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Assay of deoxy-deazapurines in DNA by 3′-phosphorylation and two-dimensional thin-layer chromatography

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ABSTRACT

Deoxy-deazapurines (deaza-dNMPs) are incorporated into cellular DNA after administration of anti-neoplastic, anti-viral, or anti-parasitic chemotherapy. Deaza-dNMPs are stable purine analogues and can be detected via ^{32}P-labeling cold DNA. Assay of analogue incorporation and normal base composition is carried out by radiolabeling DNA with all four deoxynucleotides (dNMPs) through nick translation. 3′-Monophosphate digest radiolabels representative dNMPs and deaza-dNMPs. Separation occurs in two-dimensional polyethyleneimine–cellulose thin-layer chromatography, which resolves all dNMPs. The technique was applied to human placental and calf thymus DNA, control and altered calf thymus DNA with cold stoichiometric replacement of deaza-dNMPs to include deoxy-deazaadenosine, deoxy-deazaguanosine, and deoxy-deazainosine. Scintillation detection and densitometry both accurately reflect dNMP content. This technique easily and quickly quantifies the low-molecular-mass deaza-dNMP analogues in DNA. Deaza-dNMP uptake into DNA may reflect clinical chemotherapeutic efficacy and host toxicity. The assay may therefore serve as an early biochemical dosimeter of drug effect and resistance.

INTRODUCTION

The cell maintains a number of strategies to exclude the incorporation of nucleic acid adducts and analogues into genomic DNA. Regardless, through a number of natural and unnatural (iatrogenic) chemical and biochemical circumstances, the protective enzymatic pathways are overwhelmed. Subsequently, normal DNA synthetic enzyme systems insert deoxy-deazapurines (deaza-dNMPs) in place of their corresponding deoxypurines.

We have developed a reproducible two-dimensional thin-layer chromatographic (2D-TLC) technique that identifies and separates deaza-dNMPs. The applications of this technique to detect deaza-dNMPs are many, but the most easily appreciated and focused example is its utility in cancer chemotherapy. Empirical techniques are now employed to decide how much chemotherapy a patient receives, *e.g.*, body weight and surface area. This negates both the sensitivity of the tumor to the drug or the resistance of the patient to complications. Also, patients vary in their ability to tolerate chemotherapies. This could result in under- or overdosing patients. Further, chemotherapies are expensive. This assay allows for a tailor-made regimen to be employed by gauging both host up-take or removal of adducts from a

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chemotherapy regimen by DNA repair. The quantification of deaza-dNMPs in DNA — and its ultimate toxicity — may be carried out by assaying lymphocytic DNA, sperm DNA, or by a small tissue biopsy (the assay requires nanograms of DNA, available from milligrams of tissue). Further, tumor resistance or sensitivity to a drug regimen can also be measured, based on drug uptake into DNA or the drugs' ability to measurably damage DNA. Lastly, most anti-viral or anti-parasitic regimens employ a similar chemotherapeutic strategy and can be assayed with this technique.

Deazapurines are a disparate group of analogues, of which this paper will not address. The 7-deaza compounds differ from the 3-deaza compounds, which amongst themselves represent different classes of drugs. The deaza-dNMPs have a number of novel functions. (1) They can serve as enzyme inhibitors and can further diminish DNA/RNA methylation [1]. (2) They are potent anti-neoplastic, anti-viral [2], and anti-parasitic agents, and cell viability is inversely related to deaza-dNMP incorporation into DNA [3,4]. (3) The deazapurines interfere with purine metabolism alter c-AMP functions including cardiac physiology (coronary vasodilatation, bradycardia, and left ventricular contractility) [5], and can inhibit intercellular adhesion molecule 1 (ICAM-1) [6]. (4) They replace their normal bases in DNA, diminish Hoogsteen base pairing, and enhance sequencing of GC rich regions and genes [7]. (5) deaza-G (queuine) and deaza-A (deazaneplanocin; tubercidin), which are incorporated into DNA/RNA, inhibit growth and cause single-strand breaks [8–10]. (6) deaza-A alters *c-myc* (up) and *c-fos* (down) expression [11]. (7) They are repair resistant [12] and interfere with restriction enzyme cutting [13]. (8) deaza-dG has a prolonged half-life, can enter the blood–brain barrier, and Phase I studies (as the mesylate) [14,15] have begun in the USA [11]. (9) In combination chemotherapy, deaza-purines may diminish *in vivo* anti-cancer drug resistance [14].

