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Synthesis and cytotoxic activity of per-acetylated and halogenated derivatives of nucleosides in breast cancer cells

Síntesis y evaluación citotoxica de derivados halogenados y peracetylados de nucleósidos en celulas de cancer de mama

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RESUMEN

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Objetivos: Sintetizar derivados halogenados sobre la base nitrogenada, sus respectivos derivados tipo éster o amida de todos los grupos hidroxilo y amina presentes en los nucleósidos uridina y citarabina, y evaluar su actividad citotóxica sobre una línea celular de cáncer de mama.

Metodología: primero se realizó la reacción de halogenación en la posición 5 de la base nitrogenada, posteriormente se formaron los ésteres y amidas de todos los grupos hidroxilos y amino presentes en los nucleósidos. Además, se preparó el derivado acetónido con catálisis ácida. Los compuestos se caracterizaron por espectroscopía de resonancia magnética nuclear (RMN ^{1}H y RMN ^{13}C) y espectrometría de masas por inyección directa en modo positivo. Los derivados se evaluaron sobre líneas celulares de tumor de Ovario de Hámster Chino (CHO) y de cáncer de mamá (MCF-7).

Resultados: Se obtuvieron 4 derivados mono-halogenados con cloro y bromo de la uridina y citarabina, respectivamente, sus respectivos derivados per-acetilados, los nucleósidos per-acetilados y el acetónido de la uridina; los compuestos se obtuvieron con rendimientos superiores a 90%. Los nucleósidos per-acetilados, y los derivados per-acetilados y halogenados no exhibieron una inhibición significativa de la viabilidad celular en ambas líneas celulares, sin embargo, de estos, los derivados per-acetilados y halogenados presentaron mayor actividad citotóxica que los respectivos nucleósidos per-acetilados. El derivado acetónido de la uridina mostró citotoxicidad significativa sobre ambas líneas celulares.

Conclusiones: se obtuvieron los nucleósidos per-acetilados y los respectivos derivados clorados y bromados de estos, con rendimientos altos, sin embargo, estos compuestos no exhibieron una actividad anti-proliferativa significativa ($p<0,05$), posiblemente debido a una baja activación intra-celular de los nucleósidos.

Palabras claves: actividad citotóxica, cáncer de mama, nucleósidos anticancerosos, reacciones de esterificación y halogenación, uridina y citarabina.

ABSTRACT

Objectives. To make the synthesis of halogenated derivatives on the nitrogenous base and their respective acyl ester and amide type derivatives for all hydroxyl and amine groups of the uridine and cytarabine nucleosides, and evaluate cytotoxicity against breast cancer cell line.

Methods. First, it was accomplished the halogenation reaction on the 5-position of the nitrogenous base, subsequently, the ester and amide derivatives were performed for all hydroxyl and amine group present in the nucleosides. Besides, the uridine acetonide derivatives as prepared by acid catalysis. The products were characterized by nuclear magnetic resonance spectroscopy (^{1}H RMN y ^{13}C RMN) and mass spectrometry in positive mode by direct injection. Derivatives were evaluated in Chinese hamster ovary (CHO-K1) and human breast cancer (MCF-7) cell lines.

Results. The four derivatives were obtained with chlorine and bromine for the uridine and cytarabine, respectively, their respective per-acetylated derivatives, the per-acetylated nucleoside and the uridine

acetonide; the compounds were obtained with efficiency over 90%. The per-acetylated nucleosides and the halogenated and per-acetylated derivatives did not show inhibitory effects on cell viability in MCF-7 cell line. However, the per-acetylated and halogenated derivatives presented a higher cytotoxic activity than their respective per-acetylated nucleoside. The uridine 3',4'-acetonide showed a significant cytotoxicity on both cell lines.

Conclusions. The per-acetylated nucleoside, and the respective halogenated derivatives with chlorine and bromine were obtained with high yields, nevertheless, these compounds did not exhibit a significant anti-proliferative activity ($p<0.05$), possibly due to a low intra-cellular activation.

Keywords: anticancer nucleoside, breast cancer, cytotoxic activity, esterification and halogenation reactions, uridine and cytarabine.

INTRODUCTION

Nucleoside and analog drugs have been widely used in cancer chemotherapy since the 1960's for soft (leukemia and lymphoma non-Hodking) and solid (lung, liver, breast, among others) tumors treatment. These drugs exhibit several action mechanisms such as: disruption of nucleic acids synthesis, incorporation and demethylation of DNA fragments and inhibition of necessary nucleoside synthesis. Furthermore, they can present activation in cytoplasm of apoptosis factors and destabilization of mitochondrial membrane[1-6].

The nucleoside anticancer drugs show several pharmacodynamic problem including low cellular incorporation across transporter channels, intracellular inactivation by nucleoside deaminase, degradation by ecto-nucleotidase enzymes, higher elimination through transporter channels and vesicles, among others. Moreover, they present very pharmacokinetic disadvantages such as low selectivity by cancer cell, higher chemical lability and biological degradation and inactivation in blood plasma[3-8]. Due to the considerable disadvantages of these drugs, it is recommendable that they are administered in higher doses by endovenous via; however, this administration form is responsible of the majority of many side effects (gastrointestinal disorder, neurotoxicity, nephrotoxicity and hepatotoxicity, among others) of the anticancer nucleoside[3-8].

In this sense, a substantial number of studies are focused on the synthesis of nucleoside derivatives with anticancer potential, that present better physicochemical and/or biological properties, by performing lipophilic derivatives with aliphatic and aromatic acids and forming conjugates with terpenoids, heterocycles and polymers, to accomplish this, aliphatic diacids and phosphate are commonly used as linkage. With these derivatives, the purpose is to avoid the enzymatic or the chemical degradation localized in the gastrointestinal tissue, blood plasma or cytoplasm and to achieve higher cellular uptake through the cell membrane transport[5-10].

