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Adduction of catechol estrogens to nucleosides

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Abstract

We report the formation, detection, quantitation and structural characterization of products resulting from the adduction of deoxynucleosides (deoxyadenosine, deoxyguanosine, deoxycytidine and 5-methyldeoxycytidine) to the catechol estrogens (CE) of estrone, estradiol-17β and estradiol-17α. The crude products are obtained in a one-pot synthesis through oxidation of catechols to quinones and subsequent Michael-type reaction with the deoxynucleosides in acidic medium.

In all experiments, adducts are detected by electrospray ionization mass spectrometry analysis after HPLC separation (LC/ESI/MS^n). The two pyrimidines deoxycytidine and 5-methyldeoxycytidine yield only CE adducts to deoxynucleosides, which correspond to stable adducts on DNA. For purines, the results depend on the CE (2,3- or 3,4-catechols) used, the function and configuration on carbon 17 (ketone for estrone, alcohol for α and β isomers of estradiol), and on the purine itself (deoxyadenosine or deoxyguanosine). Both stable adducts and deglycosylated adducts are formed, and therefore formation of stable adducts on DNA as well as the loss of purines from the DNA strands could be possible. MS^2 and MS^3 experiments prove to be relevant for further structural determinations, enabling in some cases the elucidation of the regiochemistry of adduction on the A and B rings of the steroid moiety.

Keywords: Catechol estrogens; Deoxynucleosides; DNA adducts; Steroid; Michael-type addition; LC/ESI/MS^n

1. Introduction

Catechol estrogens (CE) can be produced in human cells through aromatic hydroxylation of estrone (E_1) and estradiol-17β ($E_2\beta$) [1,2]. Estradiol-17α ($E_2\alpha$) is the main metabolite of $E_2\beta$ found in the bovine species [3,4] but is only a minor metabolite in humans [5]. This derivative is qualitatively submitted to the same main metabolic pathways as $E_2\beta$ and E_1, which result in the biosynthesis of 2-methoxy derivatives [6]. Compared to $E_2\beta$, $E_2\alpha$ is partially devoid of estrogenic activity [7,8]. Studies on the relative binding affinity of $E_2\alpha$ using uterine estrogen receptors or in vitro synthesized α or β receptor subtype are in good agreement with the low estrogenic potency of $E_2\alpha$ [9–11]. Further oxidation of the CE of $E_2\beta$ or E_1 yield semi-quinones and quinones, which are strong electrophiles that can react with nucleophilic sites of DNA [12]. However, on the contrary to biopotency evaluations, the influence of the configuration of the 17-hydroxyl group on the chemical reactivity of CE has never been determined until now, and to our knowledge, there has been no study on the reactivity of 17α-estradiol quinones towards DNA bases. Depending on the reactive site involved, adducts can be retained on DNA as stable adducts or decomposed as CE-bases adducts (deglycosylated adducts), giving rise to the occurrence of abasic sites on DNA [12]. As these processes could be implicated in the initiation step of carcinogenesis, the knowledge of the adducts, which are really produced in vivo, is of great interest. Previously, only a few estrogen–nucleic acid adducts were described [13–17], mainly with the 3,4-CE of E_1 and $E_2\beta$ in connection with their established carcinogenicity in animal models [14] or with their supposed link to mammary or endometrium cancer [18,19]. Very few studies have dealt with chemical reactions and mechanisms that could explain the formation of the different isomeric adducts [20,21]. 2,3-CE are of interest since they are produced by most

Abbreviations: CE, catechol estrogens; LC/ESI/MS, liquid chromatography–electrospray ionization–mass spectrometry; E_1, estrone (estra-1,3,5(10)-trien-3-ol-17-one); $E_2\alpha$, estradiol-17α (estra-1,3,5(10)-trien-3,17α-diol); $E_2\beta$, estradiol-17β (estra-1,3,5(10)-trien-3,17β-diol); 2-OH-$E_2\beta$, 2-hydroxyestradiol-17β (estra-1,3,5(10)-trien-2,3,17β-triol); 4-OH-$E_2\beta$, 4-hydroxyestradiol-17β (estra-1,3,5(10)-trien-3,4,17β-triol); 2-OH-$E_2\alpha$, 2-hydroxyestradiol-17α (estra-1,3,5(10)-trien-2,3,17α-triol); 4-OH-$E_2\alpha$, 4-hydroxyestradiol-17α (estra-1,3,5(10)-trien-3,4,17α-triol); 2-OH-E_1, 2-hydroxyestrone (estra-1,3,5(10)-trien-2,3-diol-17-one); 4-OH-E_1, 4-hydroxyestrone (estra-1,3,5(10)-trien-3,4-diol-17-one); RT, retention time; dA, deoxyadenosine; dG, deoxyguanosine; dC, deoxycytidine; 5-Me-dC, 5-methyl-deoxycytidine; TIC, total ionic current

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cell types in larger amounts than 3,4-CE [21,22] and have also been shown to generate CE–DNA adducts [23]. Moreover, using in vitro methodologies, such 2,3-CE adducts on oligonucleotides can induce A → T transversions, A → G or G → A transitions as well as deletions depending on the DNA-polymerases (α, β, δ) used for the elongation steps [24,25].

