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# Determination of genomic N3-methylthymidine in human cancer cells treated with nitrosamines using capillary electrophoresis with laser-induced fluorescence

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**ABSTRACT (200 words)**

Methylating substances alter DNA by forming N3-methylthymidine (N3mT), a mutagenic base modification. To develop a sensitive analytical method for the detection of N3mT in DNA based on capillary electrophoresis with laser-induced fluorescence detection (CE-LIF), we synthesized the N3mT-3'-phosphate as a chemical standard. The limit of detection was 1.9 amol of N3mT, which corresponds to one molecule of N3mT per 1,000 normal nucleotides or 0.1%. With this method, we demonstrated that the carcinogenic nitrosamine N'-nitrosonornicotine (NNN) induced N3mT in the human lung cancer cell line A549. Treatment with NNN also caused an elevated degree of 5-hydroxymethylcytidine (5hmdC) in DNA, while the methylation degree (i.e. 5-methylcytidine; 5mdC) stayed constant. According to our data, NNN could, via yet unknown mechanisms, play a role in the formation of N3mT as well as 5hmdC. In this study we have developed a new sensitive analytical method using CE-LIF for the simultaneous detection of the three DNA modifications, 5mdC, 5hmdC and N3mT.

**Abbreviations**:

5-hydroxymethylcytosin (5hmC); 5-hydroxymethylcytidine or 5-hydroxymethyl-2'-deoxycytidine (5hmdC); 5-methylcytosin (5mC); 5-methylcytidine or 5-methyl-2'-deoxycytidine (5mdC); the fluorescent dye 4,4-difluoro-5,7-dimethyl-4-bora-3a,4adiaza-s-indacene-3-propionyl ethylene diamine hydrochloride (BODIPY FL EDA); capillary electrophoresis with laser-induced fluorescence

detection (CE-LIF); limit of detection (LOD); N3-methylthymidine (N3mT); N'-nitrosonornicotine (NNN)

**Key words**: capillary electrophoresis with laser-induced fluorescence, DNA methylation, N3-methylthymidine, nitrosamines

The covalent binding of reactive agents to DNA often results in so-called DNA adducts, that can lead to mutations and initiate carcinogenesis. N3-methylthymidine (N3mT) is formed by methylating agents, such as methyl methanesulfonate (MMS) [1], while O-methylation of thymine has been found in rats treated with dimethylnitrosamine [2]. N'-nitrosonornicotine (NNN) is another carcinogenic nitrosamine that is found in tobacco smoke [3]. NNN forms covalent DNA adducts after metabolic activation [4].

N3mT in DNA has until now been detected *in vitro* [5–8] as well as *in vivo* [1, 2]. Mechanisms for the repair of N3mT are known for double-stranded DNA [9, 10] and single-stranded DNA [11, 12]. N3mT has been demonstrated to be mutagenic in repair-deficient cells, causing mainly T $\rightarrow$ A (47%) and T $\rightarrow$ C (9%) mutations [1]. However, the biological effects of N3mT-induced mutations have not yet been studied, and thus the relevance of this base modification for human disease is still unclear.

To date, only one method exists for the detection of N3mT in DNA, where the alkylating agent is marked radioactively [13]. We synthetized therefore the N3mT-3'-phosphate as a chemical standard in order to develop a sensitive non-radioactive method for the detection of N3mT in DNA using capillary electrophoresis with laser-induced fluorescence detection (CE-LIF). CE-LIF, a method which combines the separation power of capillary electrophoresis with the extremely high sensitivity of laser-induced fluorescence detection, has been successfully applied to the measurement of

several modified DNA nucleotides, e.g. 5-methylcytidine (5mdC) [14, 15] and 5-hydroxymethylcytidine (5hmdC) in human DNA [16].

