

Characterization of Covalent Adducts of Nucleosides and DNA Formed by Reaction with Levuglandin[†]

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ABSTRACT: Enhanced expression of cyclooxygenase-2 (COX-2) is associated with development of several cancers. The product of COX-2, prostaglandin H₂ (PGH₂), can undergo spontaneous rearrangement and nonenzymatic ring cleavage to form the highly reactive levuglandin E₂ (LGE₂) or D₂ (LGD₂). Incubation with LGE₂ causes DNA–protein cross-linking in cultured cells, suggesting that levuglandins can directly react with DNA. We report the identification by liquid chromatography–tandem mass spectrometry of a stable levuglandin–deoxycytidine (LG–dC) adduct that forms upon reaction of levuglandin with DNA. We found that LGE₂ reacted with deoxycytidine, deoxyadenosine, or deoxyguanosine in vitro to form covalent adducts with a dihydroxypyrrolidine structure, as deduced from selective ion fragmentation. For LG–deoxycytidine adducts, the initial dihydroxypyrrolidine structure converted to a pyrrole structure over time. Reaction of LG with DNA yielded a stable LG–dC adduct with a pyrrole structure. These results describe the first structure of levuglandinyl–DNA adducts and provide the tools with which to evaluate the potential for LG–DNA adduct formation in vivo.

The concept that cyclooxygenase-2 (COX-2) participates in the multistep evolution of many cancers is supported by a concerted body of evidence (1–5). That evidence includes epidemiological observations, demonstration of COX-2 expression in relevant neoplasms, overproduction of PGE₂ in patients with lung and other cancers, and experiments employing pharmacologic and genetic inhibition of COX-2. The immediate product of the cyclooxygenase (COX)¹ enzymes is the cyclic endoperoxide, prostaglandin H₂ (PGH₂) (6), which is metabolized enzymatically in a cell-specific manner to prostaglandins E₂, D₂, F_{2α}, and I₂ and thromboxane A₂. Approximately 20% of PGH₂ undergoes nonenzymatic rearrangement to the γ -ketoaldehydes, levuglandin E₂ (LGE₂) and LGD₂ (7, 8) (Figure 1). The levuglandins are among the most highly reactive molecules in mammalian biology, reacting almost instantaneously (7, 9) by Paal-Knorr condensation to form covalent adducts with free amines such as the ϵ -amine of lysine residues of proteins. Development of mass spectrometric methods to measure levuglandinyl–lysine adducts in cellular proteins (7, 10) has

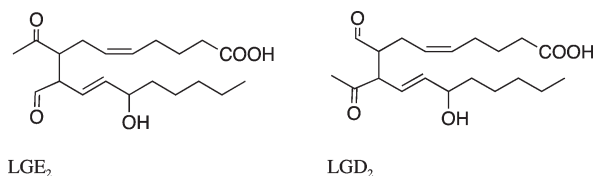


FIGURE 1: Structures of levuglandin E₂ and D₂.

permitted demonstration of COX-dependent formation of these adducts in activated platelets, in the hippocampus in Alzheimer's disease, and in neoplastic cells (11, 12).

The COXs are present in the endoplasmic reticulum, and the COX-2 isoform is particularly densely localized to the nuclear envelope (13). In that location, access of PGH₂ to the nucleus would be enhanced. In the nuclear environment, there are no synthases to dispose of PGH₂ enzymatically, thus directing PGH₂ to nonenzymatic rearrangement and formation of levuglandins within the nucleus. This raises the possibility that LG could react with available amino groups in nucleotides, and indirect evidence suggesting this possibility was the demonstration that LG can cause DNA–protein cross-links in cells (14).

We here characterize products of the reaction of LG with the nucleosides deoxycytidine, deoxyguanosine, and deoxyadenosine and demonstrate that reaction of DNA with LG produces a stable LG–deoxycytidine adduct with a pyrrole structure.

EXPERIMENTAL PROCEDURES

Materials. Methanol and acetonitrile were from Fisher Scientific (Pittsburgh, PA) and were HPLC grade or higher.

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Abbreviations: COX, cyclooxygenase; LG, levuglandin; dC, 2'-deoxycytidine; dA, 2'-deoxyadenosine; dG, 2'-deoxyguanosine; HNE, 4-hydroxynonenal; ONE, 4-oxo-2(E)-nonenal; NSAIDS, nonsteroidal anti-inflammatory drugs; HPLC, high-performance liquid chromatography; LC–MS/MS, liquid chromatography–tandem mass spectrometry; SRM, selected reaction monitoring.

