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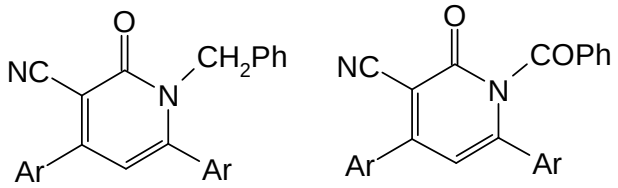
Graphical Abstract



11 **12**

Ar = *p*- ClC_6H_4-

Pyridine-3-carbonitrile derivatives possess significant antitumor activity comparable to the activity of commonly used anticancer drug, Doxorubicin

Anticancer Evaluation of Some Newly Synthesized *N*- Nicotinonitrile Derivative

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ABSTRACT

Some novel *N*-nicotinonitrile derivatives **3-14** have been synthesized starting with compound **1**. The key step of this work is the coupling between compound **1** and activated sugars to afford the corresponding cyclic nucleosides **3-6**. Moreover, the cytotoxicity and *in vitro* anticancer evaluation of the prepared compounds have also been assessed against breast MCF-7 cancer, liver HepG2 cancer and lung A549 carcinoma cell lines with investigation the effect of the synthesized compounds on the expression of urokinase plasminogen activator (uPA). The results revealed that, although all the compounds showed no anticancer activity against A549 cells without showing any effect on the expression of uPA, the tested compounds exhibited remarkable cytotoxicity activity against MCF-7 and HepG2 cell lines. Among the tested compounds, compounds **11** and **12** revealed promising anticancer activity compared to the activity of the commonly used anticancer drug, doxorubicin with inhibiting the expression of uPA.

***Keywords*:** Nicotinonitrile/ nucleosides/ Anticancer / Urokinase.

1. Introduction

Targeted therapies have a high specificity toward tumor cells, providing a broader therapeutic window with less toxicity. They are also often useful in combination with cytotoxic chemotherapy or radiation to produce additive or synergistic anticancer activity because their toxicity profiles often do not overlap with traditional cytotoxic chemotherapy. Thus, targeted therapies represent a new and promising approach to cancer therapy, one that is already leading to beneficial clinical effects. There are multiple types of targeted therapies available, including monoclonal antibodies, inhibitors of tyrosine kinases and antisense inhibitors of growth factor receptors [1].

Urokinase plasminogen activator (uPA) is a serine protease that functions in the conversion of the circulating plasminogen to the active, broad-spectrum, serine protease plasmin. uPA is secreted as an inactive single-chain proenzyme by many different cell types and exists in a soluble or cell-associated form by binding to a specific membrane uPA receptor (uPAR) [2,3]. The uPA is involved in many physiological functions and, along with members of the matrix

metalloproteinases (MMPs) family; it has been implicated in cancer invasion and metastatization [4-6]. Besides the proteolytic function, upon binding to uPAR, uPA is involved in initiating versatile intracellular signal pathways that regulate cell proliferation, adhesion, and migration through its interaction with various integrins and vitronectin [7]. Urokinase is implicated in a large number of malignancies, e.g. cancers of breast, lung, bladder, cervix, kidney, stomach and brain [8,9]. Also, the expression of urokinase is associated with tumor growth, invasion and may be a useful prognostic factor for hepatocellular carcinoma [10]. The role of uPA in human cancer progression is further supported by clinical evidences demonstrating that high tissue levels of its components correlate with a poor prognosis in different types of cancer as breast, gastrointestinal cancers [11].

On the other hand, the chemistry of cyclic and acyclic nicotinonitrile nucleosides has attracted attention during the last few decades because of its interesting pharmacological activities as antiviral [12], antitumor [13], antibacterial [14,15], anticancer [16,17] and other biological activity [18-25]. In the same direction and in continuation of our previous work for the synthesis of different nucleosides [26-28], to find more potent and selective anticancer compounds, we synthesized a series of cyclic nucleosides of pyridine derivatives.

