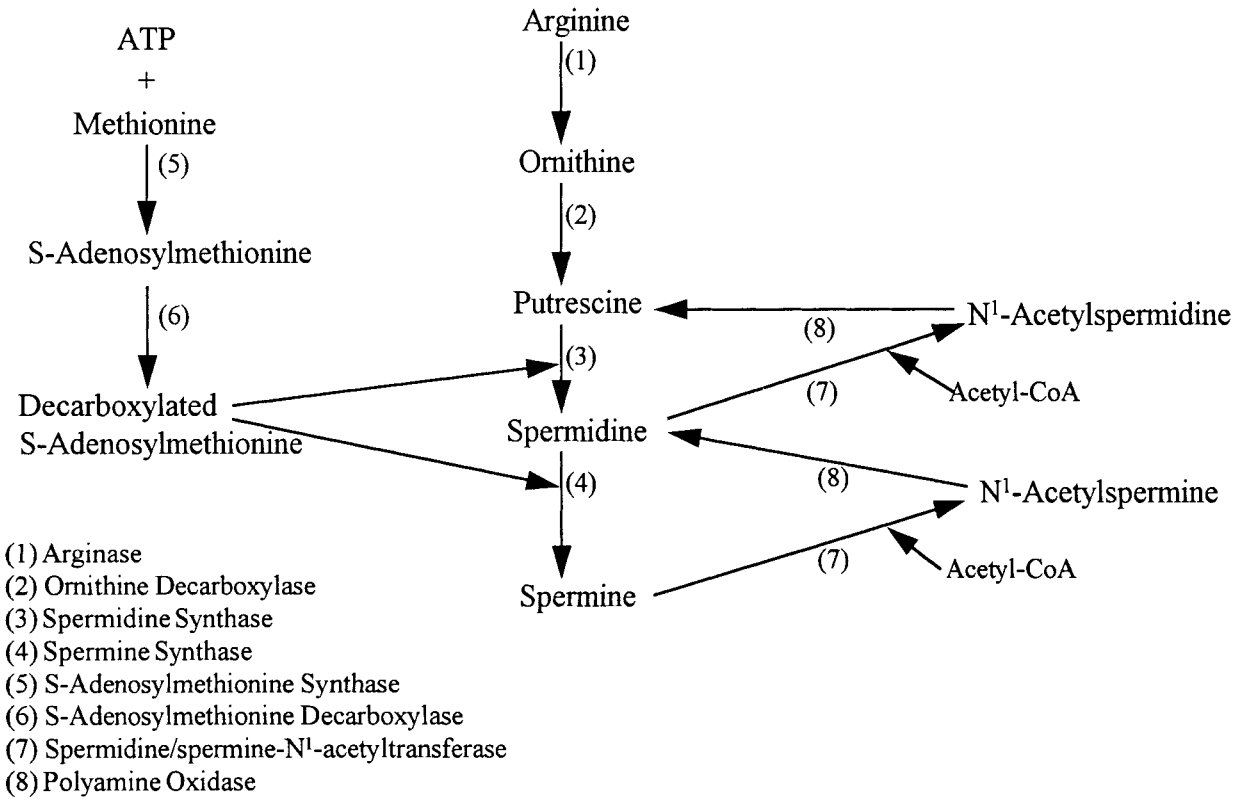

Fig. 1. Polyamine biosynthesis in mammalian cells.

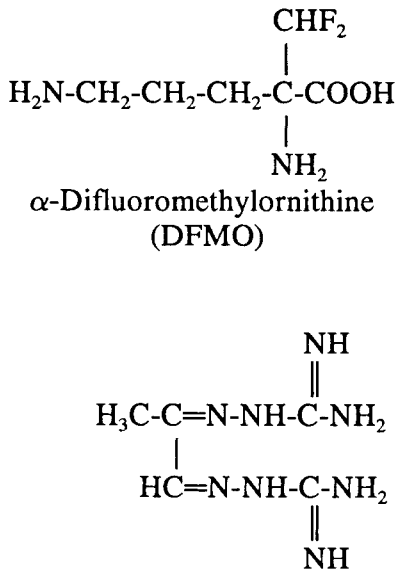

Fig. 2. Chemical structures of DFMO and MGBG.