In order to fully characterize chemical structures, we have synthesized various adducts by chemical synthesis, leading to a larger amount of products than by biological means. To obtain an easier structural analysis of the isomeric adducts synthesized, we have used deoxynucleosides instead of deoxynucleotides. We have re-examined the reactivity of 2,3- and 3,4-CE of E_1, $E_2\alpha$ and $E_2\beta$ towards four different deoxynucleosides on a systematic basis in order to determine which kind of adduct could be formed, and to assess their quantitative distribution. Because these adducts and the reactants are sensitive to oxidation, the crude reaction mixtures were directly analyzed by liquid chromatography–electrospray ionization mass spectrometry (LC/ESI/MS^n) as described before [23]. General schemes are proposed to explain the regiochemistry of the adduction process by comparison with previously published results [12,20,21,23].

2. Experimental

2.1. Materials

2.1.1. HPLC

Preparative HPLC was performed on a Thermo Separation Products P1000XR pump equipped with a UV 3000 detector (Thermo Quest, Les Ulis, France).

2.1.2. LC/ESI/MS^n

Structural analyses were carried out using HPLC on a Thermo Separation Products P4000 pump fitted with a Rheodyne 7725i injector. UV detection was achieved at 280 nm with a Thermo Separation Products UV 1000 detector. Mass spectrometry was performed on a Finnigan LCQ (Thermo Quest) quadrupole ion trap mass spectrometer fitted with an ESI source.

2.2. Chemicals

E_1 (estra-1,3,5(10)-trien-3-ol-17-one), $E_2\alpha$ (estra-1,3,5(10)-trien-3,17α-diol), $E_2\beta$ (estra-1,3,5(10)-trien-3,17β-diol), monohydrated deoxynucleosides [deoxyadenosine (dA), deoxyguanosine (dG), deoxycytidine (dC), 5-methyldeoxycytidine (5-Me-dC)], Fremy's salt and activated manganese (IV) dioxide were purchased from Sigma–Aldrich Company (L'Isle d'Abeau, France). The 2,3- and 3,4-CE were prepared from the corresponding estrogens by oxidation with Fremy's salt, according to previously published methods [26]. We set up CE purification by preparative HPLC, using a Diol column (Lichrospher OH, 250 mm × 21.2 mm, 5 μm, Interchim, Montluçon, France) and pre-column (Lichrospher OH, 33 mm × 7.8 mm, 5 μm, Interchim). Isocratic elution was achieved at 10 ml/min using dichloromethane/isopropanol (97:3, v/v). UV detection was recorded at 280 nm.

2.3. Syntheses

Adducts were prepared as previously described [15,21]. 2,3-CE (resp. 3,4-CE) were oxidized to quinones in acetonitrile at −40 °C (resp. 0 °C) with 8 eq. of activated manganese (IV) oxide. After 10 min, the suspension was filtered under vacuum through a Whatman GF/B filter on a solution of monohydrated nucleoside (6 eq.) in acetic acid/water (1:1, v/v). Stirring was maintained for 6 h at room temperature (+26 °C) under argon. After addition of 1 mg of L-ascorbic acid, the solution was evaporated to dryness, and the orange oil obtained was kept under argon at −20 °C until analysis.

2.4. LC/ESI/MS^n analyses

An Ultrabase C_{18} reversed phase column (250 mm × 2 mm, 5 μm, SFCC, Eragny, France) was used at a flow rate of 0.2 ml/min with the following gradient: from 0 to 5 min, linear gradient from 0 to 15% B and then, linear gradient to get 50% B at 30 min and then 100% B at 40 min. The latter was maintained until 60 min. Eluent compositions were as follows: (A) $CH_3OH/H_2O/CH_3COOH$ (10:90:0.2, v/v/v) and (B) $CH_3OH/H_2O/CH_3COOH$ (90:10:0.2, v/v/v). Quantification of adducts was achieved by integration of the corresponding chromatographic peaks on the appropriate reconstituted ion chromatogram. The results were expressed as percentages of the total adducts detected considering that all the adducts display the same mass spectrometric response under positive electrospray ionization conditions. Electrospray ionization was achieved using the following conditions: needle voltage (5 kV), heated capillary temperature (230 °C), heated capillary voltage (26 V), and lens tube offset (0 V). MS^n experiments were carried out using typical isolation window widths from 1.0 to 1.5 amu and excitation voltages from 0.75 to 1.25 V. All data were recorded using the AGC (automated gain control) mode. Helium was used as the collisional gas for MS^n experiments.