2. Results and Discussion

2.1. Chemistry

Heating of 4,6-Bis-(4-chloro-phenyl)-2-oxo-1,2 -dihydro-pyridine-3-carbonitrile (**1)** [27] with hexamethyldisilazane (HMDS) in the presence of ammonium sulfate gave the silyloxypyridine derivative **2**, which was subsequently treated with 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-*β*-D-ribofuranose in the presence of $SnCl_4$ according to the method of Niedball and Vorbrüggen [29] to afford the corresponding *N*-riboside **3** (Scheme 1). The ^{1}H NMR spectrum of compound **3** showed a doublet at δ 6.05 ppm ($J_{1',2'}$ = 4.30 Hz) assigned to the anomeric proton indicating the *β*- configuration [28,30] and the other sugar protons resonate at δ 3.37–3.55(m, 1H, 4'-H); 4.43–4.76 (m, 4H, 2'-H, 3'-H, 5'-H_2). Similarly, when the silyloxypyridine derivative **2** was treated with *β*-D-glycopyranose pentaacetate in the presence of $SnCl_4$, it afforded the corresponding *N*-glycoside **4**. The ^{1}H NMR spectrum of compound **4** showed a doublet at 5.26 ppm ($J_{1',2'}$ = 10.40 Hz) assigned to the anomeric proton of the glucose moiety with a diaxial orientation of H-1' and H-2' indicating the *β*-configuration. The other protons of the glucopyranose ring resonate at 3.91–4.09 (m, 2H, 6'-H2); 4.26– 4.69 (m, 4H, 2'-H; 3'-H, 4'-H, 5'-H), while the four acetoxy groups appear as four singlets in the 2.01-2.23 ppm region providing further verification of the structure [31]. Debenzoylation of the blocked riboside **3** was achieved in methanolic ammonia at 0°C to afford the desired free 1-*β*-D-ribofuranosyl derivative **5**. The IR and ^{1}H NMR spectrum of the latter compound showed the presence of hydroxyl groups and the ^{13}C NMR spectrum providing further verification of the structure (see Experimental). Also, deprotection of compound **4** with methanolic ammonia at room temperature afforded the free *N*-glycoside **6** (Scheme 1). The ^{1}H NMR spectrum of the latter compound showed the anomeric proton as a doublet at δ 6.02 ($J_{1',2'}$ = 9.80 Hz) indicative for the *β*-D-configuration, and the signals of the other six carbon-bonded glucose protons appear as multiplets at δ 3.72–4.01 and 4.27–4.59, while the signals of the four hydroxyl groups of the glucose moiety are observed at δ 5.01, 5.12, 5.20 and 5.34 (exchangeable with D_2O). The ^{13}C NMR spectrum of compound **6** is characterized by the appearance of the C=O signal and the presence of a signal at δ 90.23 corresponding to the C-1' atom. Another five signals at δ 59.77, 64.21, 67.01, 70.68, and 74.11 are assigned to C-6', C-4', C-2', C-3' and C-5', respectively (see Experimental). Moreover, the IR and ^{13}C NMR

spectra of compounds **3-6** indicated that the site of attack was on the nitrogen since it behaves as a nucleophile which attacks an electrophilic carbon atom of an alkyl halide.

Moreover, the sodium salt of derivative **1** was treated with ethyl iodide, allyl bromide, propagyl bromide, chloro acetonitrile, benzyl chloride, benzoyl chloride, epichlorohydrine or 1,3-dichloro-2-prpanol to afford the corresponding N-substituted derivatives **7–14**, respectively (Scheme 2). The IR and ^{13}C NMR spectra of the latter compounds revealed the appearance of the C= O signal and the ^{1}H NMR spectra indicated the absence of the NH group and the presence of ethyl, allyl, prop-2ynyl, cyanomethyl, benzyl, benzoyl, oxiran-2ylmethyl and 3-chloro-2-hydroxypropyl signals, respectively (see Experimental). This due to the fact that the nitrogen atom behaves as a nucleophile attacks an electrophilic carbon atom of an alkyl halide [27].