15]. Auvinen *et al.* [14] further showed that blocking endogenous ODC activity with DFMO prevented transformation of rat fibroblasts by the temperature-sensitive v-*src* oncogene. These results suggest that the gene encoding ODC is a protooncogene central to regulation of cell growth and transformation. At the *in vivo* level, DFMO has been shown to be effective in many animal chemoprevention models [16,17].

Based on these findings, our hypotheses with regard to polyamine-directed chemoprevention are two-fold: to inhibit transformation against field cancerization, and to remove cells already transformed through apoptosis. Since human papillomavirus (HPV) is considered an etiologic agent in cervical oncogenesis [18], DFMO may be capable of inhibiting HPV-induced transformation. Regarding the second hypothesis, in our preliminary study we demonstrated that methylglyoxal *bis*guanylhydrazone (MGBG) (Fig. 2), an inhibitor of SAMDC, is capable of inducing apoptosis in human tumor cells *in vitro* [19]. In view of these data, we report preliminary prog-

ress in a Phase I chemoprevention study of CIN patients using DFMO.

MATERIALS AND METHODS

Patients

Patients with CIN I–III were identified for this study. Informed consent was obtained. Referred Papanicolaou smears were reviewed and found to show CIN. A complete history, including documentation of tobacco consumption, was recorded, and a physical examination was done. A repeat Papanicolaou smear was sent for cytology. Colposcopy was then performed using 3–6% acetic acid. Colposcopically directed biopsies were performed from normal and abnormal areas for polyamine studies. Patient tissues were also cultured for *Chlamydia* and *Neisseria gonorrhoeae*, and HPV typing was carried out using the Virapap/Virotype kit (Digene Diagnostics, Inc., Silver Springs, MD).

Polyamine Studies

Tissues obtained were frozen and kept at –70°C. To prepare samples, a 25% tissue homogenate was prepared in ODC buffer using a Polytron homogenizer (Brinkman Instruments, Westbury, NY) as described previously [20]. A portion of homogenate (20 µl) was mixed with 80 µl of 5% sulfosarysylic acid, sonicated and microcentrifuged (13,000 x g) for 15 min at room temperature to obtain a clear supernatant for polyamine analysis. The remaining portion of homogenate was centrifuged (700 x g for 15 min at 4°C), and the supernatant analyzed for ODC activity [20] and protein levels. Protein concentrations were determined using Bio-Rad (Richmond, CA) protein assay kits.

Since we recognized the need to analyze our samples for polyamine precursor amino acids such as arginine and ornithine, DFMO, and acetylpolyamines in addition to free polyamines, we decided to develop a new procedure using a Dionex BioLC high-performance liquid chromatograph (HPLC) equipped with an HPIC-CS2 column and a postcolumn detection system using *O*-phthalaldehyde (Dionex, Inc., Sunnyvale, CA). Four different elution buffers were prepared and filtered. Each buffer contained 1 ml phenol per liter with the following compositions: buffer A, 1 mM potassium citrate, pH 4.70, adjusted with HCl; buffer B, 0.1 M potassium citrate, pH 4.70, adjusted with HCl; buffer C, 0.2 M KCl, 10 mM KOH, 1.34 mM disodium EDTA, 11.7 mM HBO_3, pH 9.20; and buffer D, 1.8 M KCl, 90 mM KOH, 12.1 mM EDTA, 0.105 M HBO_3, pH 9.20. The column was equilibrated with buffer A starting at 0 min, and various buffers were introduced as described in Table II. A sample was injected at 20 min, and recording commenced at 30 min. An elution profile of all standards is illustrated in Figure 3.