Other studies focused on the nucleoside and their analogs search, show better pharmacokinetic and pharmacodynamics advantages as well as an enhancement as an antimetabolite. These studies demonstrate that the natural and synthetic derivatives of the uridine (mainly phosphorylated, sulphated and glycosylated compounds), a natural and necessary nucleoside, have cellular regulatory functions, for instance, glycosyltransferase cofactors inhibition, purinergic receptor agonists, energetic reserve (as UTP can accomplish ATP functions), DNA polymerase inhibition, among others[10-13]. Uridine synthetic derivatives with lipophilic substituents (C-3'-ethynyluridine, sterols, aromatic and aliphatic acids, among others) and diverse monosaccharide positions have exhibited cytotoxic activities in several tumor cell lines or better physicochemical properties with biomembrane models interaction[14-18].

Furthermore, the uridine triacetate has shown modulating effects on the neurotoxicity, gastrointestinal disorders and cardiotoxicity when this is used in combinate chemotherapy with antiviral (Stavudine) and anticancer (Capecitabine, better known as fluorouracil) drugs. This uridine triacetate, known as Vistogard®, was approved by US Food and Drugs Administration (FDA) to the treatment of overdoses with these antiviral and anticancer nucleosides when they are administered for long-term[19,20].

EXPERIMENTAL

Chemical

All solvents were employed at reagent grade. Uridine and cytarabine at 99% purity (Alfaesar) were employed as substrate, acetic anhydride at 99% (Merck) as agent for per-esterification reaction,4-*N*,*N*-dimethylamino-pyridine (DMAP) at 99% (Alfaesar) as nucleophilic catalyst, *N*-chloro-succinimide (NCS) and *N*-bromo-succinimide (NBS) were used for the halogenation reaction. The halogenated and/or per-acetylated derivatives of uridine and cytarabine were purified by column chromatography and thin layer chromatography using normal phase with dichloromethane/methanol and ethyl acetate/acetone mixtures as eluents of the halogenation reaction and per-acetylation, respectively. All nucleoside derivatives, halogenated and/or per-acylated, were characterized by a nuclear magnetic resonance spectroscopy on the Bruker NMR-300 and the Bruker Ascend III HD 600 MHz spectrometers, where tetramethylsilane (TMS) was used as internal standard, using deuterated dimethyl sulfoxide (DMSO-*d*6) and deuterated chloroform ($CDCl_3$) as solvents. The mass spectrometry analysis was performed on the Agilent 6300-LC/MS

(electrospray ionization (ESI) and Ion-Trap analyzer) and on the Agilent 6100-LC/MS (electrospray ionization (ESI) and Single Quadrupole analyzer) equipment's, by direct injection in positive mode.

Uridine 3′,4′-acetonide (Csint)

Uridine (300 mg) was aggregated to an acetone acidulated solution with sulfuric acid[4,13,17]. The product (figure 1) was obtained as yellow solid (90% yield). ^{1}H NMR (300 MHz, DMSO-*d*6), δ ppm (multiplicity; integration; *J* (Hz); position): 1.26 (s; 3H; acetonide ring), 1.48 (s; 3H; acetonide ring), 3.57 (m; 2H; H-6′ of ribose ring), 4.06 (q; 1H; *J*=4.2; H-5′ ribose ring), 4.74 (dd; 1H; *J*=6.3 y 3.5; H-4′ ribose ring), 4.89 (dd; 1H; *J*=6.3 y 2.6; H-3′ ribose ring), 5.63 (d; 1H; *J*=8.0; H-5 uracil ring), 5.82 (d; 1H; *J*=2.6; H-2′ ribose ring), 7.79 (d; 1H; *J*=8.1; H-6 uracil ring). Other NMR (^{13}C and 2D correlations) spectroscopy characterization as was reported elsewhere[17]. ESI-MS (positive mode) m/z: Calculated for $C_{12}H_{16}N_2O_6$: 284.2652; found, 307.1 $[M+Na]^+$.