Scheme 1

Scheme 2

2.2. Anticancer Evaluation

2.2. 1. In vitro cytotoxicity activity

The cytotoxicity of the synthetic compounds **1, 3-14** were tested using SRB assay as described by Skehan *et al*. [32] in breast cancer cell line MCF-7, liver cancer cell line HepG2, and lung carcinoma cell line A549. For comparison, doxorubicin was also tested. The results revealed that all the compounds did not exert any activity against lung carcinoma cell line A549.

In case of breast cancer cell line MCF-7 and liver cancer cell line HepG2 although compounds **1** and **10** showed no activity in the two cell lines, compound **12** (IC_{50}: 2.60±0.27 and 3.75±0.44 µg/ml, respectively) exhibited similar activity to doxorubicin (IC_{50}: 2.80±0.24 and 3.75±0.35µg/ml, respectively) in both MCF-7 and HepG2. The order of cytotoxicity activity of the tested compounds was **12, 11, 14, 13, 3, 5, 4, 6, 9, 8,** and **7** in a descending order.

2.2. 2. The level of uPA protein expression

To identify the mechanism of action responsible for the cytotoxicity of prepared compounds **1, 3-14** the level of uPA protein expressed in the two cell lines (breast cancer cell line MCF-7 and liver cancer cell line HepG2) were estimated quantitatively. The result revealed that the data of uPA expression were in consistent with the cytotoxicity activity.

In case of breast cancer cell line MCF-7 and liver cancer cell line HepG2, compounds **1** and **10** have no effect on the expression of uPA, the level of uPA decreased in compounds by the following percent in MCF-7 and HepG2 respectively **3 (**66, 60%**), 4 (**54, 48%**), 5 (**60, 56%**), 6 (**9, 6%**), 7 (**3, 2%**), 8 (**3, 5%**), 9 (**7, 5%**), 11 (**86, 83%**), 12 (**90, 87%**), 13 (**77, 63%) and **14 (**82, 67%). From the results, compound **12** exhibited a good activity in MCF-7 (90%) and HepG2 (87%) similar to doxorubicin (91% and 88%, respectively) (Table 1). in both MCF-7 and HepG2. The order of uPA activity inhibition of the tested compounds was **12, 11, 14, 13, 3, 5, 4, 6, 9, 8,** and **7** in a descending order which are in accordance with the cytotoxicity activity.

Taken together, these findings suggested that there are correlation between the cytotoxicity of the tested compounds and inhibition of the urokinase activity. The tested compounds exert anti-

carcinogenic activity in MCF-7 breast and HepG2 liver cancer cells through inhibiting the activity of urokinase enzyme which may reduce the cell proliferation and resulted in significant growth inhibitory.

Table 1.

2.3. Conclusion

In conclusion, the present results suggested that there are correlation between the cytotoxicity of the synthesized compounds **1**, **3-14** and inhibition of the urokinase activity. The tested compounds exert anti-carcinogenic activity in hepatic HepG2 and breast MCF-7 cancer cell lines through down-regulation the activity of urokinase enzyme which may reduce the cell proliferation and resulted in significant growth inhibitory, especially, compounds **11** and **12** which revealed promising activity compared to the activity of the commonly used anticancer drug, doxorubicin.