LGE₂ and [¹³C]₃LGE₂ were synthesized by V. Amernath as described previously (15). All other chemicals and reagents were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise noted.

Formation of LG Adducts. A 1:1 mixture of [¹²C]LGE₂ and [¹³C]₃LGE₂ was incubated in 5-fold molar excess with 2'-deoxycytidine or other nucleoside for 2 h unless indicated. All incubations were conducted at 37 °C, in phosphate-buffered saline (pH 6.5) using DMSO as a vehicle for LGE₂. For LG–DNA adducts, a 1:1 mixture of [¹²C]LGE₂ and [¹³C]₃LGE₂ was mixed in 2-fold molar excess with 200 μg of salmon sperm DNA, in 10 mM MOPS buffer (pH 7). The mixture was allowed to react for 2 h at 37 °C. After incubation,

DNA was digested to nucleosides over a period of 24 h by serial hydrolysis. DNase I (10 units) was added along with its associated 10× buffer (New England Biolabs, Ipswich, MA) and allowed to digest for 2 h at 37 °C. Following this, 15.5 units of nuclease P1 was added, along with ZnCl₂ (final concentration of 1 mM), and the digestion allowed to proceed overnight at 37 °C. Finally, alkaline phosphatase (30 units) was added in 50 μL of MOPS buffer (pH 10) and incubated for 1 h at 37 °C. The resulting digestion was filtered through a Spin-X (Costar) column and analyzed by LC–MS.

Mass Spectrometry. Samples were analyzed by liquid chromatography tandem mass spectrometry (LC–MS/MS) on a TSQ Quantum triple quadrupole machine (Thermo Fisher Scientific,

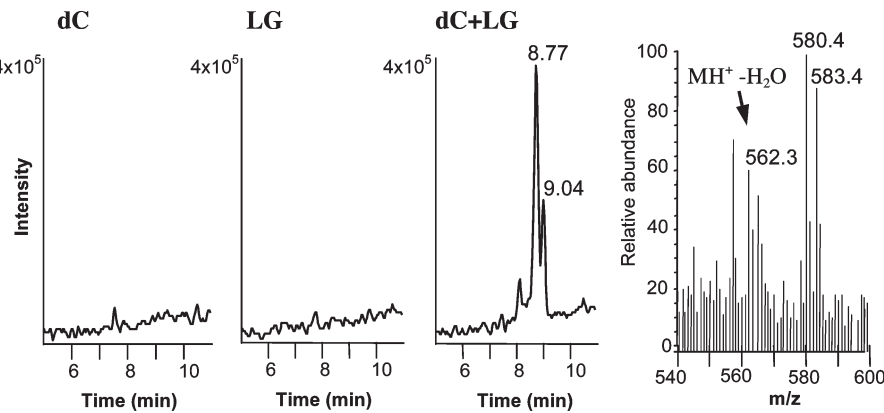
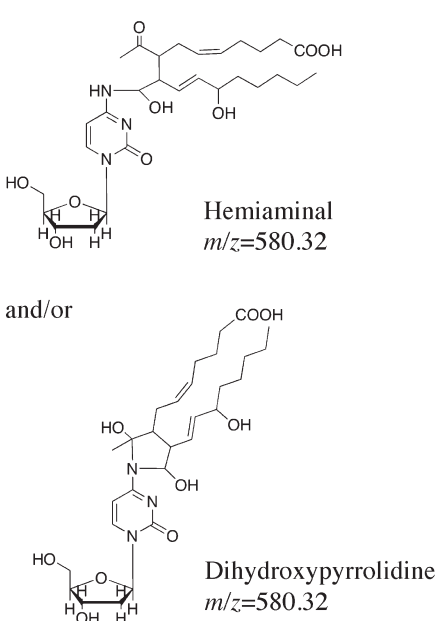
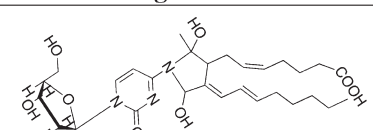
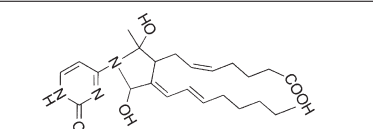
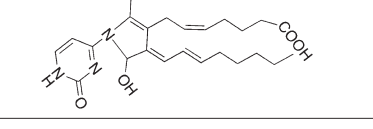
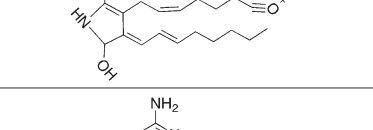
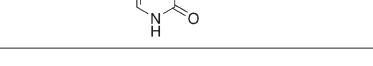


FIGURE 2: Unique ion formed in the reaction between dC and LG. A 50:50 mixture of [¹²C]- and [¹³C]₃LGE₂ was incubated with dC in a 5:1 ratio for 2 h before ESI–LC–MS analysis in full scan, positive ion mode. Equal amounts of LGE₂ or dC alone in reaction buffer containing vehicle were used as controls. Shown are the current chromatograms for the ion at *m/z* 580.3, as well as the ion spectrum for the peak at 8.77 min within the LG–dC reaction. The ions at *m/z* 580 and 583 correspond to [¹²C]LG and [¹³C]₃LG adducts, respectively.