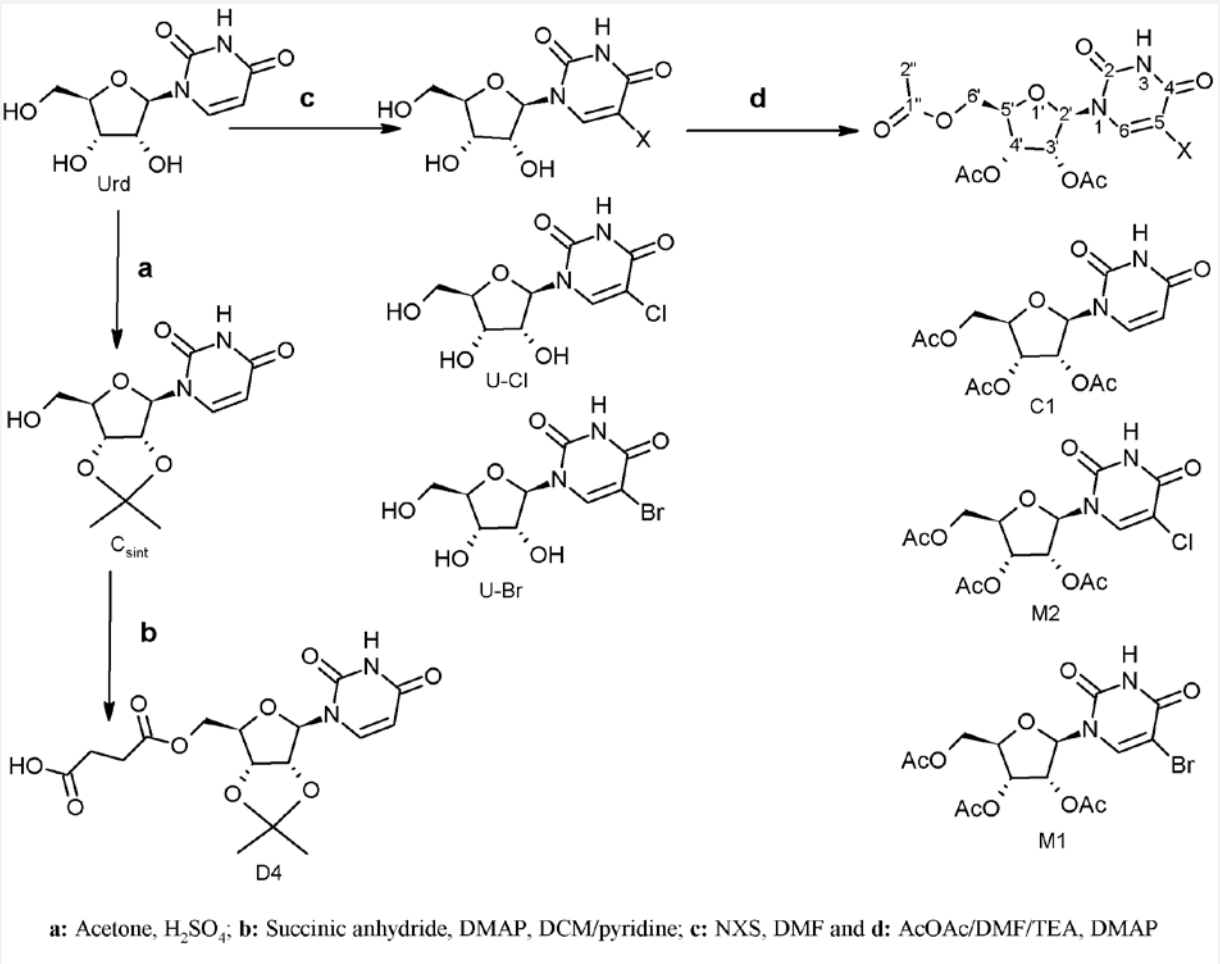

Figure 1. Reaction scheme and chemical structures of uridine derivatives.

3′,4′-acetonide-6′-O-succinyl uridine (D4)

1.0 equivalents of succinic anhydride and DMAP in catalyst amount was added to uridine acetonide (300 mg) solution in DCM/pyridine mix[4,9,17]. The product (figure 1) was obtained as pale-yellow solid (quantitative yield). ^{1}H NMR (300 MHz, DMSO-*d*6), δ ppm (multiplicity; integration; *J* (Hz); position): 1.26 (s; 3H; acetonide), 1.48 (s; 3H; acetonide), 2.60 (m; 4H; succinic acid), 4.40 (m; 3H; H-6′ y H-5′ ribose ring), 4.78 (m; 1H; H-4′ ribose ring), 4.93 (m; 1H; H-3′ ribose ring), 5.62 (d; 1H; *J*=8.0; H-5 uracil ring), 5.81 (d; 1H; *J*=2.6; H-2′ ribose ring), 7.80 (d; 1H; *J*=8.1; H-6 uracil ring). Other NMR (^{13}C and 2D correlations) spectroscopy characterization as was reported previously[17]. ESI-MS (positive mode) m/z: Calculated for $C_{16}H_{20}N_2O_9$: 384.338; found, 407.3 $[M+Na]^+$.

Per-acetylation reaction

The nucleoside (300 mg) was dissolved in an acetic anhydride/DMF/TEA (1:1:1) mixture, followed by the addition of DMAP (5 mol-%). The reaction mixture was stirred at room temperature between 6-8 hours [4,9,16]. For chemical reaction see figures 2 and 3.

3′,4′,6′-O-triacetyl-uridine or uridine triacetate (C1)

The product was obtained as yellow solid (quantitative yield). ^{1}H-NMR (300 MHz; DMSO-*d*6), δ ppm (multiplicity; integration; *J* (Hz); position): 2.04 (d; 6H; H-2″ acetyl linked to O-3′ and O-6′ ribose ring (R.R)), 2,07 (s; 3H; position H-2″ acetyl linked to O-4′ ribose ring), 4.25 (m; 3H; H-6′ and H-5′ ribose ring), 5.33 (m; 1H; H-4′ ribose ring), 5.44 (m; 1H; H-3′ ribose ring), 5.72 (d; 1H; *J*=8.0; H-5 uracil ring), 5.87 (d; 1H; *J*=5.1; H-2′ ribose ring), 7.69 (d; 1H; *J*=8.1; H-6 uracil ring). Other NMR (^{13}C and 2D correlations) spectroscopy characterization as was reported elsewhere[16]. ESI-MS (positive mode) m/z: Calculated for $C_{15}H_{18}N_2O_9$: 370.3114; found, 393.1 $[M+Na]^+$.

Halogenation reaction

The halogenation agent (NCS or NBS) was added (1.1 equivalents) on a nucleoside solution (1 mmol) in DMF, the reaction mix was stirred at room temperature for 8 hours (for chemical reaction see figures 2 and 3)[21].

5-Bromo-uridine

^{1}H-NMR (600 MHz, *d*6), δ ppm (multiplicity; integration; *J* (Hz); position): 3.57 (d, 1H; *J*=12.1; H-6′ ribose ring), 3.69 (d; H; *J* = 12.1; H-6′ ribose ring), 3.86 (d; *J* = 2.5; 1H; H-5′ ribose ring), 3.98 (q; *J* = 4.8; 1H; H-4′ ribose ring), 4.04 (q; *J* = 4.6; 1H; H-3′ ribose ring), 5.73 (d; *J* = 4.3; 1H; H-2′ ribose ring), δ 8.48 (s; 1H; H-6 uracil ring).

5-Chloro-uridine

^{1}H-NMR (600 MHz, *d*6), δ ppm (multiplicity; integration; *J* (Hz); position): 3.58 (d; *J* = 10.4; 1H; H-6′ ribose ring), 3.68 (d; *J* = 12.0; 1H; H-6′ ribose ring), 3.86 (d; *J* = 2.2; 1H; H-5′ ribose ring), 3.99 (dt; *J* = 14.4 and 7.1 Hz; 1H; H-4′ ribose ring), 4.02 (dd; *J* = 9.3 and 4.5; 1H; H-3′ ribose ring), 5.73 (d; *J* = 4.1; 1H; H-2′ ribose ring), δ 8.40 (s; 1H; H-6 uracil ring).