Detection and identification of DNA modifications was achieved by digesting 100 ng of DNA with a mixture of micrococcal nuclease (MN; Sigma) and spleen phosphodiesterase (SPD; Callbiochem) to yield the 2'-deoxyribonucleoside-3'-monophosphates (dNps). The monophosphates were then labeled with the fluorescent dye 4,4-difluoro-5,7-dimethyl-4-bora-3a,4adiaza-s-indacene-3-propionyl ethylene diamine hydrochloride (BODIPY FL EDA), which binds covalently by its amino linker to the phosphate group of a deoxyribonucleotide once the latter has been activated with a carbodiimide reagent [17]. After removing the excess of carbodiimide and BODIPY FL EDA by precipitation, the diluted reaction mixture was directly subjected to CE-LIF analysis, thus detecting several DNA modifications, namely 5mdC, 5hmdC and N3mT in the same DNA sample in the same analytical run.

N3-methylthymidine-3'-phosphat (N3mTp) was synthesized according to *Figure 1*. Starting reagent was dimethoxytrityl (DMT)-protected thymidine-3'-phosphoramidite **1**. The exchange of the amide group by two cyanoethyl ester groups to the cyanoethylated product was performed according to Dahl et al. [18]. The cyanoethylated product was immediately, without any further purification step, reacted with cumene hydroperoxide to **2**, purified with silica gur chromatography (82% yield) and identified by mass spectrometry and $^{1}$H-NMR spectroscopy. Methylation at the nitrogen atom N3 of the pyrimidine ring with methyl iodide resulted in product **3**, according to Adams et al. [19]. The product was purified on silica gur (29% yield) and identified by mass spectrometry and $^{1}$H-NMR spectroscopy. The cleavage of the DMT group was achieved not classically, using ammonium- or methylamine hydroxide [20] or acetic acid [21], but rather with

$ZnBr_2$ and nitromethane, due to easier purification of the reaction mixture. Product **4** was isolated in 84% yield, identified, and reacted with concentrated ammonia in order to cleave the cyanoethyl protection group [22]. The final product **5** was purified using semi-preparative HPLC (77% yield) and identified by mass spectrometry and $^{1}$H-NMR spectroscopy as N3mTp **5**.

Detection and identification of N3mTp by CE-LIF was achieved by fluorescence labeling of 100 ng of synthesized N3mTp with a large excess of BODIPY FL EDA, as described earlier [16]. The resulting electropherogram (*Figure 2A*) showed base-line separation of a new signal tentatively identified as the BODIPY conjugate of N3mTp eluting at 33 min. To observe effects on enzymatic digestion, N3mTp was spiked into calf thymus DNA at different concentrations and digested by a mixture of micrococcal nuclease (MN) and spleen phosphodiesterase (SPD). *Figure 2B* shows an electropherogram of 100 ng calf thymus DNA spiked with N3mTp in a ratio of 50:1, prior to MN/SPD digestion and fluorescence labelling.

However, the obtained peak areas of the dNPs conjugates do not reflect correctly the composition of the nucleotides in the DNA sample, and therefore correction factors are needed. These correction factors take into account the differential extent of enzymatic hydrolysis, the variable chemical reactivities of each deoxynucleoside-3'phosphate towards the fluorescence marker, the different fluorescence quantum yields of the dNp-BODIPY conjugates and differential recoveries after sample work-up. Correction factors for the individual nucleotides are calculated by comparing the experimentally obtained composition of a synthetic oligonucleotide to the theoretical composition. For other DNA modifications such as 5mdC and 5hmdC [16], a synthetic DNA fragment was digested, and peak areas of the dNp-BODIPY conjugates were time-corrected and results were expressed as the percentage of the total peak area. The mean values obtained were then calibrated against the

proportion of nucleotides present in a synthetic DNA fragment to encompass nucleotide specific correction factors. For an even more precise establishment of correction factors regarding the hydrolysis and derivatisation step, longer DNA molecules of known base composition could be used. Wirtz et al. [14] have obtained correction factors for 5mdC by methylating λ-DNA *in vitro* using the enzyme M.HpaII methylase that methylates a total of 656 cytosines on defined positions. In this way, it is possible to generate a complex double stranded DNA sequence with a defined amount of 5mdC that can be used for establishing the correction factors in a more realistic way compared to the oligonucleotides. In our case, no oligonucleotide was available as we have synthesized the N3mT monophosphate, thus no correction factor could be implemented. However, we took into account that the signal of N3mT-BODIPY-conjugate was 25% lower than the signal of unmodified thymidine BODIPY-conjugate. The less efficient derivatisation of N3mT-dNp is possibly explained by a reduced reactivity of the methylated thymidine compared to the unmodified nucleotide. The authors understand that exact quantification of N3mT in DNA might be difficult to obtain with the presented method. Therefore, the method should be rather used as a semi-quantitative measurement for comparison of different samples, i.e. the amount of N3mT in DNA of untreated versus treated cells.