Table 1: Structures of the Products of Fragmentation of the LG–dC Ion at *m/z* 580.4^a

Precursor ion	<i>m/z</i> =	Fragment ion
 <p>Hemiaminal <i>m/z</i>=580.32</p> <p>and/or</p> <p>Dihydroxypyrrolidine <i>m/z</i>=580.32</p>	562.4	
	446.4	
	428.2	
	317.1	* 
	112.1	

^aLG was reacted with dC in a 5:1 ratio for 2 h at 37 °C, and the resulting mixture was analyzed by ESI–LC–MS/MS. On the basis of the product ions obtained, the precursor ion at *m/z* 580.4 could be either a hemiaminal or an open ring, dihydroxypyrrolidine structure as shown. The asterisk denotes a previously reported LG product ion from ref 16.

Waltham, MA) using electrospray ionization. Samples were separated on a reversed-phase C₁₈ column (Phenomenex Prodigy, 5 μ m, 150 mm \times 1 mm) using mobile phase A (deionized water containing 0.1% glacial acetic acid) and mobile phase B (acetonitrile) and eluted at a flow rate of 0.1 mL/min with a linear gradient from 5 to 80% solvent B over 10 min. The mass spectrometer was operated in positive ion mode, and selected reaction monitoring (SRM) data were collected in profile mode. Nitrogen was used for both the sheath and auxiliary gases, which were set to 31 and 25 (arbitrary units), respectively. The source collision energy was 5 V; all other collision energies were as noted. The capillary temperature was 300 °C, and the electrospray needle was maintained at 4400 V. For full scans, using Quad 1, the mass spectral resolution was set to a full width at half-maximum (fwhm) of 0.7 μ m, and the scan time was 2 s. Full scan data were collected in centroid mode.

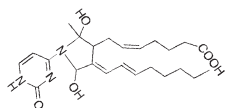
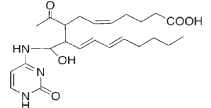
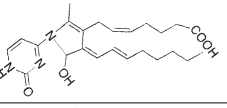
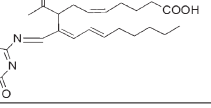
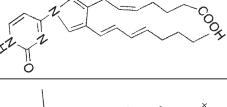
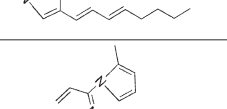
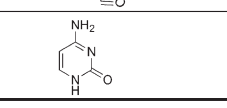
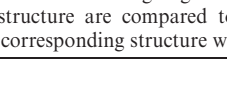
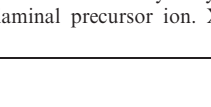
RESULTS

Formation and Structure of LG–Nucleoside Adducts. We examined whether LG has the capability of reacting with individual nucleosides. To better identify specific reaction products, we used a 1:1 mixture of [¹²C]LG and [¹³C₃]LG, which produces a characteristic ion doublet when analyzed by full scan mass spectrometry. The reaction of LG with deoxycytidine yielded a unique [¹²C]LG product at m/z 580.4 together with a [¹³C₃]LG product at m/z 583.4 (Figure 2), which cochromatographed with a lesser dehydration product (Figure 2). Fragmentation of the LG–dC adduct at m/z 580 (Table 1) generated an ion fragment at m/z 112.1, corresponding to the cytosine base, as well as an ion fragment at m/z 317.1, corresponding to a previously described fragment ion of LGE₂ (16). Larger fragment ions were consistent with the loss of deoxyribose at relatively low voltages, followed by a second dehydration product. We were able to assign two possible structures, equal in molecular weight, to the reaction product ion at m/z 580 (Table 1). The first structure, a hemiaminal, is an intermediate in the formation of a Schiff base; the second, a dihydroxypyrrolidine, is a pentyl ring formation closed around the nitrogen of deoxycytidine. Both have been previously described as intermediates in the formation of the final pyrrole structure in the reaction of γ -ketoaldehydes with lysine (9).