5-Bromo-3′,4′,6′-O-triacetyl-uridine or 5-Bromo-uridine triacetate (M1)

The derivative was obtained coupling the halogenation and per-acetylation reactions (see reaction scheme, figure 1), this was isolated as pale-yellow solid (quantitative yield). ^{1}H-RMN (300MHz, $CDCl_3$), δ ppm (multiplicity; integration; *J* (Hz); position): 2.14 (d; 6H; H-2″ (α protons) acetyl linked to O-3′ and O-6′ ribose ring), 2.18 (s; 3H; H-2″ acetyl linked to O-4′ ribose ring), 4.41 (m; 3H; H-5′ and H-6′ ribose ring), 5.38 (m; 2H, H-3′ and H-4′ ribose ring), 6.12 (d; 1H; H-2′ ribose ring), 7.87 (s; 1H; H-6 uracil ring). ^{13}C-RMN (75MHz, $CDCl_3$), δ ppm (integration; position): 20.42 (1C; C-2″ (α carbon) acetyl linked to O-6′ ribose ring), 20.52 (1C; position C-2″ acetyl linked to O-3′ ribose ring), 20.94 (1C; C-2″ acetyl linked to O-4′ ribose ring), 62.96 (1C; C-6′ ribose ring), 70.07 (1C; C-4′ ribose ring), 73.14 (1C; C-3′ ribose ring), 80.37 (1C; C-5′ ribose ring), 87.37(1C; C-2′ (anomeric carbon) ribose ring), 97.98 (1C; 5-C (halogenated position) uracil ring), 138.62 (1C; 6-C uracil ring), 149.70 (1C; 2-C uracil ring), 158.71 (1C; 4-C uracil ring), 169.71 (2C; C-1″ acetyl linked to O-3′ and O-4′ ribose ring), 170.16 (1C; C-1″ acetyl linked to O-6′ ribose ring). Calculated for $C_{15}H_{17}BrN_2O_9$: 449.2074; found, 472.2 $[M+Na]^+$.

5-Chloro-3′,4′,6′-O-triacetyl-uridine or 5-Chloro-uridine triacetate (M2)

The derivative was obtained coupling the halogenation and per-acetylation reactions (see reaction scheme, figure 1), this was isolated as pale-yellow solid (quantitative yield). ^{1}H-RMN (300MHz, $CDCl_3$), δ ppm (multiplicity; integration; *J* (Hz); position): 2.16 (d; 6H; H-2″ (α protons) acetyl linked to O-3′ and O-6′ ribose ring), 2.22 (s; 3H; H-2″ acetyl linked to O-4′ ribose ring), 4.42 (m; 3H; H-5′ and H-6′ ribose ring), 5.38 (m; 2H; H-3′ and H-4′ ribose ring), 6.11 (d; 1H; H-2′ ribose ring), 7.78 (s; 1H; H-6 uracil ring).^{13}C-RMN (75MHz, $CDCl_3$), δ ppm (integration; position): 20.41 (1C; C-2″ (α carbon) acetyl linked to O-6′ ribose ring), 20.51 (1C; C-2″ acetyl linked to O-3′ ribose ring), 20.85 (1C; C-2″ acetyl linked to O-4′ ribose ring), 62.91 (1C; C-6′ ribose ring), 70.03 (1C; C-4′ ribose ring), 73.11 (1C; C-3′ ribose ring), 80.23 (1C; C-5′ ribose ring), 87.37(1C; C-2′ (anomeric carbon) ribose ring), 110.13 (1C; 5-C (halogenated position) uracil ring), 135.99 (1C; 6-C uracil ring), 149.41 (1C; 2-C uracil ring),

158.61 (1C; 4-C uracil ring), 169.70 (2C; C-1″ acetyl linked to O-3′ and O-4′ ribose ring), 170.13 (1C; C-1″ acetyl linked to O-6′ ribose ring). Calculated for $C_{15}H_{17}ClN_2O_9$: 404.7564; found, 427.8 $[M+Na]^+$.

4-N-acetyl-3′,4′,6′-O-triacetyl-cytarabine (D7)

The product was obtained as described in per-acetylation reaction (see scheme reaction in figure 2) and was isolated as yellow solid in quantitative yield. ^{1}H NMR (300 MHz; $CDCl_3$), δ ppm (multiplicity; integration; *J* (Hz); group; position) 1.89 (s; 3H; H-2″ acetyl linked to O-3′ arabinofuranosyl ring (AF.R)), 2.09-1.98 (m; 6H; H-2″ acetyl linked to O-4′ and O-6′ AF.R), 2.21 (s; 3H; H-2″ acetyl linked to N-4 cytosine ring), 4.16 (t; 1H; *J*=10.5; H-5′ AF.R), 4.33 (t; 2H; *J*=12.3; H-6′ AF.R), 5.04 (d; 1H; *J*=10.8; H-4′ AF.R), 5.49 (d; 1H; *J*=2.5; H-3′ AF.R), 6.29 (d; 1H; *J*=3.4; H-2′ AF.R), 7.43 (d; 1H; *J*=7.6 Hz; H-5 cytosine ring), 7.88 (d; 1H; *J*=7.6 Hz; H-6 cytosine ring). ^{13}C NMR (75 MHz, $CDCl_3$), δ ppm (integration; position): 20.38 (1C; C-2″ acetyl linked to O-3′ of AF.R), 20.60 (1C; C-2″ acetyl linked to O-4′ of AF.R), 20.70 (1C; C-2″ acetyl linked to O-6′ of AF.R), 24.75 (1C; C-2″ acetyl linked to 4-N cytosine ring), 62.77 (1C; C-6′ AF.R), 73.97 (1C; C-3′ AF.R), 76.32 (1C; C-4′ AF.R), 81.05 (1C; C-5′ AF.R), 85.71 (1C; C-2′ AF.R), 96.45 (1C; 5-C cytosine ring), 144.77 (1C; 6-C cytosine ring), 154.72 (1C, 2-C cytosine ring), 163.25 (1C, 4-C cytosine ring), 168.26 (1C; C-1″ acetyl linking to O-4′ of AF.R), 169.64 (1C; C-1″ acetyl linked to O-3′ AF.R), 170.55 (1C; C-1″ acetyl linked to O-6′ AF.R), 171.63 (1C; C-1″ acetyl linked to 4-N cytosine ring). ESI-MS (positive mode) m/z: Calculated for $C_{17}H_{21}N_3O_9$: 411.3634; found, 434.1 $[M+Na]^+$.