3. Results

3.1. General considerations for elucidating the adduction of deoxynucleosides

The reaction of $E_2\beta$-2,3-quinone with deoxynucleosides was extensively studied in previous works [23,27]. In this work, we have studied the possible Michael-type adduction of four deoxynucleosides (dA, dG, dC, 5-Me-dC) with the 2,3-quinones of E_1 and $E_2\alpha$, and with the 3,4-quinones of

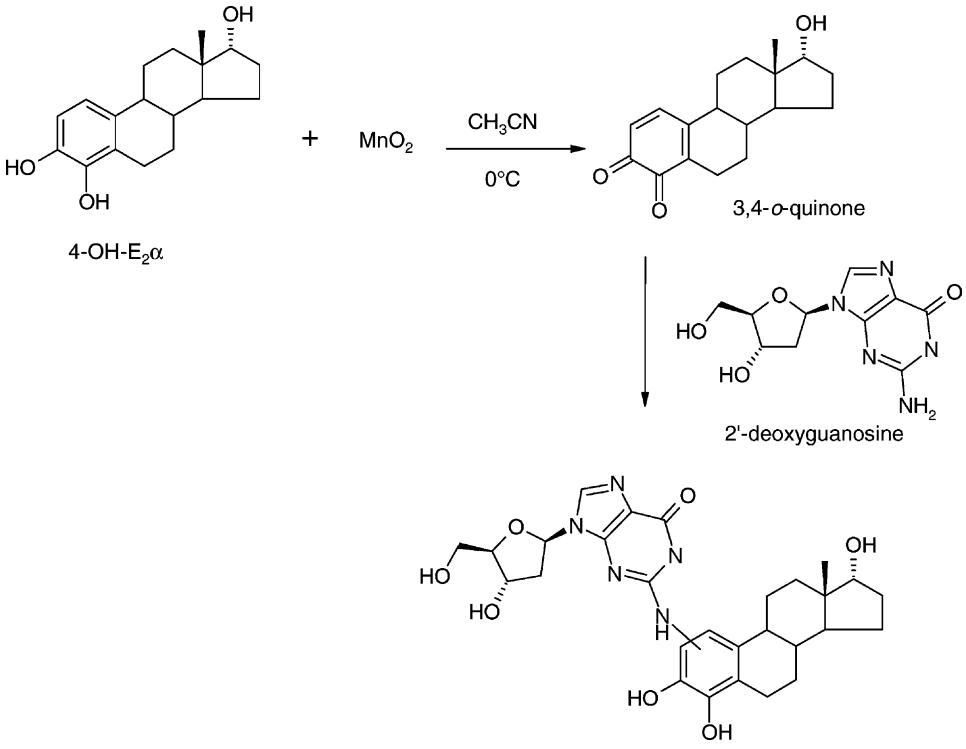

Fig. 1. Adduction of nucleosides with CE: reaction of dG with $E_2\alpha$-3,4-quinone.

E_1, $E_2\beta$ and $E_2\alpha$, in acidic medium (Fig. 1). These quinones were obtained in situ by oxidation of the corresponding catechols (CE), using one of the two methods employed in the literature [20,21]. LC/ESI/MS and MS^n experiments were carried out for the detection and analysis of adducts in crude mixtures.

According to the nucleophilic site involved in the Michael-type reaction, two different types of adducts could be formed: CE-nucleosides and CE-bases (Fig. 2), the latter resulting from the glycosidic bond breaking during the reaction. From each crude reaction mixture, TIC chromatograms were obtained by LC/ESI/MS (Fig. 3a), and various adducts were separated. Their characterization was based on the reconstituted ion chromatograms corresponding to the *m/z* ratio of their quasi-molecular MH^+ ions (Fig. 3b and c). The repeatability of the experiments was assessed with $E_2\alpha$-2,3-Q on the basis of duplicate experiments for each nucleoside used. The qualitative composition of the reaction mixtures was found to be the same, although slight variations in the relative quantities of adducts were observed with variation coefficients being less than 12%. For every quinone/deoxynucleoside pair studied, the relative

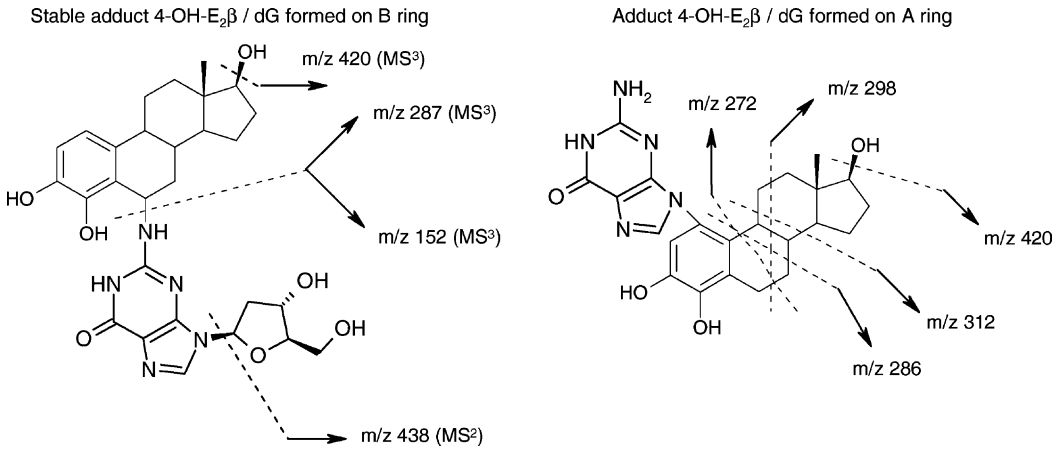

Fig. 2. Fragmentation patterns of adducts synthesized from 4-OH-$E_2\beta$ and dG.