TABLE II. Elution Buffer Schedule

Time	Flow Rate	Buffer Composition			
(min)	(ml/min)	%A	%B	%C	%D
0.0	1.7	100	0	0	0
4.0	1.7	100	0	0	0
4.1	1.7	0	100	0	0
11.0	1.7	0	100	0	0
11.1	1.7	98	2	0	0
16.0	1.7	98	2	0	0
16.1	1.2	98	2	0	0
26.0	1.2	98	2	0	0
26.1	1.2	95	5	0	0
34.0	1.2	95	5	0	0
34.1	1.2	80	20	0	0
39.0	1.2	80	20	0	0
39.1	1.2	0	0	100	0
48.0	1.2	0	0	100	0
48.1	1.2	0	0	88	12
57.8	1.2	0	0	88	12
57.9	1.2	0	0	60	40
70.0	1.2	0	0	0	100

RESULTS

For this preliminary study, we obtained abnormal cervical tissues (colposcopically directed biopsies) from 10 patients with CIN III. The amounts of tissue by weight varied from 1.3 to 11.1 mg. We analyzed these for ODC activity and

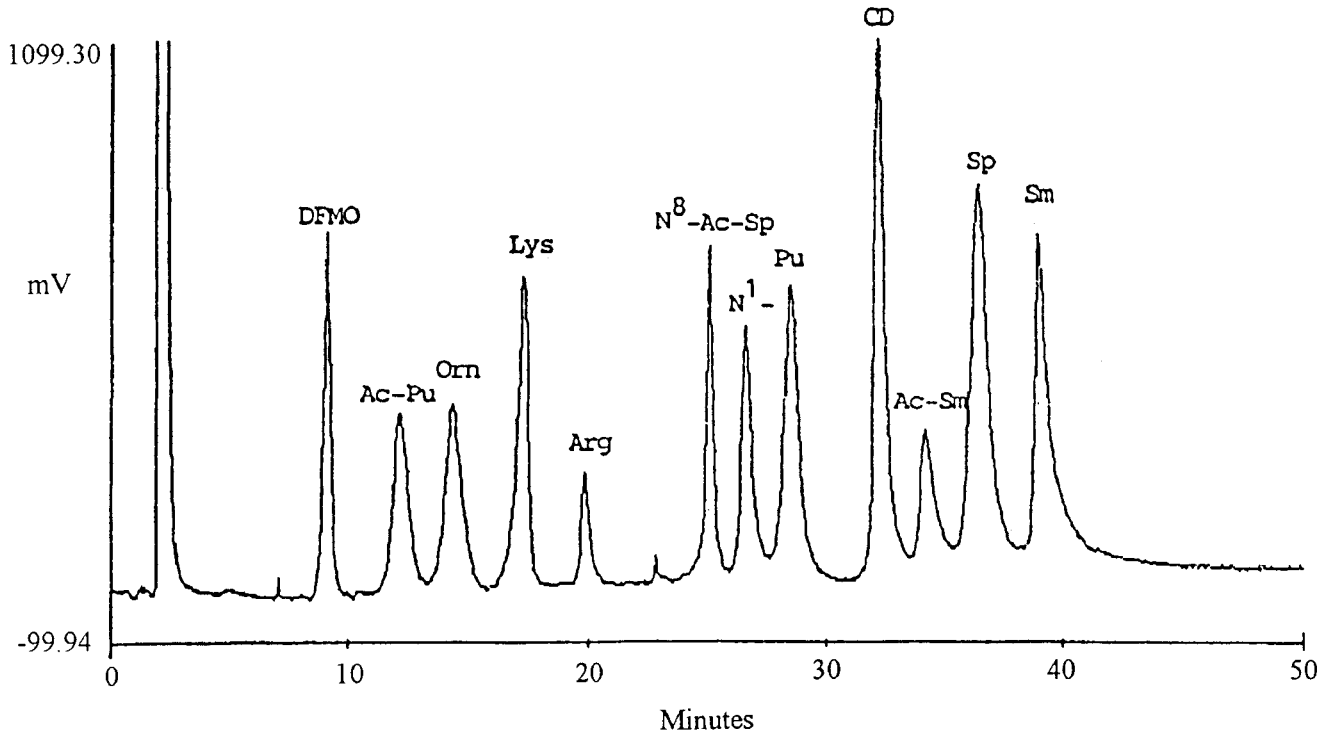

Fig. 3. An elution profile of all standard polyamines and related compounds (200 pmol each). Ac-Pu, acetylputrescine; N^8-Ac-Sp, N^8-acetylspermidine; N^1-, N^1-acetylspermidine; Pu, putrescine; CD, cadaverine; Ac-Sm, acetylspermine; Sp, spermidine; Sm, spermine.

TABLE III. Polyamine Studies of CIN III Patients (Tissue ODC and Polyamines)

Patient	Tissue (mg)	ODC pmol/mg*/hr	Putrescine pmol/mg*	Cadavarine pmol/mg*	Spermidine pmol/mg*	Spermine pmol/mg*	*Chlamydia*	*Neisseria gonorrhoeae*	HPV
A	2.2	83.0	–	–	2,535.4	2,618.2	–	–	+
B	6.0	15.6	56.8	–	685.6	729.6	–	–	+
C	6.1	26.1	1,014.3	147.2	1,187.4	908.5	–	–	+
D	2.5	55.0	523.0	42.6	1,219.2	1,247.0	–	–	+
E	5.4	94.9	882.2	305.3	903.6	510.7	+♦	–	–
F	11.1	49.9	83.1	–	850.0	975.4	–	–	+
G	1.7	240.0	–	–	602.4	624.3	–	–	+