The present 2D-TLC technique which enhances the ability to detect deaza-dNMPs is based on a technique that has been presented in detail [16]. The technique labels representative fractions of all deoxynucleotides in DNA, *in situ*, affording adduct and analogue detection. The TLC separation retains normal deoxynucleotides retention values — which allows rapid visual assessment by characteristic R_F values — of deaza-dNMPs content in DNA. This will subsequently allow a better opportunity to characterize the quality of experimental DNAs as substrate, quantify endogenous dNMPs, adducts and analogues in DNA, and better monitor drug effect, resistance, and host toxicity.

EXPERIMENTAL

Materials

Nucleic acids, enzymes, chromatographic solvents and plates, reagents, kits, and equipment were purchased and used from sources indicated in previous publications [16–29]. All deaza-dNMPs replaced nitrogen (N-7) for carbon at position 7 (C-7).

Methods

Substrate DNA, ^{32}P-labeling and nick translation methods for TLC analysis of formed DNA adducts and analogues were as delineated in prior publications and explained in detail [16–29].

For partial replacement of dNMPs by deaza-dNMPs 2 μl of unlabeled deaza-dNMPs as the cold triphosphate ($1.8 \cdot 10^{-9}$ mol/μl) were added to standard nick translation of calf thymus DNA.

DNA includes freshly extracted human placental DNA and purchased calf-thymus DNA.

Ambis computer-assisted TLC scintillation counting and Beckmann laser densitometry computer-assisted analysis of the TLC XAR-5 autoradiograms were carried out directly as indicated in a prior publication [16]. All R_F values are presented as the consequent two-dimensional R_X values {X,Y coordinates} and easily converted to approximate R_F values by the formula $(R_X - 1)/19$.

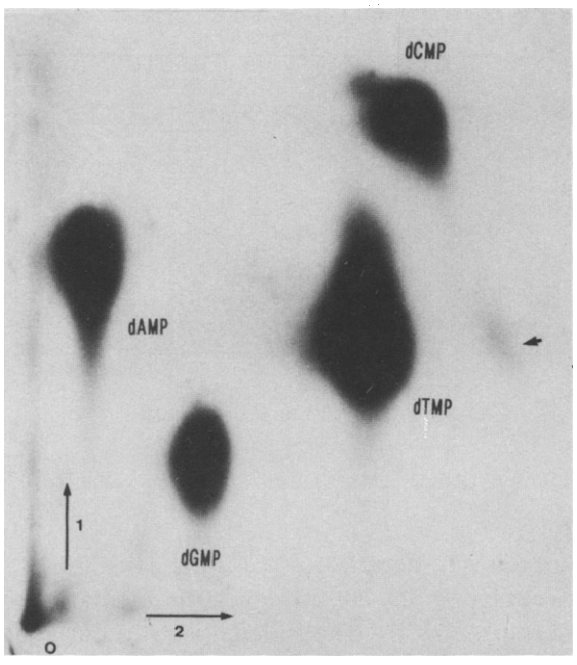


Fig. 1. Control calf thymus DNA (24-h autoradiogram). Autoradiogram demonstrates, in clockwise position, the major bases of DNA as their nucleotides: adenine (R_X = 1.4,9.4), thymine (R_X = 9.0,7.4), guanine (R_X = 4.6,4.4), and cytosine (R_X = 10.0,13.5).

RESULTS

The results of the following TLCs are presented and discussed to include: (1) freshly prepared calf thymus and human placental DNA; (2) cold stoichiometric replacement of deaza-dAMP for dAMP; (3) replacement of deaza-dGMP for dGMP; (4) replacement of deaza-dIMP for dGMP; (5) densitometry *versus* scintillation counting.