3. Experimental

3.1. Chemistry

All melting points are uncorrected and measured using Electro-Thermal IA 9100 apparatus (Shimadzu, Japan). Infrared spectra were recorded as potassium bromide pellets on a Perkin-Elmer 1650 spectrophotometer (Perkin-Elmer, Norwalk, CT, USA), National Research Center, Cairo, Egypt. ^{1}H NMR and ^{13}C NMR spectra were determined on a Jeol-Ex-400 NMR spectrometer (JEOL, Tokyo, Japan) and chemical shifts were expressed as part per million; (δ values, ppm) against TMS as internal reference (Organic Chemistry Department , Center of Molecular and Macromolecular Studies, Polish Academy of Science, Łódź, Poland). Mass spectra were recorded on VG 2AM-3F mass spectrometer (Thermo electron corporation, USA), National Research Center, Cairo, Egypt. Microanalyses were operated using Mario El Mentar apparatus, Organic Microanalysis Unit, and the results were within the accepted range (± 0.20) of the calculated values. Follow up of the reactions and checking the purity of the compounds was made by TLC on silica gel-precoated aluminum sheets (Type 60 F254; Merck, Darmstadt, Germany). Column Chromatography was performed on (Merck) Silica gel 60 (particle size 0.06–0.20 mm). Compound 1 was prepared according to a reported method [27].

3.1.1. Synthesis of 4,6-bis(4-Chlorophenyl)- 2-oxo-1-(2',3',5'-tri-O-benzoyl-β-D-ribofuranosyl)-1,2dihydro pyridine-3-carbonitrile (**3**)

Compound **1** (0.340 g, 1 mmol) was refluxed by stirring under anhydrous condition for 12 hours with hexamethyldisilazane (60 mL) and ammonium sulfate (0.02 g). The obtained clear solution was evaporated under reduced pressure and the resulting trimethylsilylated pyridine **2** was dissolved in dry 1,2-dichloromethane (40 mL) and a solution of 1-*O*-acetyl- 2,3,5-tri-*O*-benzoyl-*β*-D-ribofuranose (1 mmol) and $SnCl_4$ (1.6 mL) in dry 1,2-dichloromethane (10 mL) was then added dropwise with stirring under inert atmosphere (nitrogen gas) until the reaction was judged complete by thin layer chromatography. The reaction mixture was poured into saturated $NaHCO_3$ solution and extracted with $CHCl_3$ (2 × 30 mL). The extracts were collected and dried over anhydrous Na_2SO_4, filtered and concentrated producing the crude nucleoside that was purified on silica gel column using n-hexane: ethyl acetate (4:1) as an eluent to give **3**. Yield 73%; oil; IR (KBr, ν, cm^{-1}): 2214 (CN), 1716, 1682 (C=O); ^{1}H NMR ($CDCl_3$, δ ppm): 3.37-

3.55(m, 1H, 4'-H), 4.43–4.76 (m, 4H, 5'-H_2, 2'-H, 3'-H), 6.05 (d, 1H, $J_{1',2'}$ = 4.30 Hz, 1'-H), 7.49–8.15 (m, 24H, 23Ar-H + pyridine-H5); ^{13}C NMR ($CDCl_3$, δ ppm): 60.77 (C-5'), 67.28 (C-3'), 68.93 (C-2'), 73.01 (C-4'), 93.25 (C-1'), 114.37 (CN), 129.33–136.16 (Ar-C), 164.37, 174.34, 179.84, 185.12 (4C=O); Anal. Calcd. For $C_{44}H_{30}Cl_2N_2O_8$ (785.64): C 67.27, H 3.85, Cl 9.03, N 3.57. Found: C 66.97, H 3.61, Cl 8.74, N 3.21.

3.1.2. Synthesis of 4,6-bis(4-Chlorophenyl)- 2-oxo-1-(2',3',4'5'-tetra-O-acetyl-β-D-glucopyranosyl)-1,2-dihydro pyridine-3-carbonitrile (**4**)