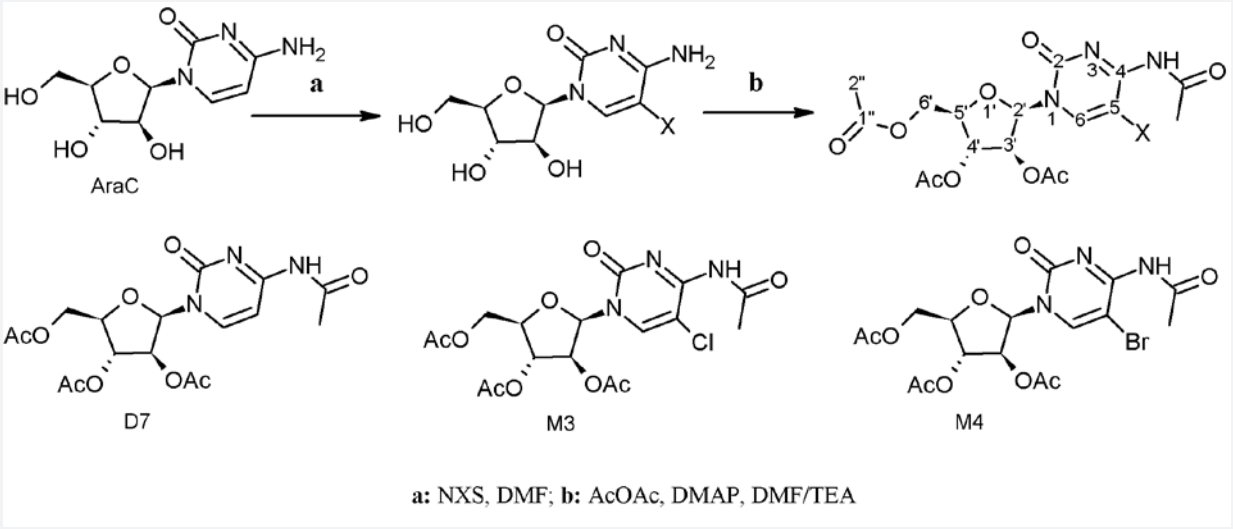

Figure 2. Reaction scheme and chemical structures of per-acetylated cytarabine.

5-Bromo-4-N-acetyl-3′,4′,6′-O-triacetyl-cytarabine (M3)

The derivative was obtained coupling the halogenation and per-acetylation reactions (see reaction scheme, figure 2) and was isolated as yellow solid (quantitative yield). ^{1}H NMR (300 MHz; $CDCl_3$), δ ppm (multiplicity; integration; *J* (Hz); group; position) 1.88 (s; 3H; H-2″ of acetyl linked to O-3′ arabinofuranosyl ring (AF.R)), 2.06-1.99 (m; 6H; H-2″ acetyl linked to O-4′ and O-6′ AF.R), 2.22 (s; 3H; H-2″ acetyl linked to N-4 cytosine), 4.29 (m; 3H; H-5′ and H-6′ AF.R), 5.09 (d; 1H; *J*=10.7; H-4′ AF.R), 5.52 (d; 1H; *J*=2.4; H-3′ AF.R), 6.27 (d; 1H; *J*=3.4; H-2′ AF.R), 8.01(s; 1H; H-6 cytosine ring). ^{13}C NMR (75 MHz, $CDCl_3$), δ ppm (integration; position): 20.49 (1C; C-2″ acetyl linked to O-3′ AF.R), 20.72 (1C; C-2″ acetyl linked to O-4′ AF.R), 20.81 (1C; C-2″ acetyl linked to O-6′ AF.R), 24.88 (1C; C-2″ acetyl linked to 4-N cytosine ring), 62.84 (1C; C-6′ AF.R), 73.92 (1C; C-3′ AF.R), 76.42 (1C; C-4′ AF.R), 81.12 (1C; C-5′ AF.R), 85.53 (1C; C-2′ AF.R), 96.33 (1C; 5-C cytosine ring), 144.67 (1C; 6-C cytosine ring), 155.04 (1C, 2-C cytosine ring), 163.12 (1C, 4-C cytosine ring), 168.38 (1C; C-1″ acetyl linking to O-4′ of AF.R), 169.72 (1C; RO-C=O; position C-1″ of acetyl linked to O-3′ of AF.R), 170.63 (1C; C-1″ acetyl linked to O-6′ AF.R), 171.45 (1C; C-1″ acetyl linked to N-4 cytosine ring). ESI-MS (positive mode) m/z: Calculated for $C_{17}H_{20}BrN_3O_9$: 490.2594; found, 513.3 $[M+Na]^+$.

5-Chloro-4-N-acetyl-3′,4′,6′-O-triacetyl-cytarabine (M4)