Control calf thymus or human placental DNA

Similar reproducible findings from earlier publications [16] for calf thymus DNA digest include (Fig. 1) normal retention times of all radiolabeled monophosphates to cold UV markers. Autoradiogram demonstrates, in clockwise position, the major bases of DNA as their deoxyribonucleotides (Table I) adenine [retention factor is given as a bi-coordinate location (R_X) X,Y (in cm) = 1.4,9.4], thymine (R_X = 9.0,7.4), guanine (R_X = 4.6,4.4), and cytosine (R_X = 10.0,13.5). Our percentages of dNMP label of calf thymus DNA are listed in tabular and graphic forms (Table I and

TABLE I
DENSITOMETRY

Nucleotide	Control DNA	7-Deaza-dATP	7-Deaza-dGTP	7-Deaza-dITP
R_X values				
dAMP	1.4/9.4	1.5/9.5	1.5/10.0	1.5/10.0
dCMP	10.0/13.5	10.6/13.9	10.3/14.2	9.4/13.9
dGMP	4.6/4.4	4.4/3.8	4.6/4.0	4.5/4.1
dTMP	9.0/7.4	8.7/7.5	8.7/8.3	8.6/7.8
7-Deaza-dAMP	–	1.6/14.8	–	–
7-Deaza-dGMP	–	–	2.6/3.3	–
7-Deaza-dIMP	–	3.7/5.8	–	3.2/6.9
Area (mm^2)				
dAMP	485	138	406	382
dCMP	421	260	297	250
dGMP	371	202	–	–
dTMP	968	644	690	574
7-Deaza-dAMP	–	403	–	–
7-Deaza-dGMP	–	–	170	–
7-Deaza-dIMP	–	267	–	401
Percent volume ($AUmm^2$)				
dAMP	31.44	3.16	31.99	27.41
dCMP	8.49	10.67	5.00	5.86
dGMP	14.04	4.93	–	–
dTMP	46.02	45.82	48.22	47.16
7-Deaza-dAMP	–	20.43	–	–
7-Deaza-dGMP	–	–	14.73	–
7-Deaza-dIMP	–	14.91	–	19.58

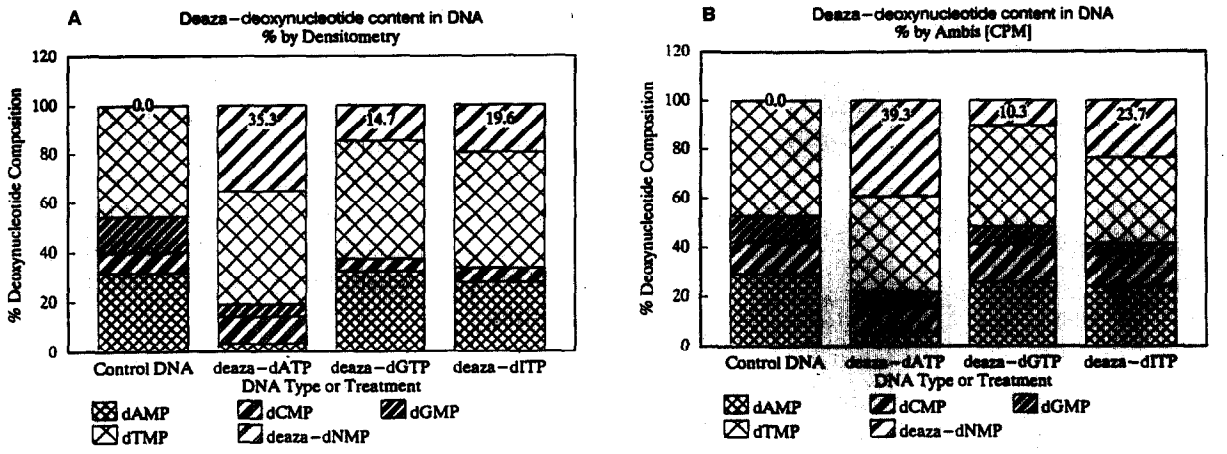


Fig. 2. Tables I and II presented as histograms of DNA dNMPs and adduction. Densitometry (A) *versus* scintillation counts (B).

Fig. 2A). Deoxyuridylate exists after 24-h autoradiography which may represent an artefactually chemically altered product of dCMP, or is likely real (Table II and Fig. 2B). Further, some variation in CPM exists due to differences in quenching at lower counts. Lastly, densitometry integrates area, and these numbers appear in Table I and Fig. 2A.