The same procedure as in preparation of compound **3** was performed, but 1,2,3,4,6-penta-*O*-acetyl-*β*-D-glucopyranose (1 mmol) was used to give product **4**. Yield 59%; m.p. 183–185°C; IR (KBr, ν, cm^{-1}): 2222 (CN), 1713, 1688 (C=O). ^{1}H NMR ($CDCl_3$, δ ppm): 2.01, 2.09, 2.13, 2.23 (4s, 12H, 4CH_3CO), 3.91–4.09 (m, 2H, 6'-H2), 4.26–4.69(m, 4H, 5'-H, 4'-H, 3'-H, 2'-H), 5.26 (d, 1H, $J_{1',2'}$ = 10.40 Hz, 1'-H), 7.36–8.01(m,9H, 8Ar-H+ pyridine-H5); ^{13}C NMR (DMSO, δ ppm): 20.30–20.5 (4CH_3CO), 61.00 (C-6'), 66.49 (C-2'), 68.22 (C-3'), 71.69 (C-4'), 75.90 (C-5'), 81.80 (C-1'), 115.22 (CN), 125.11 – 139.02 (Ar-C), 167.90, 169.44, 169.72, 170.02,170.60 (5C=O); Anal. Calcd. For $C_{32}H_{28}Cl_2N_2O_{10}$ (671.49): C 57.24, H 4.20, Cl 10.56, N 4.17. Found: C 56.98, H 3.89, Cl 10.31, N 3.82.

3.1.3. Synthesis of 4,6-bis(4-Chlorophenyl)- 1-(1-β-D-ribofuranosyl)-2-oxo-1,2-dihydropyridine-3-carbonitrile (**5**)

Dry ammonia gas was passed into a solution of protected nucleoside **3** (1 mmol) in dry methanol (20 mL) at 0°C for 30 minute, then the reaction mixture was stirred at room temperature until the reaction was judged complete by TLC. The resulting mixture was then concentrated under reduced pressure at 40°C to afford a solid residue that was purified on silica gel column using chloroform: methanol (4:1) as an eluent to give **5**.Yield 53%; oil; IR (KBr, ν, cm^{-1}): 3444–3232 (OH), 2220 (CN), 1670 (C=O); ^{1}H NMR (DMSO-d_6, δ ppm): 3.53–3.57 (m, 3H, 4'-H, 5'-H_2), 3.88–4.89 (m, 2H, 2'-H, 3'-H), 4.60–4.62 (m,1H, OH, D_2O exchangeable), 4.72–4.74 (m, 1H, OH, D_2O exchangeable), 4.83– 4.87(m, 1H, OH, D_2O exchangeable), 6.53 (d, 1H, $J_{1',2'}$ = 4.60 Hz, 1'-H),7.61–8.31 (m, 9H, 8Ar-H+ pyridine-H5); ^{13}CNMR (DMSO, δ ppm): 62.01(C-5'), 71.22(C-3'), 76.21(C-2'), 81.55 (C-4'), 88.33 (C-l'), 115.51 (CN), 124.46 – 133.65 (Ar-C), 167.22 (C=O); Anal. Calcd. For $C_{23}H_{18}Cl_2N_2O_5$ (473.32): C 58.37,H 3.83, Cl 14.98, N 5.92. Found: C 58.09, H 3.63, Cl 14.71, N 5.60.

3.1.4. Synthesis of 4,6-bis(4-Chlorophenyl)- 1-(1-β-D-glucopyranosyl)-2-oxo-1,2-dihydropyridine-3-carbonitrile (**6**)

The same procedure as in preparation of compound **5** was used to give product **6** from **4**.Yield 42%; 284–286°C; IR (KBr, ν, cm^{-1}): 3460–3220 (OH), 2217 (CN), 1680 (C=O); ^{1}H NMR (DMSO-d_6, δ ppm): 3.72–4.01(m, 3H, 6'-H_2, H-5'), 4.27–4.59 (m, 3H, H-4', H-3', H-2'), 5.01 (m, 1H, OH,D_2O exchangeable), 5.12 (m, 1H, OH, D_2O exchangeable), 5.20 (m, 1H, OH, D_2O exchangeable), 5.34 (m, 1H, OH, D_2O exchangeable), 6.02 (d, 1H, $J_{1',2'}$ = 9.80 Hz, H-1'), 7.21 (s, 1H, pyridine-H5), 7.45–8.39 (m, 8H, Ar-H); ^{13}C NMR (DMSO, δ ppm): 59.77 (C-6'), 64.21 (C-2'), 67.01 (C-3'), 70.68 (C-4'), 74.11 (C-5'), 90.23 (C-1'), 115.28 (CN), 129.22–138.69 (Ar-C), 169.68 (C=O); Anal. Calcd. for$C_{24}H_{20}Cl_2N_2O_6$ (503.34): C 57.27, H 4.01, Cl 14.09, N 5.57. Found: C 56.89, H 3.74, Cl 13.81, N 5.22.