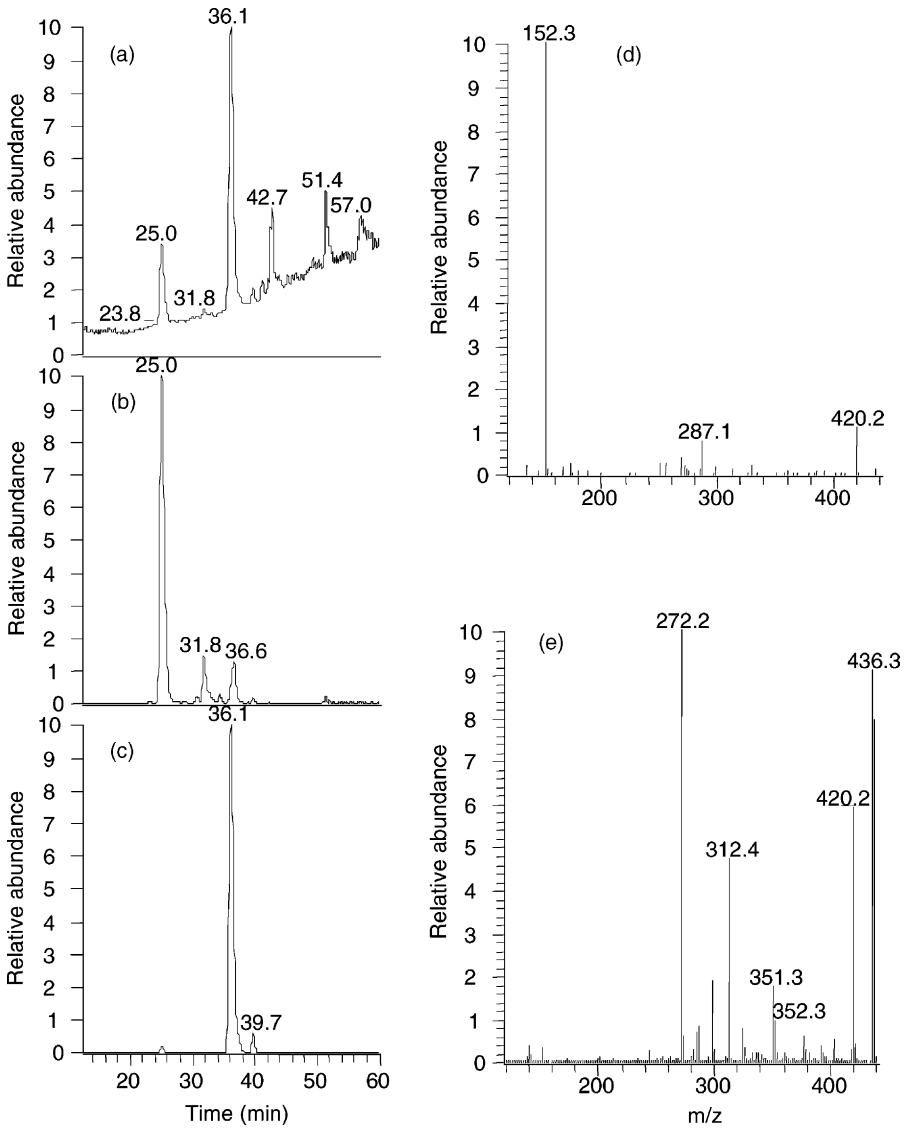

Fig. 3. LC/ESI/MS and LC/ESI/MS^n analyses of adducts synthesized from 4-OH-$E_2\beta$ and dG. Recordings are obtained for TIC (a), reconstituted ion chromatogram of *m/z* 554 quasi-molecular ions (CE-nucleoside) (b), reconstituted ion chromatogram of *m/z* 438 quasi-molecular ions (CE-base) (c), MS^3 spectrum of *m/z* 554 ions (d), and MS^2 spectrum of *m/z* 438 ions (e).

quantification of each adduct was carried out according to the HPLC procedure described in the experimental section.

Due to the very low reaction yield, structural information was generated by mass spectrometry. Moreover, direct LC/ESI/MS analyses of the crude mixtures were performed in order to avoid the degradation of adducts that occurred while trying to isolate them for further structural identification. Taking these limitations into account, our conclusions about the regiochemistry of the adduction were deduced from the well-known chemistry of the 1,4- and 1,6-Michael-type reactions, and from the interpretation of the experimental MS^n data. Considering the estrogen quinones studied in this work, the Michael reaction theoretically allows five possible sites of adduction on the A and B rings of the steroid skeleton, as indicated in Fig. 4. As a matter of fact, most of the adducts can be further identified by comparing their fragmentation patterns determined by MS^2 and MS^3 experiments to those of similar adducts unambiguously identified in previous works [21,23]. Schematically, for adducts in which the nucleoside is linked to the B ring of the steroid, the CAD spectra displays ions yielded by the breaking of the steroid–base bond, which corresponds to the protonated nucleoside and the steroid cation, as described in Figs. 2 and 3d. In the case of a steroid–base linkage on A ring, the bonding between the two partners is stronger and charge-remote cross-ring breakings are observed, as shown in Figs. 2 and 3e [21,23].