Fragmenting the LG–dC adduct at m/z 580 predominantly yielded dehydration products, which prevented us from distinguishing between the dihydroxypyrrolidine and hemiaminal structures. Therefore, we fragmented the precursor ion corresponding to the LG–dC dehydration product (m/z 562), which had an identical retention time of the major ion at m/z 580, suggesting that the loss of water occurred within the mass spectrometer. As expected, at increasing fragmenting voltages, there was a loss of deoxyribose, followed by several successive dehydrations (Table 2). Three fragment ions at m/z 410.3, 299, and 162 are possible products of a dihydroxypyrrolidine structure but cannot derive from an initial hemiaminal structure (Table 2), suggesting that the LG–dC reaction product at m/z 580 is a dihydroxypyrrolidine. No fragment ions specific to a hemiaminal precursor ion were found.

Comparable adducts, differing only by the molecular weights of the respective nucleosides, were found after incubation of LGE₂ with dA (m/z 604.3) and dG (m/z 620.3) (Figure 3A). These unique ions, showing as a doublet when a mixture of [¹²C]LGE₂ and [¹³C₃]LGE₂ was used, were not seen in control incubations of

Table 2: Structures of the Products of Fragmentation of the Dehydrated LG–dC Ion at m/z 562.3^a

m/z	Dihydroxypyrrolidine	Hemiaminal
446.3		
428.4		
410.3		X
299.5		X
162.2		X
112.3		

^aStructures for the resulting fragment ions based on an initial dihydroxypyrrolidine structure are compared to a hemiaminal precursor ion. X indicates no corresponding structure was found.

individual nucleosides or LGE₂ alone (data not shown). Because fragment ions of the LG–dA adduct at m/z 604 and the LG–dG adduct at m/z 620 were similar to those derived from the LG–dC dihydroxypyrrolidine adduct and corresponded to a loss of deoxyribose, individual bases, and shared LG fragments at m/z 317 and 299 (data not shown), we conclude that these three nucleoside adducts have similar structures (Figure 3B). There was no individual preference of reaction in an equimolar mixture of nucleosides, and each adduct ion was detected in relatively equal abundance. Incubation of LG with thymidine, which lacks a free amino group, did not produce any unique reaction products (Figure 3A).

Formation of a Stable LG–dC Pyrrole Structure. A unique ion at m/z 544 was detected at longer incubation times in the LG–dC reaction. Fragmentation of the ion at m/z 544 yielded a loss of deoxyribose at low energies, and fragment ions including a cytosine base at high energies. The molecular ion, as well as the fragment ions generated, corresponds to a pyrrole LG–dC structure (Table 3). The major fragment ions at m/z 428 and 176 were chosen for analysis by selected reaction monitoring (SRM), to increase the sensitivity of detection. To determine whether the m/z 580 dihydroxypyrrolidine detected in the LG–dC reaction converts over time to an m/z 544 pyrrole structure, we monitored both ions over a period of 48 h using SRM. Over time, the ion at m/z 580 disappeared in parallel with the appearance of the ion at m/z 544 (Figure 4), suggesting that the dihydroxypyrrolidine is an intermediate of the final pyrrole structure. The proposed mechanism of LG–dC adduct formation and conversion to the pyrrole structure is shown in Figure 5.

Detection of LG–DNA Adducts. To determine whether LG reacts with a base in double-stranded DNA, we incubated a mixture of [¹²C]LGE₂ and [¹³C₃]LGE₂ with commercial salmon sperm DNA. This was digested to nucleosides over 24 h and