The derivative was obtained coupling the halogenation and per-acetylation reactions (see reaction scheme, figure 2) and was isolated as yellow solid (quantitative yield). ^{1}H NMR (300 MHz; $CDCl_3$), δ ppm (multiplicity; integration; *J* (Hz); group; position) 1.89 (s; 3H; H-2″ acetyl linked to O-3′ arabinofuranosyl ring (AF.R)), 2.07-1.98 (m; 6H; H-2″ acetyl linked to O-4′ and O-6′ AF.R), 2.20 (s; 3H; H-2″ of acetyl linked to 4-N AF.R), 4.27 (m; 2H; H-5′ and H-6′ AF.R), 5.06 (d; 1H; *J*=10.8; H-4′ AF.R), 5.49 (d; 1H; *J*=2.6; H-3′ AF.R), 6.27 (d; 1H; *J*=3.3; H-2′ AF.R), 7.96 (s; 1H; H-6 cytosine ring). ^{13}C NMR (75 MHz, $CDCl_3$), δ ppm (integration; position): 20.17 (1C; C-2″ acetyl linked to O-3′ AF.R), 20.18 (1C; C-2″ acetyl linked to O-4′ AF.R), 20.27 (1C; C-2″ acetyl linked to O-6′ AF.R), 24.32 (1C; C-2″ acetyl linked to N-4 cytosine ring), 62.35 (1C; C-6′ AF.R), 73.54 (1C; C-3′ AF.R), 75.97 (1C; C-4′ AF.R), 80.26 (1C; C-5′ AF.R), 84.99 (1C; C-2′ AF.R), 104.15 (1C; C-5 cytosine ring), 144.34 (1C; C-6 cytosine ring), 154.59 (1C, C-2 cytosine ring), 162.52 (1C; C-4 cytosine ring), 167.84 (1C; C-1″ acetyl linking to O-4′ AF.R), 169.22 (1C; RO-C=O; C-1″ acetyl linked to O-3′ AF.R), 170.12 (1C; C-1″ acetyl linked to O-6′ AF.R), 171.21 (1C; C-1″ acetyl linked to N-4 cytosine ring). ESI-MS (positive mode) m/z: Calculated for $C_{17}H_{20}ClN_3O_9$: 445.8084; found, 468,8 $[M+Na]^+$.

Viability assay

Cell lines employed: CHO-K1 (Chinese Hamster Ovary, ATCC N° CCL61) and MCF-7 (human breast adenocarcinoma, ATCC N° HTB22). Ham F12 (Sigma) and DMEM (Sigma) were used as culture media and fetal bovine serum (FBS, Gibco, Brazil) was implemented as a supplement. Penicillin and *streptomycin* (Gibco) were used as antibacterial and antifungal agents, respectively. The (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, thiazolyl blue) (MTT, Sigma). DMSO 99.9% purity (Sigma) and 2-propanol 98% purity (J. T. Baker), were employed at reagent grade. The absorbance was read in Multiskan FC Microplate Photometer–ThermoScientific spectrophotometer.

Cell culture

CHO-K1 and MCF-7 cell lines in exponential phase were cultured in Ham F-12 or DMEM medium, which were supplemented with FBS at 5% and treated with penicillin 100 U/mL and streptomycin 100 µg/mL. The cultures were incubated at 37°C for 48 hours. MCF-7 cell line was incubated in wet atmosphere with 5% CO_2.

MTT assay

MCF-7 and CHO-K1 cultured cells were disseminated in 96 well plates at a cell density of $5x10^3$ and $6x10^3$ cells per well, respectively. The cultures were treated with concentrations of 1, 10 and 100 µM of uridine, cytarabine or their derivatives and incubated at 37°C for 48 hours, followed by an addition of 10 µL of MTT (5 mg/mL) to each plate, which were, additionally, incubated in the dark at 37°C for 4 hours. Subsequently, 100 µL of acidified isopropanol were added to each plate and were agitated at room temperature for 1 hour. The absorbance was measured at 570 nm and cell viability percentage was calculated by three replicas of each treatment. Results were indicated with mean and standard deviation (X±SD) in at least two experiments. Statistical analysis was performed with ANOVA two-way, Bonferroni test with $p<0.05$ with GraphPad Prism Version 5.0 Software.

RESULTS

Chemical

The halogenation reaction with NCS and NBS allowed the selective substitution on the 5-position of uracil and cytosine ring of each nucleoside with quantitative conversions. As the esterification performed with acetic anhydride as reagent and solvent does not require heating or aggressive mediums (acidic or basic), it is an ideal reaction for highly acid labile substrates or thermo-labile, like nucleosides. This reaction is assisted with a nucleophilic catalyst, allowing the per-acylation of nucleosides, achieving the esters formation of all hydroxyl groups of ribose and arabinofuranosyl rings of uridine and cytarabine, respectively, and the amide formation on amine group of cytosine ring in the second nucleoside[4,21].

The three hydroxyl groups esterification in the uridine, is simple because the OH-3′ and OH-4′ position is *Z* among them, and *E* regarding the ribose ring and other hydroxyl group (position 6′); therefore, acetyl groups are oriented in the space like scissors. Nevertheless, this reaction in the cytarabine, is very complex as the hydroxyl groups of same positions are *E*, and the OH-3′ is oriented *Z* in relation to the cytosine ring; therefore, the steric interactions are strong, even so, it was possible to obtain the per-acetylated derivative of cytarabine considering that the acetyl group is small and the anhydride acetic is used in higher amounts.

The halogenated and per-acetylated derivatives were identified comparing ^{1}H-NMR, ^{13}C-NMR, ^{1}H-^{1}H COSY, HSQC and HMBC uridine and cytarabine data (300 MHz and 600 MHz; DMSO-*d*6). ^{1}H-NMR and ^{13}C-NMR signals for the monosaccharide rings and nitrogenous bases of the nucleoside derivatives, presented a significant deviation on their chemical shift from the respective substrates (uridine and cytarabine). The NMR data and masses founded with the MS spectrometry fit with the structures proposed.

Viability assay

The results of cell viability, obtained by MTT assay with uridine and cytarabine halogenated and their per-acetylated derivatives are shown in figure 3. The per-acetylated derivatives of uridine and cytarabine, did not present significant inhibition of cell viability with the cell lines implemented (CHO-K1 and MCF-7) at the lowest concentrations evaluated (1 μM and 10 μM). Nevertheless, a higher inhibition of cell viability on both cell lines was observed for the peracetylated and halogenated nucleoside evaluated. Moreover, only the uridine acetonide exhibited a significant inhibition (higher than 80%) of cellular viabilities of CHO-K1 and MCF-7 cell lines. The uridine presented a decrease (around 20%) on MCF-7 cell viability at the highest concentration tested (100 μM).