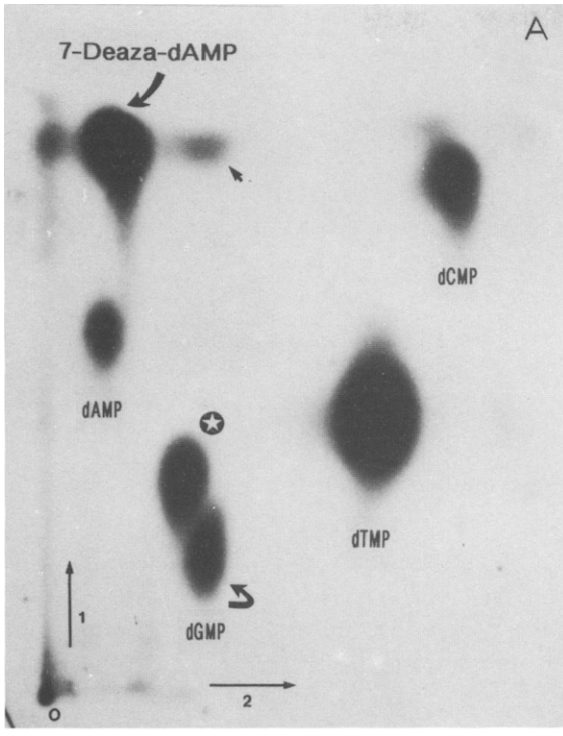
Cold stoichiometric replacement of deaza-dAMP for dAMP (Fig. 3A and B as two-dimensional autoradiogram and three-dimensional Ambis, respectively; 24-h autoradiogram)

We have easily been able to detect deaza-dAMP on our TLCs. Deaza-dAMP is easily incorporated into DNA and rapidly migrates to the front (densitometry R_X = 1.6,14.8; densitometry percentage = 20.4% at highest millimolar substitution). Figs. 3A and B demonstrates our ability to easily detect these abnormal cold nucleotides from normal. The addition of deaza-dAMP after incorporation into DNA significantly competes with dAMP labeling. Further, after the addition of deaza-dAMP, two "mouse ears" appear with characteristic R_X values that may represent methylated deaza-dAMP species. Lastly, either contamination or natural deamination of deaza-dAMP results in the analogue or adduct (black bordered five-pointed star; R_X = 3.2,6.9) contiguous to dGMP and represents deaza-dIMP. Deaza-dAMP may readily deaminate more quickly than dAMP.

TABLE II

SCINTILLATION COUNTING (AMBIS)

Nucleotide	Control DNA (%)	7-Deaza-dATP (%)	7-Deaza-dGTP (%)	7-Deaza-dITP (%)
dAMP	25701/29.2	3855/5.7	8906/25.6	15854/24.1
dCMP	11254/12.8	6392/9.4	5333/15.3	8598/13.1
dGMP	9303/10.6	4423/6.5	2476/7.1	2630/4.0
dTMP	41621/47.4	26605/39.1	14495/41.7	23080/35.1
7-Deaza-dAMP	–	21073/31.0	–	–
7-Deaza-dGMP	–	–	3565/10.3	–
7-Deaza-dIMP	–	5672/8.3	–	15566/23.7