H	5.2	17.4	386.7	–	462.5	592.6	–	–	+
I	1.3	160.6	2,104.6	458.8	2,121.2	2,538.5	–	–	+
J	2.2	90.0	1,301.5	–	1,301.5	1,638.6	–	–	+

* per mg soluble protein; ♦ treated.

TABLE IV. Polyamine Studies of Patients with CIN Tissue ODC and Polyamines: Normal vs Abnormal Tissue

Patient	Grade	Tissue Status	ODC pmol/mg*/hr	Putrescine pmol/mg*	Cadaverine pmol/mg*	Spermidine pmol/mg*	Spermine pmol/mg*	Spermidine / Spermine
1	CIN III	Normal	1,174	–	–	8,466	4,141	2.04
		Abnormal	4,341	–	–	10,584	10,258	1.03
2	CIN III	Normal	4,113	–	–	52,042	54,267	0.96
		Abnormal	1,466	–	–	21,508	23,969	0.90
3	CIN III	Normal	5,253	–	–	54,261	52,043	1.04
		Abnormal	5,694	–	–	29,785	18,658	1.60
4	CIN I	Normal	1,090	–	–	7,647	5,347	1.42
		Abnormal	1,708	–	–	3,377	–	–
5	CIN III	Normal	787	–	–	5,322	2,571	2.07
		Abnormal	474	–	–	4,119	3,658	1.13
6	CIN III	Normal	141	–	–	4,079	2,449	1.67
	CIS	Abnormal	278	8,578	–	12,582	16,382	0.77
7	CIN III	Normal	454	–	–	6,771	5,728	1.18
		Abnormal	168	–	–	9,081	9,257	0.98
8	CIN III	Normal	1,651	–	–	15,222	12,391	1.23
		Abnormal	326	–	–	5,825	6,654	0.88
9	CIN III	Normal	–	–	–	35,484	13,187	2.69
		Abnormal	29	–	–	5,545	2,077	2.67
10	CIN III	Normal	50	–	–	6,092	1,856	3.28
		Abnormal	382	–	–	30,289	75,774	0.40
11	CIN II–III	Normal	144	–	–	6,137	7,794	0.79
		Abnormal	490	–	–	39,107	19,599	2.00
12	CIN II–III	Normal	4	–	–	5,777	4,558	1.27
		Abnormal	39	–	–	3,312	3,920	0.84
13	CIN II–III	Normal	1,032	–	–	55,583	21,548	2.58
		Abnormal	1,396	–	–	42,456	28,598	1.48