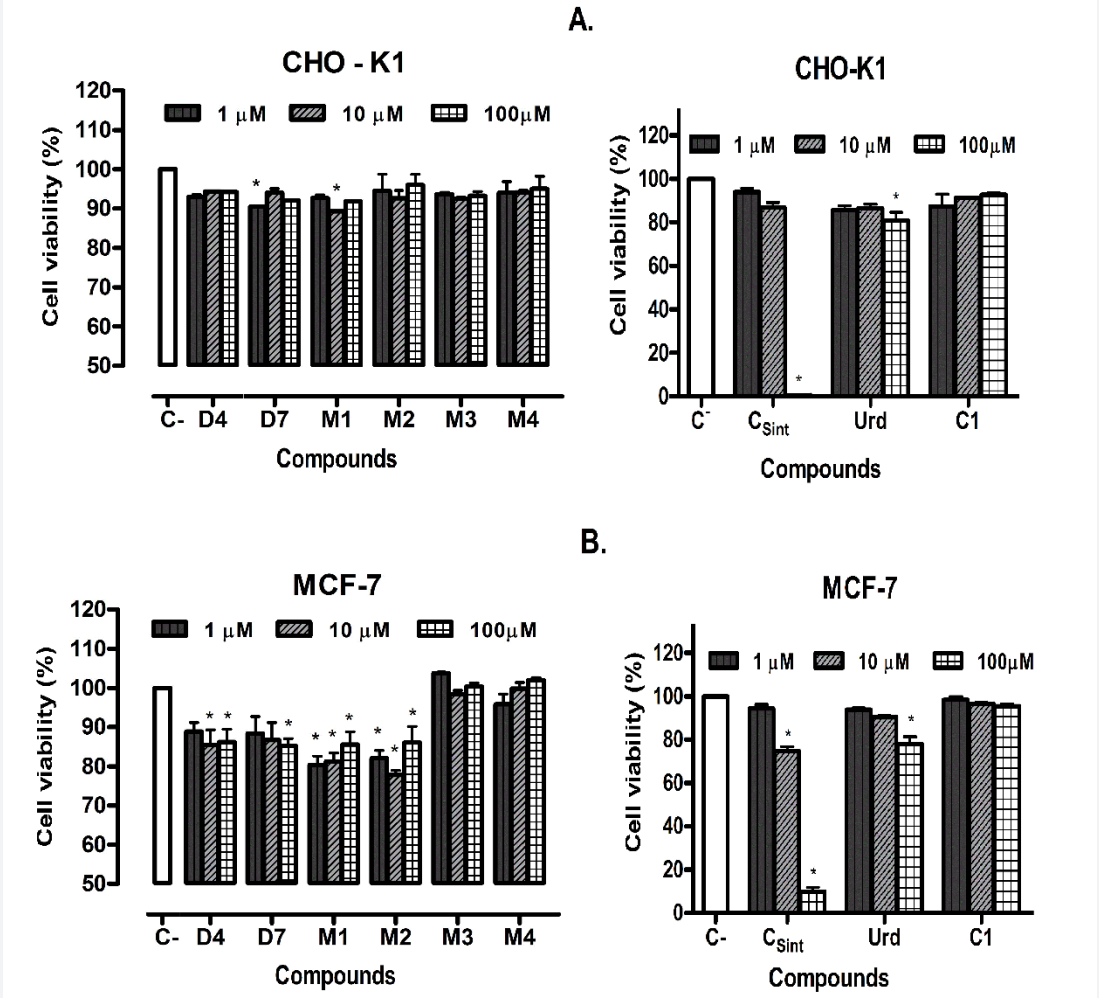

Figure 3. Cell viability of per-acetylated nucleoside (C1 and D7) an halogenated and per-acetylated derivatives (M1-M4), assessed with 1μM, 10μM and 100μM of the compounds by 48 hours. A. CHO-K1 cell line. B. MCF-7 cell line. C- (Control without treatment) and Urd: uridine. Data are shown as mean ± S.D. Statistical significance was calculated with $p<0.05$.

DISCUSSION

Chemical

The per-acetylated products were obtained in quantitative yields, possibly due to the use of acetic anhydride as reagent in the acylation and in the solvent mixture, thus the effective collisions among the substrate and reagent were increased significantly. Although the amount of substrate dissolved was low, the products are highly soluble in the reaction; this property causes the displacement to the right of the equilibrium between solid and dissolved substrates. Moreover, the use of the nucleophilic catalyst (DMAP) increases the rate reaction producing a higher product formation. As the acylation reagent was in vast amount, the intermediate performed with the DMAP, is generated quickly and continuously so is available for the nucleophilic attack by alcohol or amine groups of the nucleosides[4,21].

The COSY H-H spectrum for 3′,4′,6′-O-acetyl-uridine in DMSO-*d*6 presents at high-field, a multiplet coupling with shift δ=4.25 ppm equivalent to three protons (corresponding to overlapping of H-5′ and H-6′ protons of ribose ring) with a high deshielding signal δ=5.44 ppm (H-3′). Furthermore, this proton presents two couplings, with a shielding signal localized at δ=5.33 (H-4′) and other highly deshielded signal δ=5.87 ppm corresponding to an anomeric proton (H-2′) of the ribose ring. At down-field, a coupling between the proton appeared at δ=5.72 ppm (H-5 uracil ring) with the highest deshielding δ=7.69 (H-6 uracil ring). The COSY H-H spectrum for the 4-N-acetyl-3′,4′,6′-O-triacetyl-cytarabine in $CDCl_3$ shows at high field that the signals for the H-5′ and H-6′protons of arabinofuranosyl ring are not overlapped, neither are the H-3′and H-4′ protons. However, it is possible to observe a coupling between protons with higher shielding δ=4.16 ppm (H-5′) of arabinofuranosyl ring and the signals at δ=4.33 ppm (H-6′). Moreover, an intense coupling is observed for the proton localized at δ=5.49 ppm (H-3′) with a high deshielding signal, δ=6.29 ppm corresponding to an anomeric proton (H-2′) of the ribose ring. At down-field, it is observed a coupling between two deshielding signals, localized at δ=7.43 ppm and δ=7.88, corresponding to H-5 and H-6 protons of cytosine ring, respectively.

In the spectrums of the halogenated and per-acetylated derivatives of nucleoside the coupling at down-field disappeared therefore, the signal for the H-6 proton is see as singlete.