The limit of detection (LOD) for N3mT in DNA was determined by diluting the synthesized N3mTp standard with calf thymus DNA in the nanomolar range. CE-LIF analyses of samples (100 ng DNA) prepared in this way revealed a linear relationship between the fluorescent signal and the N3mT content over a broad range of concentrations (from 385 nM to 385 pM). The LOD for the 3'-phosphate of N3mT was 1.9 amol (~385 pM) at a signal/noise (S/N) ratio of 3 when a 5 nl volume was injected (assuming 100% yield in all reaction steps) in line with LODs for 5mdCp (1.4 amol; 280 pM) and 5hmdCp (0.45 amol; 90 pM) [16]. This corresponds to one molecule of N3mT per 1,000

normal nucleotides or 0.1% per total nucleotides, and to approximately 0.4% per total thymidines. After successful establishment of our CE-LIF method for the detection of N3mT, we applied this new technique for the analysis of DNA isolated from human lung cancer cells treated with the nitrosamine NNN. A549 cells and H1299 cells were treated with 100 µM NNN for 24 h. DNA was isolated from the cells digested, labeled and analyzed by CE-LIF. Three different DNA modifications N3mT, 5hmdC and 5mdC were detectable in the same analytical run.

No N3mTp was detected in A549 and H1299 cells treated with 0.05% dimethyl sulfoxide (DMSO; solvent control) or in untreated cells. After treatment with 100 µM NNN, a signal was found and identified as the N3mTp-Bodipy-conjugate by spiking with the synthesized standard. *Figure 3A* shows the level of N3mT in DNA of A549 and H1299 cells treated with 100 µM NNN for 24 h. In A549 cells, NNN treatment caused the formation of 0.5% N3mT (calculated per total thymidine). In H1299 cells, NNN treatment led to 0.25% N3mT (per total thymidine), that is just below the limit of detection. N3mT levels were in general close to the detection limit of 1.9 amol (0.1% total nucleotides) but spiking with the synthesized N3mTp standard allowed identification of the fluorescence signal clearly as the N3mTp-Bodipy-conjugate.

NNN also altered levels of hydroxymethylation. As shown in *Figure 3B*, NNN increased the level of 5hmdC in A549 cells from 0.15% (controls) to 0.31% (NNN 100 µM), and in H1299 from 0.03% to 0.16% 5hmdC (NNN, 100 µM, 24h). The degree of DNA methylation measured as 5mdC stayed constant. The levels of 5mdC in DMSO treated control cells were 3.5% (A549 cells) and 3.6% (H1299 cells). After treatment with NNN (100 µM, 24h) 5mdC was measured as 3.8% (per total cytidine) in A549 cells and as 4.0% in H1299 cells (*Figure 3C)*. These results suggest that treatment of both cell lines with NNN caused N-methylation of thymidine as well as hydroxymethylation of

cytidine. However, the exact mechanism which leads to the formation of these DNA modifications remains unknown.