* per mg soluble protein

polyamine levels as reported in Table III. It is clear that we can determine the enzymatic activities of ODC and polyamine levels with these amounts of cervical tissue. However, values obtained showed a great deal of variability. The tissues from patients C, D, E, and I showed measurable amounts of cadaverine. Since cadaverine is normally produced from lysine by the action of bacterial lysine decarboxylase, we evaluated several possible reasons for these results and therefore examined the tissues for venereal disease infections. Only patient E had previously had a chlamydial infection. By the time we obtained the sample, she was culture negative for *Chlamydia*. All patients were negative for *Neisseria gonorrhoeae*. As anticipated, all patients were positive for HPV with one exception, patient E. To reduce possible bacterial contamination, we subsequently rinsed tissue samples with saline prior to freezing. In the next group of patients, we obtained both normal and abnormal biopsies and analyzed for ODC activity and polyamine levels (Table IV). In most patients, we observed that abnormal tissues had greater ODC activities than normal tissues. Since Hixon *et al.* [21] showed that the ratio of spermidine to spermine in tissues is the most reliable value, we examined these ratios; in most patients the ratios obtained from normal tissues were greater than those from abnormal tissues.

We have initiated treatment of CIN III patients with DFMO for one month as a Phase I study. We are using a de-escalation schedule as described by Meyskens *et al.* [22] to determine the lowest effective dose. Patients receive an oral DMFO dose of 1.0, 0.5, 0.25, 0.125 or 0.06 g/m^2/day. We then collect blood and cervical tissues before and after each course of treatment. The following studies are performed to examine alterations in polyamine metabolism in patients with CIN III who are treated with DFMO: plasma DFMO, ornithine and arginine levels, plasma N^1-acetylspermidine levels, erythrocyte free polyamine levels, cervical tissue free polyamine levels including cadaverine, cervical tissue N^1-acetylspermidine levels, and cervical tissue ODC activity. The presence of DFMO in the plasma of these patients following treatment indicates compliance with the clinical protocol. Since the half-life of DFMO in plasma is very short (3.5–5.6 hrs) [23–25], blood specimens need to be collected soon after the last dose of DFMO, particularly from patients taking low daily doses.

DISCUSSION

We have learned that polyamines can be detected in the amount of cervical tissue obtained from routinely sized biopsies. Washing biopsies helps eliminate bacterial contamination. This study has thus allowed us to refine our techniques for the Phase I trial. In addition, our new HPLC procedure enables us to determine N^1-acetylspermidine levels. If DFMO is affecting polyamine metabolism in these patients, we expect to observe the following phenomena in post-treatment samples from the Phase I trial: increases of precursor amino acids (ornithine and arginine) in plasma; decreases of free polyamine levels in erythrocytes (carrier of free polyamines in blood) as observed in our previous high-dose DFMO study [26] and in cervical tissues; and decreases in cervical tissue ODC activity.

It is already known that transformed NIH/3T3 cells excrete N^1-acetylspermidine, which is produced by the action of SSAT [27]. N^1-Acetylspermidine is primarily found in human tumor tissues, not in normal tissues [2,28,29]. In view of this, we postulated that N^1-acetylspermidine can be detected in dysplastic (CIN III) tissues. Thus we decided to analyze for N^1-acetylspermidine in plasma and cervical tissues from patients with CIN III.

Currently CIN III patients are being treated with various doses of DFMO for one month. We expect to determine if polyamines and their precursor amino acids are effective markers in analyzing DFMO effects in this chemopreventive trial, thus functioning as pharmacodynamic parameters as well as surrogate endpoint biomarkers for transformation.

ACKNOWLEDGEMENTS

This work was supported in part by NCI contract NO1-CN-25433. We thank Ms. Alma Swisher for her excellent assistance in preparing this manuscript.

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