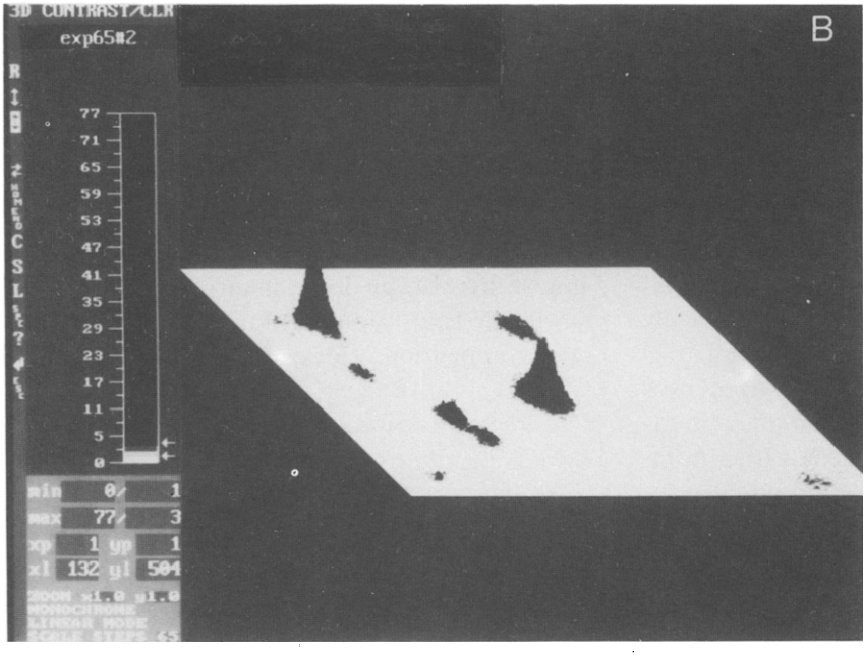


Fig. 3. (A) Cold stoichiometric replacement of deaza-dAMP for dAMP. Deaza-dAMP is easily incorporated into DNA and rapidly migrates to the front (densitometry R_X = 1.6,14.8; densitometry percentage = 20.4% at highest millimolar substitution). Either contamination or natural deamination of deaza-dAMP results in the analogue (black bordered five pointed star; R_X = 3.2,6.9) contiguous to dGMP and represents deaza-dIMP. (B) Cold stoichiometric replacement of deaza-dAMP for dAMP. The figure demonstrates three-dimensional representation of Ambis β-scintillation counts for deaza-dAMP, deaza-dIMP and control dNMPs.

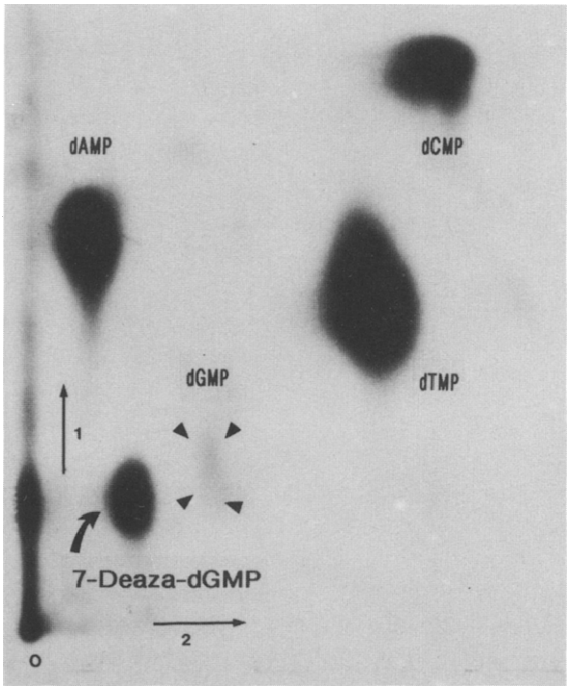


Fig. 4. Cold stoichiometric replacement of deaza-dGMP for dGMP. Deaza-dGMP has a unique autoradiographic chromatographic pattern (densitometry R_X = 2.6,3.3). Spots along the "Y"-axis likely represent inorganic monophosphate moieties (typically low R_X values) or nucleic acid monophosphates (higher R_X values) with molecular weights in excess of 800.

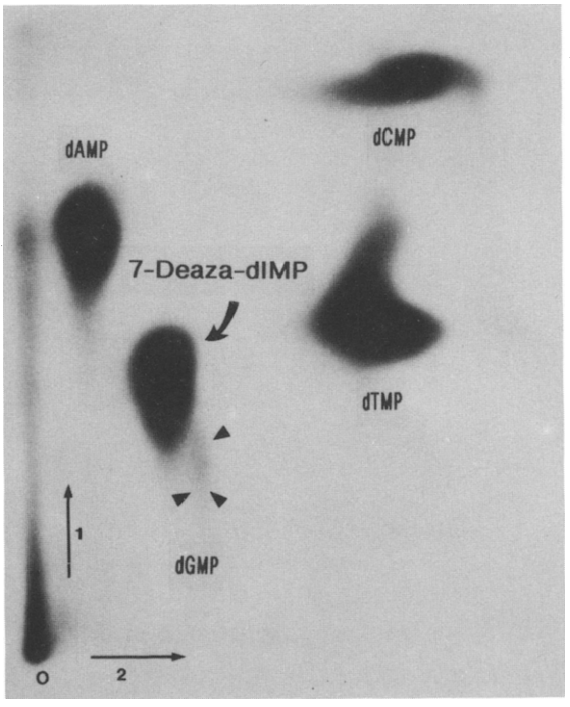


Fig. 5. Cold stoichiometric replacement of deaza-dIMP for dGMP. R_X measurements of deaza-dIMP are 3.2,6.9. Ambis further corroborates competition of deaza-dIMP for dAMP.