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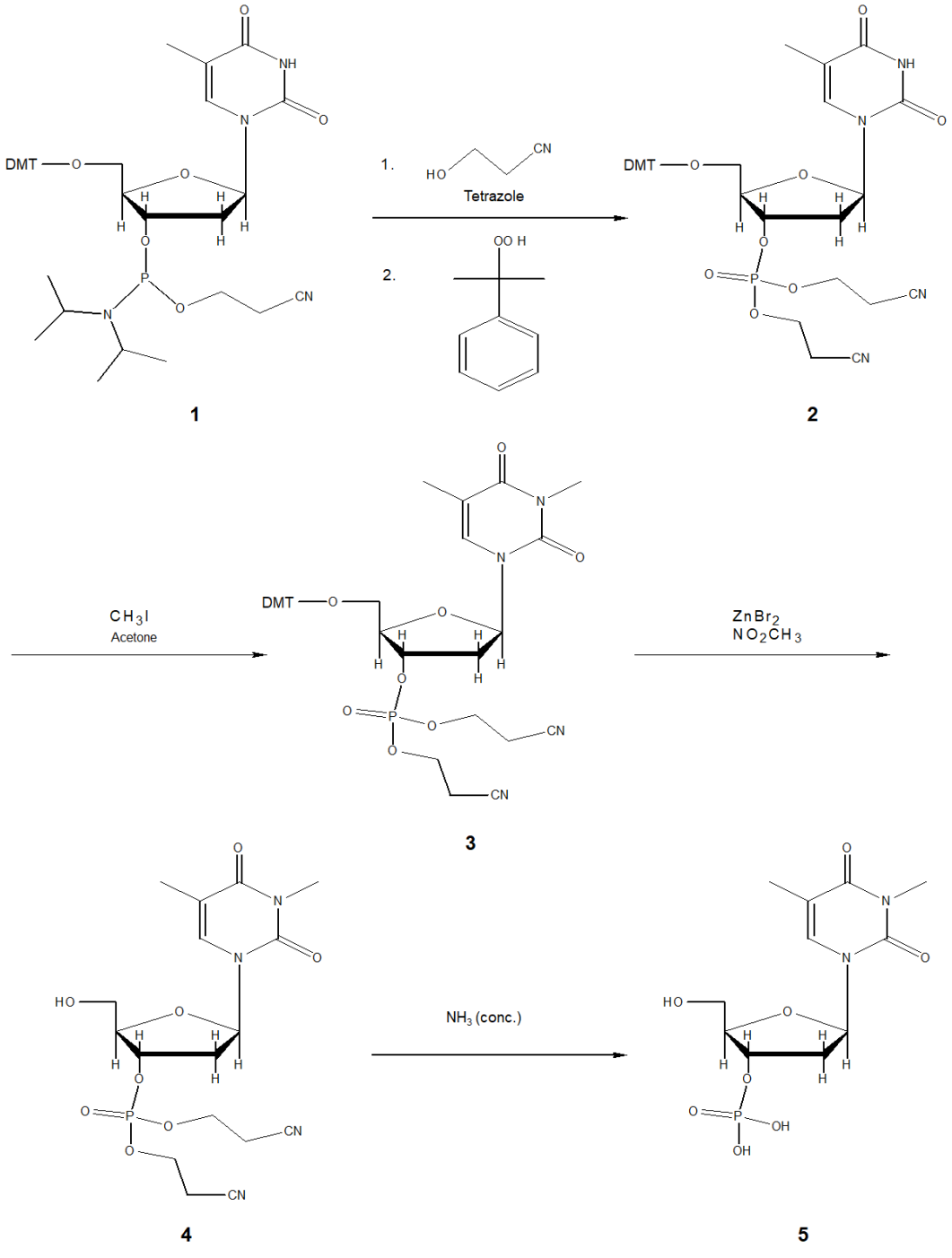