Yet, in some cases, the fragmentations observed in MS^n were unusual and we could not precisely determine which

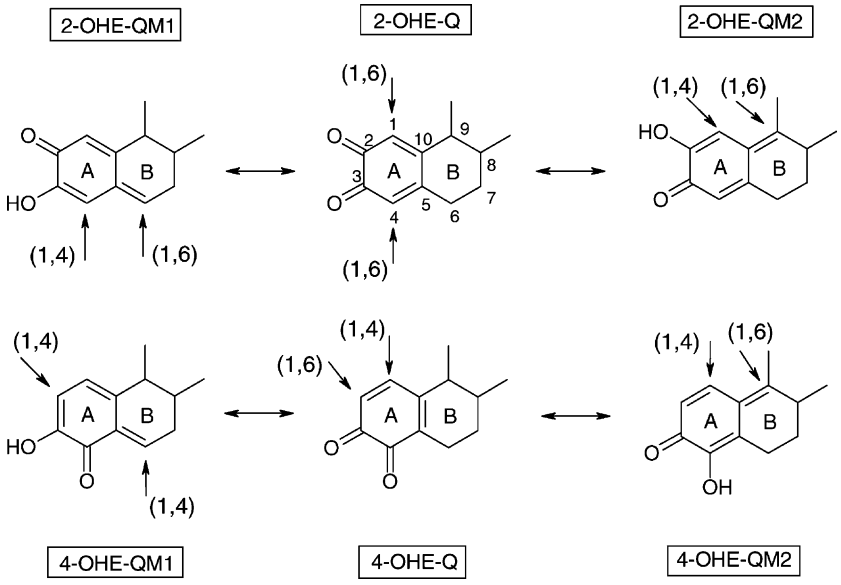

Fig. 4. Structures of 2,3-quinones, 3,4-quinones and quinone methides. Arrows indicate electrophilic sites for 1,4- and 1,6-Michael-type adductions.

site of adduction was more likely to correspond to a defined structure, especially for the pyrimidic nucleosides.

3.2. Reaction of dC and 5-Me-dC with CE

Depending on the quinone, one to five CE-nucleosides were formed whereas no CE-base was detected (Table 1). Indeed, $E_2\beta$-2,3-Q yielded only one adduct with dC and 5-Me-dC whereas four or five adducts were formed with E_1-3,4-Q, E_1-2,3-Q and $E_2\alpha$-2,3-Q, with no major adduct (Table 1). In the reaction between $E_2\alpha$-3,4-Q or $E_2\beta$-3,4-Q and dC, two and three adducts were detected, one of them being the major product accounting for 84 and 78%, respectively. Most often, the regiochemistry of adduction was difficult to determine, due to non-elucidated MS^n data (Table 1).

3.3. Reaction of dG with CE

CE-nucleosides and CE-bases were detected with all quinones (Table 2). The CE-base accounted for more than

Table 1
LC/ESI/MS^n data and quantification of the CE-nucleosides obtained from dC and 5-Me-dC and expected sites of adduction on the quinone moiety, as deduced from the chemistry of Michael-type reaction and LC/ESI/MS^n data

Pyrimidines	Estrogens																	
	3,4-CE-quinones									2,3-CE-quinones								
	E_1-3,4-Q			$E_2\beta$-3,4-Q			$E_2\alpha$-3,4-Q			E_1-2,3-Q			$E_2\beta$-2,3-Q[a]			$E_2\alpha$-2,3-Q		
dC	*m/z* 512			*m/z* 514			*m/z* 514			*m/z* 512			*m/z* 514			*m/z* 514		
	RT[b]	%	Site[c]	RT	%	Site	RT	%	Site	RT	%	Site	RT	%	Site	RT	%	Site
	24.0	34	**1**	*24.5*	78	**1**	*29.0*	16	**1**	*26.0*	31	**1**	*25.5*	100	**3**	*22.0*	29	**1**
	26.0	13	**1**	*28.5*	11	**1**	*31.5*	84	**1**	*27.0*	16	**1**				*25.8*	21	**1**
	33.0	29	**1**	*33.5*	11	**1**				*29.5*	42	**1**				*27.0*	31	**1**
	41.0	24	**1**							*31.0*	11	**1**				*35.0*	19	**1**
5-Me-dC	*m/z* 526			*m/z* 528			*m/z* 528			*m/z* 526			*m/z* 528			*m/z* 528		
	24.5	22	**2**	*39.0*	53	**2**	*29.0*	54	**2**	*28.0*	21	**2**	*26.4*	100	**2**	*27.5*	27	**2**
	35.0	13	**2**	*39.7*	47	**2**	*39.5*	25	**2**	*30.5*	35	**2**				*29.0*	17	**2**
	39.0	19	**2**				*40.0*	21	**2**	*32.0*	14	**2**				*36.0*	30	**2**
	40.0	35	**2**							*40.0*	14	**2**				*41.0*	15	**2**
	41.5	11	**2**							*40.5*	16	**2**				*41.5*	11	**2**

[a] Reported in [23,27].
[b] Retention times (RT) are given in minutes.
[c] **1**: C_1, C_2, $C_{6\alpha}$, $C_{6\beta}$, C_9; **2**: C_1, C_4, $C_{6\alpha}$, $C_{6\beta}$, C_9; **3**: $C_{6\alpha}$, $C_{6\beta}$, C_9 (B ring).