Cold stoichiometric replacement of deaza-dGMP for dGMP (Fig. 4)

Enzymatic insertion of deaza-dGMP is efficient (14.73% in AUmm^2) almost completely obliterating dGMP's autoradiographic spot at 24 h. Ambis detects significant counts of dGMP (2476 dpm). Deaza-dGMP has a unique autoradiographic chromatographic pattern (densitometry R_X = 2.6,3.3). Few other confounding autoradiographic densities are evident. Spots along the "Y"-axis likely represent inorganic monophosphate moieties (typically low R_X values) or nucleic acid monophosphates (higher R_X values) with molecular masses in excess of 600–800 Da.

Cold stoichiometric replacement of deaza-dIMP for dGMP (Fig. 5)

Deaza-dIMP successfully competes for and almost completely replaces dGMP. Densitometric replacement of dGMP by deaza-dIMP is 19.58% further representing some competition for dAMP — now reduced approximately 4% (almost 10% overall) from expected. R_X measurements of deaza-dIMP are 3.2,6.9. Ambis both detects dGMP - almost invisible by densitometry — with 4% detectability (2630 dpm). Ambis further corroborates competition of deaza-dIMP for dAMP.

Densitometry versus scintillation counting (Tables I and II)

Ambis correlated closely with densitometry, but exceeded densitometry by 11% overall when viewed by dNMP across DNA samples (inter-DNA variability). Variability in analysis of DNAs (all dNMPs in one DNA *versus* another DNA) was much less and densitometry here exceeded Ambis by only 4%. Correlation coefficients still tend to be extremely small, yet very significant ($p < 0.001$). Linear regression coefficients predict interchangeable results from densitometry to Ambis.

Other phosphates within the field may represent normal minor base nucleotides present in DNA, *e.g.*, normally methylated dNMPs, or adducts, by-products of oxygen stress. Autoradiography not only demonstrates four primary spots, but is accompanied by a specific "fingerprint" for each replaced deaza-dNMP.

DISCUSSION

Deoxynucleotide analogues are typically introduced into DNA by the use of anti-metabolite chemotherapies, *e.g.*, halopyrimidines and deoxy-deazapurines. The present 2D-TLC technique can successfully quantify many dNMP adducts — any altered base in DNA — that occur through environmental, dietary, oxygen stress, aging processes. This TLC technique assesses base composition and adduct formation. The procedure requires labeling extracted cold DNA (so no radioactivity is injected into the patient) from a patient by "shot-gun" radioactive 5'-phosphorylation of representative ^{32}P-α-dNMPs (deoxynucleotides). Subsequent 3'-monophosphate digests the DNA and "sister exchanges" (transfers) a radioactive $^{32}PO_4^{2-}$ to the neighboring cold nucleotide, now radioactively labelling the cold DNA deoxynucleotides from the patient. Separation is easily and reproducibly carried out with 2D-TLC. Our experience with this technique exceeds 2000 chromatograms with outstanding and statistically significant reproducibility, *e.g.*, R_F variation under 5%.

The clinical utilization of this technique is as a measure of tumor drug resistance or extreme host sensitivity to deaza-dNMPs. The assays would occur after an initial course of chemotherapy, followed by tumor biopsy to measure incorporation of deaza-dNMPs into its DNA. High uptake of deaza-dNMPs into DNA would be predictive of tumor sensitivity to the chemotherapy. Conversely, low DNA up-take of deaza-dNMPs in the tumor/host microenvironment would imply drug resistance (apparent: microenvironment prevents uptake; or intrinsic: true drug resistance by, *e.g.*, gene amplification) many months before clinical or radiographic indicators demonstrate drug failure.