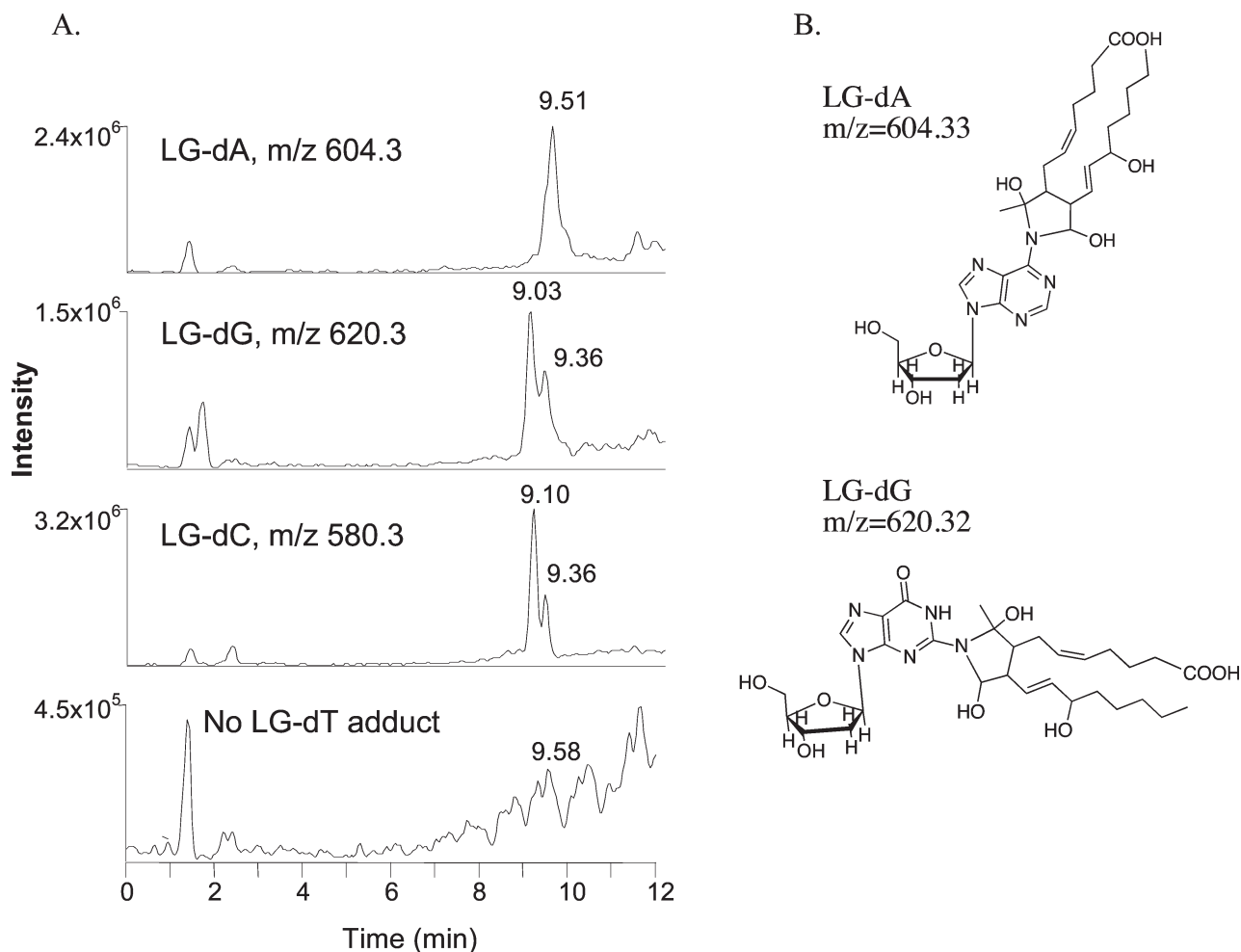


FIGURE 3: Unique ions formed after reaction of LG with an equimolar mix of nucleosides. A 50:50 mixture of [^{12}C]- and [$^{13}\text{C}_3$]LGE₂ was incubated at 37 °C with an equimolar nucleoside mix, with LG in a 3:1 ratio per individual nucleoside. After 2 h, the reaction and individual controls (not shown) were analyzed by ESI-LC-MS in full scan, positive ion mode. (A) Ion current chromatograms for the LG-dA, LG-dG, and LG-dC adducts within the LG-nucleoside reaction, along with the chromatogram for the predicted ion at m/z 595 of an LG-dT adduct. (B) Deduced dihydroxypyrimidine structures of the LG-dA and LG-dG adducts.

initially analyzed by LC-MS for the LG adducts previously identified through incubation with individual nucleosides. The unique reaction product identified (Figure 6A) corresponded by mass to the pyrrole LG-dC adduct (m/z 544.3). This identity was confirmed through ion fragmentation, which yielded a cytosine base as well as fragment ions identical to those generated through extended reaction with individual deoxycytidine. SRM analysis of the LG-DNA adduct, using these fragment ions, yielded a chromatogram similar to that from analysis of the LG-dC pyrrole adduct (Figure 6B).

DISCUSSION

Levuglandins, reactive byproducts of prostaglandin synthesis, have been shown to form covalent adducts with proteins (17, 18) and cross-link proteins and DNA (14). Here we characterize the structures of covalent adducts of LG with nucleosides and demonstrate that LGE₂ reacts with double-stranded DNA to form adducts of deoxycytidine (dC). Characterization of the LG-dC adduct of DNA now provides an experimental approach to determining whether the catalytic activity of COX-2 can lead to formation of this DNA adduct in cells.