Table 2
LC/ESI/MS^n data and quantification of adducts obtained from dG and dA and expected sites of adduction on the quinone moiety, as deduced from the chemistry of Michael-type reaction and LC/ESI/MS^n data

Purines		Estrogens																	
		3,4-CE-quinones									2,3-CE-quinones								
		E_1-3,4-Q			$E_2\beta$-3,4-Q			$E_2\alpha$-3,4-Q			E_1-2,3-Q			$E_2\beta$-2,3-Q[a]			$E_2\alpha$-2,3-Q		
dG	CE-nucleosides	*m/z* 552			*m/z* 554			*m/z* 554			*m/z* 552			*m/z* 554			*m/z* 554		
		RT[b]	%	Site[c]	RT	%	Site	RT	%	Site	RT	%	Site	RT	%	Site	RT	%	Site
		26.0	25	**5**	*25.0*	15	**1**	*31.2*	69	**5**	*32.5*	95	**3**	*29.5*	14	**4**	*29.0*	77	**3**
					31.8	2	**1**							*30.7*	48	**4**	*34.0*	20	**3**
														35.6	4	**6**			
	CE-bases	*m/z* 436			*m/z* 438			*m/z* 438			*m/z* 436			*m/z* 438			*m/z* 438		
		35.5	75	**5**	*36.1*	83	**5**	*39.5*	31	**5**	*29.5*	5	**1**	*26.6*	6	**4**	*26.0*	3	**3**
														27.8	22	**4**			
														36.3	6	**6**			
dA	CE-nucleosides	*m/z* 536			*m/z* 538			*m/z* 538			*m/z* 536			*m/z* 538			*m/z* 538		
		26.0	92	**3**	*27.0*	11	**5**	*29.0*	27	**1**	*40.0*	18	**3**	*36.7*	81	**4**	*36.0*	75	**3**
		40.5	8	**3**	*29.5*	80	**5**	*34.8*	16	**1**	*41.0*	78	**3**	*39.3*	16	**4**	*40.5*	22.5	**3**
					39.0	9	**3**	*36.5*	42	**1**									
								42.5	11	**1**									
	CE-bases	*m/z* 528			*m/z* 528			*m/z* 528			*m/z* 528			*m/z* 528			*m/z* 528		
		–	–	–	–	–	–	*32.0*	4	**3**	*34.5*	4	**3**	*38.2*	2	**4**	*30.0*	2	**3**
														38.7	1	**4**	*37.5*	0.5	**3**

[a] Reported in [23,27].
[b] Retention times (RT) are given in minutes.
[c] **1**: C_1, C_2, $C_{6\alpha}$, $C_{6\beta}$, C_9; **3**: $C_{6\alpha}$, $C_{6\beta}$, C_9 (B ring); **4**: $C_{6\alpha}$, $C_{6\beta}$ (B ring); **5**: C_1, C_2 (A ring); **6**: C_1, C_4.

75% for reactions involving E_1-3,4-Q and $E_2\beta$-3,4-Q. In all the other experiments, CE-nucleosides corresponded to more than 66% of the total amount of adducts. Moreover, we noticed one major adduct for E_1-2,3-Q and $E_2\alpha$-2,3-Q (95 and 77%, respectively). The adduction reaction of dG seemed to be very selective with all the quinones used, except $E_2\beta$-2,3-Q, since we detected no more than one or two CE-nucleosides and one CE-base (Table 2). On the contrary, the reaction of $E_2\beta$-2,3-Q was less selective: three CE-nucleosides and three CE-bases were produced. Indeed, the 2,3-quinones tended to give alkylation preferentially through their B ring, whereas the regiochemistry was on the A ring of 3,4-quinones. For quinones such as E_1-3,4-Q, $E_2\alpha$-3,4-Q, $E_2\beta$-2,3-Q and $E_2\alpha$-2,3-Q, the general regiochemistry proposed was the same for the CE-nucleosides and their corresponding CE-bases (Table 2).

3.4. Reaction of dA with CE

We detected at least two and at the most four CE-nucleosides that represented more than 96% of the total amount of adducts (Table 2). $E_2\alpha$-3,4-Q qualitatively yielded the larger number of CE-nucleosides with four adducts and was the only 3,4-quinone to yield a CE-base. Indeed, no CE-base was detected for E_1-3,4-Q and $E_2\beta$-3,4-Q. For E_1-3,4-Q, $E_2\beta$-3,4-Q, and their corresponding 2,3-quinones and also for $E_2\alpha$-2,3-Q, there was one major CE-nucleoside, accounting for 92, 80, 78, 81, and 75%, respectively, of the total amount of adducts detected (Table 2). Concerning the deduced regiochemistry, adductions occurred on the B ring except for two of the CE-nucleosides formed with $E_2\beta$-3,4-Q (Table 2).