The HSQC spectrum for 3′,4′,6′-O-triacetyl-uridine in DMSO-*d*6, presents at high-field the expected couplings between the proton signals with highest shielding of the methyl groups and the highest shielding carbons, corresponding to alpha position of the acetyl groups. Additionally, two couplings by the multiplet corresponding to H-5′ and H-6′ protons, with two kinds of carbons: a highly shielding carbon δ=62.61 ppm and another highly deshielding carbon δ=80.18 ppm, corresponding to C-6′ and C-5′ carbons of the same monosaccharide, respectively. Furthermore, it is possible to perceive the expected couplings for signals of the H-4′, H-3′ and H-2′ protons of the ribose ring with three kinds of deshielding carbons, localized at δ=70.47 ppm, δ=72.61 ppm and δ=87.17, corresponding to C-4′, C-3′ and C-2′ carbons of the monosaccharide, respectively. At down-field, it is noted a coupling among the highest deshielding proton (H-6) and a high deshielded carbon, localized at δ=139.12 ppm (C-6 carbon of uracil ring). The HSQC spectrum for the 4-N-acetyl-3′,4′,6′-O-triacetyl-cytarabine in $CDCl_3$, shows at high field a coupling by the shielding proton at δ=4.16 ppm (H-5′) of the arabinofuranosyl ring with a high deshielding carbon δ=81.05 ppm, corresponding to C-5′ of the monosaccharide. In addition, it is possible to notice a coupling between the protons with chemical shift at δ=4.33 ppm (H-6′of arabinofuranosyl ring) and the shielding carbon at δ=62.77 ppm, corresponding to C-6′ of the monosaccharide, as well the expected couplings between the proton signals H-4′, H-3′ and H-2′ of arabinofuranosyl ring and the carbons corresponding to the same position of the monosacharide. At down-field, a coupling is detected for the proton signal of the H-5 citosine ring with deshielding carbon localized at δ=96.45 ppm, corresponding to C-5 of the same nitrogenus base and a second coupling between the proton signal with highest deshielding of the citosine ring and the highest deshielding carbon with chemical shift at δ=144.77 ppm (C-6 of nitrogenus base).

In the spectrums for the halogenated and per-acetylated compounds at down field, it is noticed only the coupling between the highest deshielding proton (H-6) of the nitrogenous base ring and a high deshielding carbon with chemical shift at δ=135-145 ppm corresponding to C-6 nitrogenous base ring.

Viability assay

The cytotoxic activity exhibited for the halogenated nucleoside and its per-acetylated derivatives has a non-significant effect. The negative result obtained is probably indicative of a phenomenon of resistance to the nucleoside for the MCF-7 cell line. This phenomenon may respond to three factors: an overexpression of receptors of tyrosine kinase (ErbB) that can accelerate the cell cycle and deactivate the cell cycle detainment performed by nucleosides[22]; an overproduction of the enzymatic targets pyr1-3 (the multifunctional polypeptide which comprises carbamyl-phosphate synthetase (EC 2.7.2.5), aspartate transcarbamylase (EC 2.1.3.2), dihydro-

orotase (EC 1.3.3.1); pyr5,6 (enzyme complex comprising orotate phosphoribosyltransferase (EC 2.4.2.10) and orotidine-5′-phosphate decarboxylase (EC 4.1.1.2), in which generally participates in the novo synthesis of pyrimidine nucleosides and in which the larger amounts of enzymes cause the inhibition of nucleoside savage route[23]; and when the transmembrane channels transport (of phosphoglycoprotein nature) for the nucleosides are overexpressed, as this can cause a reduction in the nucleoside cellular uptake from the exterior of the cell[24].

The uridine acetonide showed a higher inhibition effect on viability of MCF-7 cell line than the uridine. This can be explained by the simple activation (phosphorylation) over OH-6′ and the hydrolysis of acetonide group, also the substituent in the acetonide derivate can provide a lipophilicity ($LogP_{(uridine\text{-}acetonide)}$ = 0.20, $LogP_{(uridine)}$ = -1.61, $LogP_{(cytarabine)}$ = -1.93, predicted by ACD/Labs and ChemAxon®), hence the solubility and/or interaction with the lipophilic phase of the cellular membrane can increase. In the per-acetylated and halogenated nucleoside, the adjacent chains are very small, thus the hydrolysis of the acyls groups can be viable and thermodynamically favorable, but this process may result slower than the phosphorylation in the same position[3,4,8-10]. Furthermore, the per-acetylated derivatives of uridine and cytarabine did not show a higher lipophilicity ($LogP_{(uridine\text{-}triacetate)}$ = -0.11, predicted by ACD/Labs and ChemAxon®) than the nucleosides, therefore the interaction with the lipophilic phase of membrane cell was lower than in the acetonide[8-10,14]. Additionally, some of the nucleoside derivatives, particularly with voluminous substituents, present a great steric impediment, which can have a inhibitory effect on the hydrolysis of 6'-acyl-ester group of the ribose, exerted by esterase enzyme and the phosphorylation over OH-6′ of the ribose ring, is essential for the activation and action mechanism of the nucleosides[3,4,8,15].

CONCLUSIONS

The methods used allowed to obtain the acetylation of all hydroxyl and/or amine groups of substrates of the nucleoside type and the mono-halogenation of the nitrogenous base.

No significant effects on cell viability for the nucleoside and derivatives halogenated and per-acetylated were observed with $p<0.05$. Moreover, these compounds did not present a dose-response relationship, the uridine 3′,4′-acetonide showed a differential effect and a higher activity on cell viability on the MCF-7 human cancer breast cell line than on the CHO-K1 cell line. Nevertheless, the derivate 3′,4′-acetonide 6'-O-succinyl uridine showed lower activity on both cell lines than the uridine 3′,4′-acetonide. Furthermore, the breast cancer cell line MCF-7 used, according to the results, exhibits possible resistance to nucleosides and/or analogs. The per-acetylated and halogenated derivatives of the nucleosides are voluminous and present low activity, hence the decreased activation and the subsequent cytotoxic activity.

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