Table 3: Structure and Fragment Ions of the LG-dC Ion at m/z 544.3^a

m/z	Fragment ion	Precursor ion at $m/z=544.3$
428.2		
410.1		
176.1		
162.0		
112.1		

^aLG was reacted in a 3:1 ratio with dC for 48 h at 37 °C, and the results were analyzed by ESI-LC-MS/MS. On the basis of the product ions seen, the precursor ion is most likely a pyrrole dC-LG structure, similar to the pyrrole-lysyl adduct seen in ref 19. The fragment ions at m/z 428 and 176 were chosen for SRM analysis in later experiments.

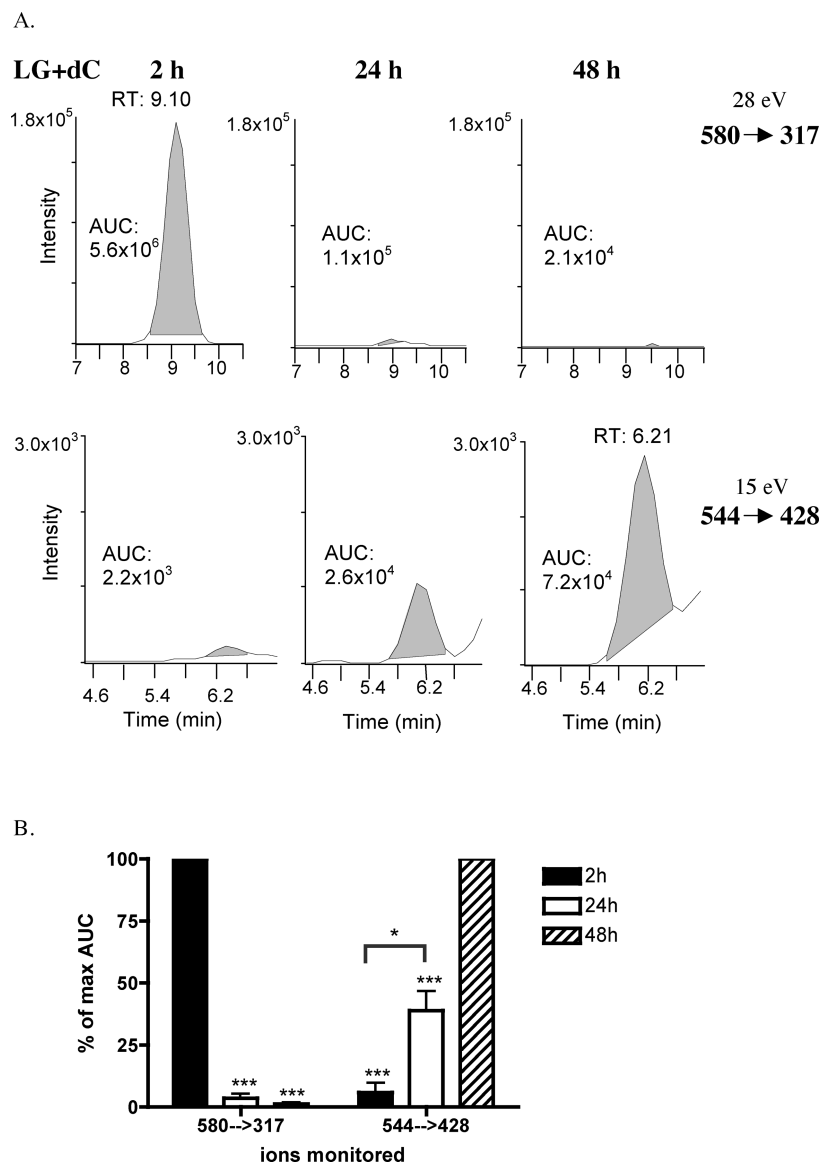


FIGURE 4: Ion at m/z 580 that disappears over time while the ion at m/z 544 appears. LGE₂ was incubated in a 2:1 ratio with dC at pH 7 and allowed to incubate for 2, 24, or 48 h at 37 °C. Samples were immediately frozen following the time point and analyzed at a later date by ESI-LC-MS/MS, using SRM. The parent ions at m/z 580 and 544 were analyzed in the same run. (A) Shown is the chromatograph of one fragment ion for each precursor ion from one experiment, along with the results of peak integrations. (B) The results of three separate experiments were expressed as a percent of the maximum integrated area from each experiment, corresponding to the 2 h time point for the ion at m/z 580 and the 48 h time point for the ion at m/z 544. These values are summarized by the mean ($n = 3$) and standard error. Three asterisks indicate $p < 0.001$ from maximum AUC, and one asterisk indicates $p < 0.05$ by one-way ANOVA followed by Bonferroni's multiple-comparison test.