4. Discussion

4.1. Analysis of the chemical reactivity

In the past, the reaction of deoxynucleosides on steroid quinones was not investigated on CE derivatives of $E_2\alpha$, probably because these compounds were considered to be very minor metabolites in humans. Therefore, we acquired new results concerning the 17α isomers of 2,3- and 3,4-CE and provided additional observations concerning CE of E_1 and $E_2\beta$. In our experiments, we used the deoxynucleosides dA, dG, dC, and also 5-Me-dC. 5-Me-dC is obtained on DNA through enzymatic methylation of dC. It plays a role in the transcription regulation during the cellular differentiation and prevents the expression of specific genes [28,29]. Although much less occurring than dA, dG and dC, its chemical modification can be hazardous, and then, determine profiles of mutational hot spots [30].

Analysis of the chemical reactivity was based on the structural characteristics of both reagents: the quinone and the deoxynucleoside. On one hand, quinones are double electrophilic enones for the 1,4- and 1,6-Michael-type reactions. They have two theoretical activated sites each (Fig. 4), since the adduction on positions 5 and 10 is unlikely because of the junction between A and B rings. There also exists two tautomeric quinoid forms called quinone-methides [12] for each quinone (QM1 and QM2). These are simple enones, with two possible C=O/C=C conjugations. Because of the junction between A and B rings, only two activated sites are free for 1,4- and 1,6-Michael-type reactions on 2-OHE-QM1, 2-OHE-QM2 and 4-OHE-QM2. 4-OHE-QM1 has two available sites for the 1,4-adduction. Concerning the C_6 position, the stereoselectivity of adduction can be α or β. Therefore, quinones have five possible sites of adduction which are C_1, C_4, $C_{6\alpha}$, $C_{6\beta}$, C_9 for 2,3-quinones, and C_1, C_2, $C_{6\alpha}$, $C_{6\beta}$, C_9 for 3,4-quinones. C_2 position could be disfavored because it is a soft electrophile while the attacking nucleophile has hard character. We can suppose that the C_9 position is disfavored for steric hindrance since it corresponds to the junction between B and C rings. Yet, we recently demonstrated the formation of a CE-nucleoside adduct involving a linkage at the C_9 position between dG and E_2β-2,3-Q deuterated on $C_{6\alpha}$, $C_{6\beta}$, and $C_{7\alpha}$ [31]. Moreover, the possible adduction at the C_9 position has been reported by Bolton for glutathione conjugates [32]. On the other hand, nucleosides possess several nucleophilic sites: exocyclic N^4, intracyclic N^3 and O for dC. Both purines have nucleophilic intracyclic N^1, N^3, N^7, C^8 and an exocyclic amine (N^6 for dA, N^2 for dG). In addition, dG has a supplementary nucleophilic oxygen atom. We considered that it was the exocyclic N that was responsible for the formation of CE-nucleoside adducts, while the involvement of the intracyclic N^7 of purines could explain the formation of CE-bases by breaking the glycosidic bond [21].

The systematic use of LC/ESI/MS^n for performing analyses of the crude reaction mixtures has enabled us to answer the following questions:

(i) How many adducts are formed for a given pair quinone/nucleoside?
(ii) Which type of adduct is detected (CE-nucleoside or CE-base)?
(iii) What is the regiochemistry of the adduction?

4.2. Adduction of dC and 5-Me-dC with CE-quinones

Adduction of 5-Me-dC with CE-quinones was never mentioned in the literature until now. Concerning dC, no adduction was reported in acidic medium [21]. Under reductive conditions, C_1–N^4 and C_2–N^4 adducts of dC with E_1-3,4-Q were described [20]. We previously reported the characterization of adduct of dC with E_2β-2,3-Q involving the B ring of the steroid moiety [23]. In this work, we have observed the exclusive formation of CE-nucleoside adducts with dC and 5-Me-dC (Table 1). This indicates that the exocyclic nitrogen is preferentially involved in the chemical reaction (more nucleophilic and less hindered alkylation site). Thus, on a DNA strand, dC could be chemically modified by covalent bonding of a steroid. Moreover, when exposed to the following quinones: E_2β-2,3-Q, E_2β-3,4-Q and E_2α-3,4-Q, a specific adduction could occur since we have obtained only one major adduct (Table 1). On the contrary, up to five isomers could be obtained on exposition of dC or 5-Me-dC to the other quinones.