Normal tissue, in particular lymphocytic DNA, would measure acceptable host sensitivity. A high uptake of deaza-dNMPs into normal tissues would predict likely complications. Low uptake of deaza-dNMPs into lymphocytic DNA may indicate the possibility of tolerating higher doses of chemotherapy, regardless of the patient's weight, skin surface area, or body cell mass considerations. This would better assure tumor cell kill, and patient salvage. This schema would represent a more focused, "tailor-made" chemotherapy regimen designed for the individual patient.

Deaza-dNMPs effects on DNA can occur at the base level, within a sequence, in three-dimensional conformation, and in DNA's interaction with regulatory proteins. The possibility exists with the present technique to demonstrate unequivocal incorporation of deaza-dNMPs in DNA, which have only been carried out previously with radiolabeled deazanucleic acids or deaza-dNMPs [30]. Deaza-nucleoside analogues, in many ways, are treated similar to normal nucleosides. Deazanucleosides are converted to their triphosphates by mouse leukocytes [31]. DNA containing 7-deaza-dG,AMP is resistant to restriction enzyme cutting, in a similar way that methylated DNA resists restriction digestion [32,33].

Yet, differences in analogues chemistry from normal nucleosides account for their toxicity. Deaza-dNMPs may block salvage pathways, and may be employed to enhance combination chemotherapy [34]. Based on the deaza-dNMP's metabolic inhibition, tubercidin is a potent anti-neoplastic and anti-parasitic [35] agent isolated from culture filtrates of microorganisms [36]. These studies can also demonstrate cell-specific drug resistance. Surprisingly, these altered nucleotides are also naturally synthesized since analogues of 7-deaza-GMP (c7GMP) are present in tRNA of prokaryotes and eukaryotes (queuosine) [37]. Yet, deaza-dGMP does increase single-strandedness as demonstrated by S_1 nuclease activity.

Deaza-dNMPs are commonly employed in the laboratory to diminish sequencing gel compres-

sions that occur through Hoogsteen base pairing in GC-rich genes. Further, these transmethylation inhibitors diminish Hoogsteen bonding and ultimate GC compression making GC-rich gene sequencing possible. Indeed, human N-*myc* has a very high GC content (85%), as does *Herpes simplex* virus type I, and bovine basic fibroblast growth factor, and cannot be sequenced without the addition of 7-deaza-dGMP [7]. Possible unanticipated interconversion of deazapurines for alternative dNMPs other than intended may lead to sequencing or restriction fragment errors. As demonstrated in our results with deaza-dAMP, deaza-dIMP is also present in DNA. Their estimated replacement of normal dNMP sequences is important to measure, since this may lead to sequencing errors. These unique analogues may have importance in protecting and preserving sequences from restriction enzymes in experimental protocols. The present technique is therefore adjunctive in quantifying effects by calculating replacement of dNMPs with deaza-dNMPs.

The benefits of this TLC technique have been outlined previously. In brief, they are [16]: (1) the improved ability to radiolabel nucleotide adducts *versus* standard post-labeling techniques; (2) high-resolution on TLC: one employs a new TLC plate with each TLC; (3) ease of technique with "off-the-shelf" materials; (4) excellent quantification of low-molecular-mass adducts.

The TLC separation between the deaza-dNMPs and standard dNMPs contrasts the mobilities of C-7 *versus* N-7. Weakly C-7-adsorbing moieties migrate farther. The TLC cellulose binds N-7, better than C-7, since saturated hydrocarbons are poorly adsorbed. Adsorption is highest for –COOH> –OH> $-NH_2$ > C–O> O–alkyl> $-CH_3$ for the lowest [38]. Also, tautomeric N-7 shifts by a transient rearrangement of bonding may shift usual configuration [39]. These chemical rearrangements further enhance and contrast separations that effect C-7 *versus* N-7.

CONCLUSION

Our conclusions from these TLC studies are that we can determine deaza-dNMP composition in genomic DNA or in kilobase size fragments or motifs. The technique is able to measure DNA chemotherapy analogue accumulation in tissues. It offers a rapid test of tumor cell drug resistance and host sensitivity, and may affect chemotherapeutic regimen. Also, the assay serves as a mode to quickly analyze incorporated deaza-dNMP content in DNA fragments prior to sequencing. The possibility of the application of this assay as a marker of chemotherapeutic and metabolic consequences of disease or chemical injury to tissue samples and biopsy specimens is feasible.

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