*Figure 1: Synthesis steps of N3-methylthymidine-3'-phosphate (N3mTp).*

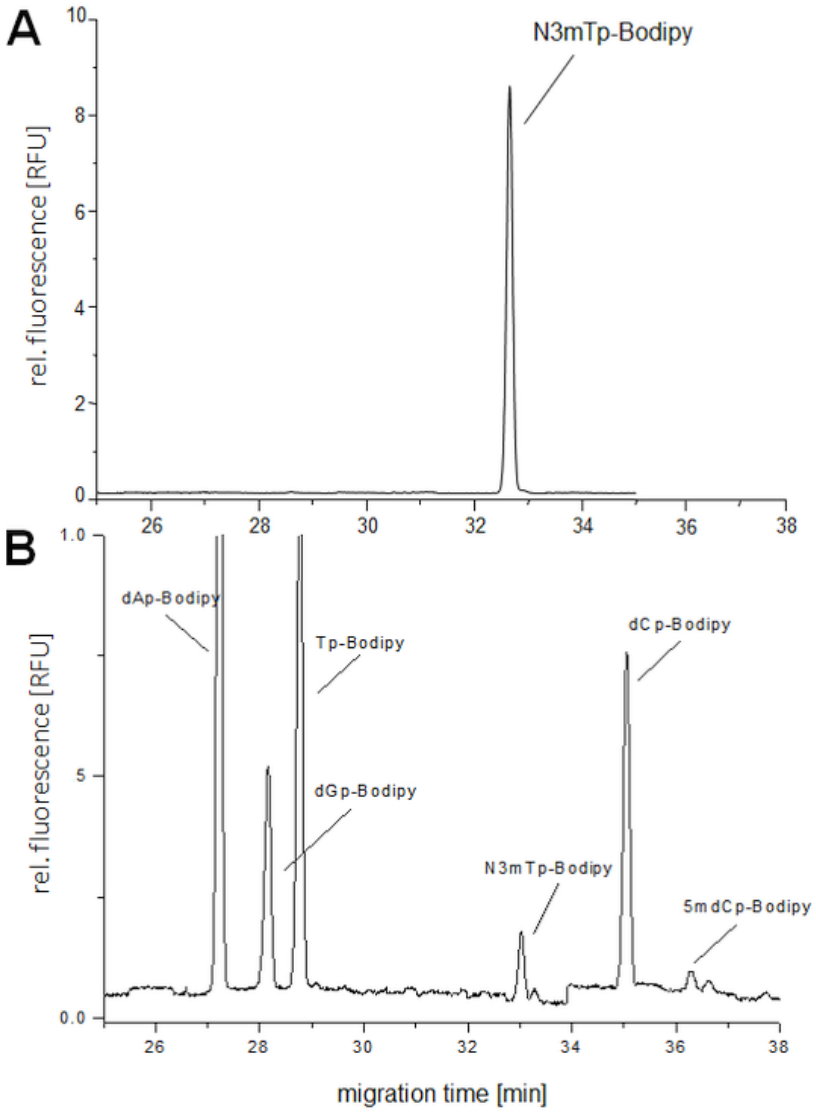


*Figure 2: CE-LIF analysis of 100 ng N3mTp (A) and 100 ng of calf thymus DNA spiked with N3mT (50:1) (B), after MN/SPD digestion and fluorescence labeling of the 3'-phosphates. Separation buffer: 90 mM SDS, 18 mM $Na_2HPO_4$, 10% methanol*

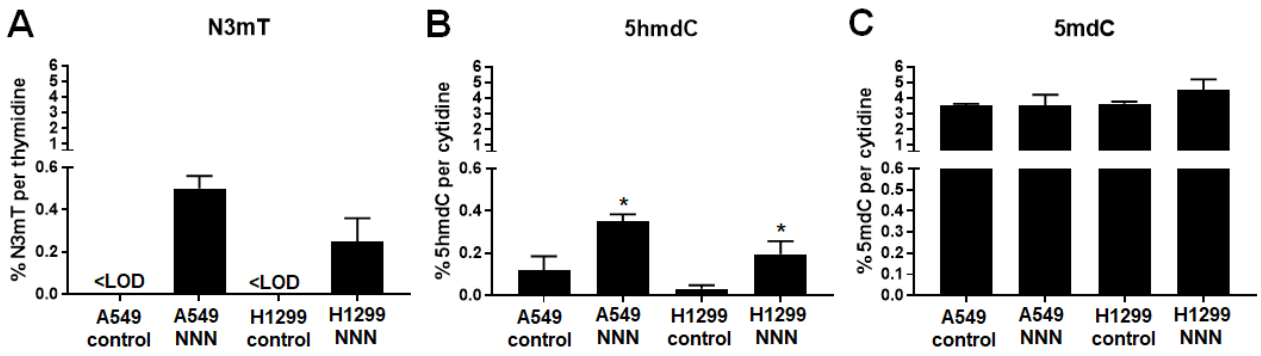


*Figure 3: Levels of N3mT (per thymidine) (A), 5hmdC (B) and 5mdC (per cytidine) (C) in DNA of human lung cancer H1299 and A549 cells after treatment with 100 µM of NNN for 24 h. Values represent mean ± SD of 3 biological replicates that were separately analyzed at least 3 times each.*

** p<0.05, difference compared to control incubations.*