We have found that LGE₂ reacts with the free amino group of dC to form an immediate covalent adduct at m/z 580 with a likely dihydroxypyrrolidine structure, according to specific fragment ions. The occurrence of an additional hemiaminal structure, of equal m/z , is also possible. The LG-dC dihydroxypyrrolidine at m/z 580 is a probable intermediate of the pyrrole structure, as during extended incubation of LG with dC, the ion at m/z 544 ion emerges while the ion at m/z 580 declines over time. Because of the possibility of unequal ionization between the ions at m/z 580 and 544, at this time we are not able to determine the percentage of dihydroxypyrrolidine adduct that successfully converts to a stable end pyrrole.

A pyrrole structure, proceeding from a Schiff base, is a hypothesized intermediate in the formation of LG-lysyl lactam adducts (19). However, unlike LG-lysyl adducts (10),

after reaction with dC there does not seem to be oxidation to a final lactam structure. We were unable to detect any ion corresponding to an LG-dC lactam either in extended LG-dC reactions or within the LG-DNA reaction. This is likely due to the stabilizing effect of the pyrimidine ring. The stability of the LG-dC pyrrole structure is demonstrated by our ability to detect it after the lengthy enzymatic digestion necessary to reduce DNA to nucleosides. Whereas LG reacts with the individual nucleosides, dA and dG, an examination of the LG-DNA reaction spectra did not reveal any products corresponding to putative LG-dA or LG-dG pyrrole or lactam structures.

Adducts of DNA have the potential for producing genomic instability (20–22). Genomic lesions have been shown to result from lipid adducts of DNA. Lipid peroxidation produces aldehydic products that react covalently with DNA (23). The

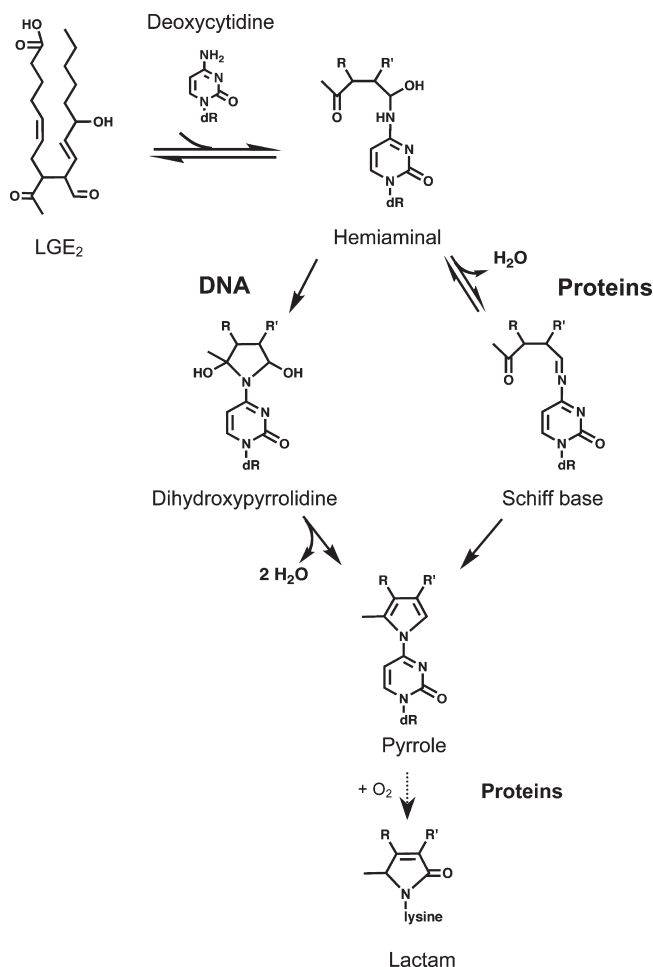


FIGURE 5: Proposed mechanism of the LG-dC reaction. The final pyrrole structure proceeds from an intermediate dihydroxypyrrolidine as suggested in ref 36. In contrast, the LG-lysyl adduct in proteins proceeds via an intermediate Schiff base and the pyrrole is further oxidized into a terminal lactam structure (37).

most-studied of these is 4-hydroxynonenal (HNE), which reacts with deoxyguanosine in DNA to form a mutagenic 1,*N*²-propa-*n*-2'-deoxyguanosine (HNE-dG) adduct (20, 24). This adduct is preferentially formed at the third base of codon 243 in the p53 gene, a frequent mutation spot in many human cancers (25). Another lipid peroxidation product that also can be generated as a byproduct of COX-2 (26), malondialdehyde, reacts with guanine residues to create a stable M1G adduct that has been shown to be mutagenic in a number of systems (27–29) and carcinogenic in rats (30). However, direct oxidation of DNA leading to base propenal formation is responsible for almost all of the M1G (31), making MDA derived from COX-2 an unlikely source of this adduct. 4-Oxo-2(*E*)-nonenal (ONE) also forms following lipid peroxidation, COX-2 or lipoxygenase activity, and reacts with deoxyguanosine in DNA to form heptanone-etheno-deoxyguanosine adducts (32) that experience an increase in number in the small intestine of a mouse colorectal cancer model (33).