4.3. Adduction of dG with CE-quinones

Stack et al. [21] have described a CE-nucleoside (C_6–N^2) with E_1-2,3-Q (40% yield) and a CE-base (C_1–N^7) with E_1-3,4-Q (10% yield). Three CE-nucleosides and three CE-bases on both rings A and B of E_2β-2,3-Q were also previously described [23]. In this work, both types of adducts were present, indicating that both types of DNA modifications could happen. Indeed, the results obtained for 3,4-quinones (mainly CE-bases for E_1-3,4-Q and E_2β-3,4-Q, CE-nucleoside for E_2α-3,4-Q, A ring regiochemistry) indicated that abasic sites could be generated preferentially on dG sites of DNA when exposed to E_1-3,4-Q or E_2β-3,4-Q. Besides, we showed they were not the only possible alterations, since CE-nucleosides were also detected, thus providing complementary information to what was previously described [21]. For 2,3-quinones, CE-nucleosides are considered as the main type of resulting adducts. Moreover, there is a preponderant adduct that involves the B ring of E_1-2,3-Q, as described previously [21], and E_2α-2,3-Q. Yet, no major adduct has been detected with E_2β-2,3-Q (Table 2). We deduce that these quinones can produce a specific covalent modification of the dG sites on DNA and that the β isomer of estradiol shows specific results.

The fact that nucleosides add preferentially on the B ring of 2,3-quinones while adducts can be formed from adduction on A ring of 3,4-quinones as well, can be explained by the involvement of different quinoids (Fig. 4). For 3,4-quinones, the quinone methide 4-OHE-QM1, which could lead to adduction at C_6 position, is said to be disfavored [12], therefore enabling the reaction to occur on the A ring. However, this regioselectivity has been mainly observed with dG, showing the influence of the nucleoside rather than that of the quinoid. Quinones of E_2β gave CE-nucleosides on both A and B rings while E_2α and E_1 seemed to be more regioselective on one ring, i.e. B ring for 2,3-quinones and A ring for 3,4-quinones (Table 2). This result is in favor of a long distance effect of the C_{17} configuration (ketone or α/β hydroxyl).

4.4. Adduction of dA with the CE-quinones

A single CE-nucleoside (C_6–N^6) with E_1-2,3-Q (80% yield) and no adduct with E_1-3,4-Q were reported [21]. Besides, formation of two CE-nucleosides and two CE-bases (diastereoisomers on positions $C_{6\alpha}$ and $C_{6\beta}$), was previously

evidenced with $E_2\beta$-2,3-Q [27]. In this work, we have obtained more than 96% of CE-nucleosides in all our experiments with dA, but we have detected no CE-bases at all with E_1-3,4-Q and $E_2\beta$-3,4-Q. Consequently, we can notice that the reaction of dA with E_1-3,4-Q yields adducts that are exclusively CE-nucleosides. This brings complementary results on the reactivity of 3,4-quinones on dA, compared to what was expected from the literature [21]. $E_2\alpha$-3,4-Q shows a different reactivity from the other 3,4-quinones (one CE-base with a B ring regiochemistry, four major CE-nucleosides of undetermined regiochemistry), thus underlining the specific behavior of the 17α isomer of estradiol. The formation of one and two CE-bases is also detected on the B ring of 2,3-quinones, indicating that abasic sites could also result from the reaction of dA sites on DNA with estrogen–2,3-quinones, although to a much lesser extent. From all these observations, the stable covalent modification of dA on DNA could be indeed the major result of the exposition to all the quinones.

From the results obtained with 22 out of 24 experiments, we can conclude that, among the total number of adducts detected, CE-nucleosides correspond to the most frequently obtained adducts. Thus, we can suspect that the major alteration of DNA results from the covalent adduction of the nucleosides dC, 5-Me-dC, dA and dG with CE-quinones, and that different regioisomers can be produced. Yet, in 8 out of these 22 experiments, we have also detected a significant amount of CE-bases. This suggests that the alteration of DNA by generation of apurinic sites can also be possible. From this work, it can be deduced that pyrimidic dC and 5-Me-dC cannot eliminate a CE-base from a DNA strand. The two experiments for which the CE-base adducts are preponderant have implicated dG and the 3,4-quinones of E_1 and $E_2\beta$. Besides, for 34 adducts detected by LC/ESI/MS^n, we are able to predict the steroid ring (A or B) involved in the linkage between the quinone and the nucleoside used. These general conclusions evidence the influence of various factors on the adduction reaction such as the nature of the nucleoside/quinone pair used or the C_{17} configuration of steroids, but remain difficult to explain. However, these results could be particularly useful for comparison with adducts purified from biological samples obtained from biopsies, cell incubation or with adducts coming from in vitro adduction experiments performed on calf thymus DNA. Indeed, the reaction conditions used in our chemical model studies have been described to accurately predict the DNA adducts formed in vivo [12,14,21]. Although some long distance specific effects on the alkylation process that are linked to the hydroxyl stereochemistry at the C_{17} position of CE have been revealed, we can hypothesize that 17α isomers of CE could represent a valuable chemical model to perform in vitro DNA alkylation studies. In fact, due to the weak estrogenic potency of $E_2\alpha$ [10,11], this method could be an easy way to uncouple such cellular metabolic disruptions and DNA repairing steps due to CE adduction processes from other cellular events mediated via estrogen receptors as it is the case with $E_2\beta$ incubation. Such cellular events could appear in parallel to DNA damage as recently shown with 4-hydroxyequilenin [33].

Acknowledgments

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