The evidence from investigations of these other lipid adducts of DNA engenders a hypothesis that LG adducts of DNA also could cause genomic instability. The proximity of COX-2 to the nucleus, the absence of PGH₂-metabolizing enzymes in the nucleus, and the fact that LG is far more reactive than HNE (9) together provide additional support for this hypothesis. Previous research into the involvement

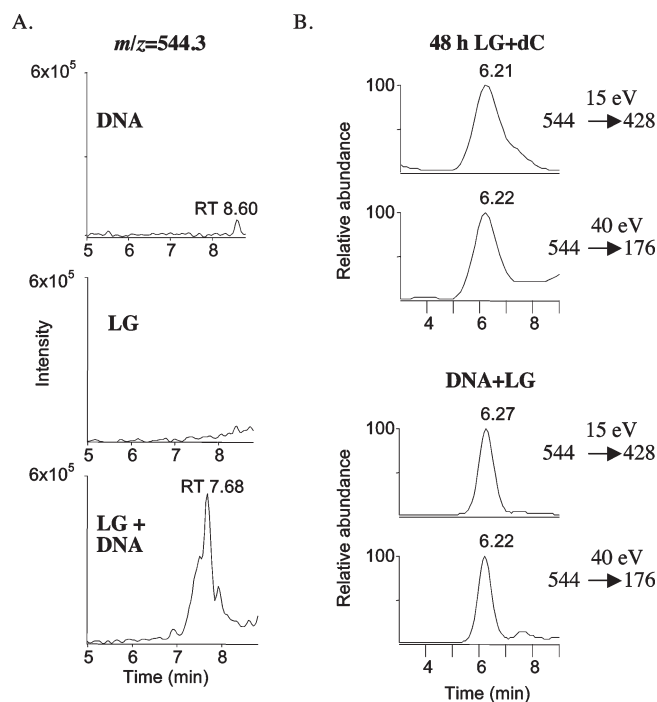


FIGURE 6: LG-dC species formed in a reaction between LG and DNA. A mixture of [¹²C]- and [¹³C₃]LGE₂ was incubated in a 2:1 ratio with 200 μg of salmon sperm DNA at 37 °C for 2 h. Equal amounts of LGE₂ or DNA alone in reaction buffer containing vehicle were used as controls. DNA was digested to nucleosides over a period of 24 h using DNase I, nuclease P1, and alkaline phosphatase. (A) Samples were analyzed by ESI LC-MS in full scan, positive ion mode. Shown are the chromatographs for the ion at *m/z* 544.3. (B) The LG-DNA sample (bottom) was analyzed by SRM, monitoring the ion transition of the ion at *m/z* 544 to fragments of ions at *m/z* 428 and 176, and the resulting chromatograph was compared to identical SRM analysis of a 48 h LG-dC reaction (top).

of COXs in cancer has provided evidence demonstrating that prostaglandins such as PGE₂ lead to an increased level of proliferation, protection from apoptosis, angiogenesis, suppression of the immune response, and enhanced metastasis (34). These effects result from the action of PGE₂ on G-protein-coupled receptors (GPCR) to initiate the relevant signaling pathways, which promote propagation of the malignant genotype and the dissemination of cancer cells, but do not directly explain the massive genomic instability derived from successive somatic mutations and chromosomal instability that occur during the evolution of the malignant phenotype. However, the combination of somatic mutations and chromosomal instability caused by levuglandin adducts of DNA together with prostaglandin-initiated mechanisms that promote clonal expansion and spread would synergize to contribute to the evolution of highly malignant neoplasms with poor prognosis. This severe malignant phenotype is associated with cancers of the colon, lung, breast, and pancreas and other neoplasms that express COX-2 that account for more than half of the 560,000 cancer deaths in the United States annually (35).

The results presented here identify for the first time the structure of a stable covalent modification of DNA by levuglandin E₂, a product of the cyclooxygenases and of lipid peroxidation. This provides a basis for investigating the biological significance of formation of LG-DNA adducts and constitutes the basis for a novel hypothesis for the involvement of COXs and/or oxidative stress in carcinogenesis.

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