General for synthesis compounds **7-14**

To a solution of compound **1** (0.340 g, 1 mmol) in dry dimethylformamide (50 ml), 50% oil-immersed sodium hydride (0.20 g) was added. Thereafter the reaction mixture was stirred at room temperature for 1 hour, then ethyl iodide, allyl bromide, propagyl bromide, chloro acetonitrile, benzyl chloride, benzoyl chloride, epichloro hydrine, or 1,3-dichloro-2-propanol (1 mmol) was added. The reaction mixtures were stirred at 70°C for 3, 6, 4, 3, 5, 6, 2 and 2 hours, respectively. After evaporation under reduced pressure, the residues were purified on silica gel column using chloroform: methanol (9:1) as an eluent to give compounds **7**, **8**, **9**, **10**, **11**, **12**, **13** or **14**, respectively.

3.1.5. 4,6-bis(4-Chlorophenyl)-1-ethyl-2-oxo-1,2-dihydropyridine-3-carbonitrile (**7**)

Yield 64%; m.p. 149–151°C; IR (KBr, ν, cm^{-1}): 2218 (CN), 1678 (C=O); ^{1}H NMR ($CDCl_3$, δ ppm): 1.33 (t, 3H, *J* = 6.71Hz, $NCH_2\underline{CH_3}$), 3.33 (q, 2H, *J* = 7.20 Hz, $N\underline{CH_2}CH_3$), 6.97(s, 1H, pyridine-H5), 7.36–8.01(m, 8H, 8Ar-H). MS m/z (%): 372 (M^++4, 2), 370 (M^++2, 11), 368 (M^+, 21). Anal. Calcd. For $C_{20}H_{14}Cl_2N_2O_2$ (369.25): C 65.06, H 3.82, Cl 19.20, N 7.59 Found: C 64.84, H 3.49, Cl 18.92, N 7.33.

3.1.6. 1-Allyl-4,6-bis(4-chlorophenyl)-2-oxo-1,2-dihydropyridine-3-carbonitrile (**8**)

59% yield; m.p. 197–199°C; IR (KBr, ν, cm^{-1}) 2220 (CN), 1680(C=O); ^{1}H NMR ($CDCl_3$, δ ppm): 4.36 (d, *J*=5.1 Hz, 2H, NCH_2), 3.95 (d, *J*=9.8 Hz, 1H, CH_a), 4.03 (d, *J*=16.2 Hz, 1H, CH_b), 5.60 (q, *J*=16.2 Hz, 1H, CH), 7.67 (s, H, pyridine-H5), 7.76–8.31 (m, 8H, Ar-H); ^{13}C NMR ($CDCl_3$, δ ppm): 68.0 (NCH_2), 112.6 (CH_2), 115.51 (CN), 119.11 (CH), 128.31–138.15 (13Ar-C). MS m/z (%): 339 (M^+- 41, 100). Anal. Calcd for $C_{21}H_{14}Cl_2N_2O$ (381.26): C 66.16, H 3.70, Cl 18.60, N, 7.35. Found: C 65.77, H 3.39, Cl 18.41, N 7.09.

3.1.7. 4,6-bis(4-Chlorophenyl)-2-oxo-1(prop-2ynyl)-1,2-dihydropyridine-3-carbonitrile (**9**)

51% yield; m.p. 215–217°C; IR (KBr, ν, cm^{-1}): 2218 (CN), 1685 (C=O); ^{1}H NMR (300 MHz; DMSO-d_6, δ ppm): 2.81 (t, *J*=2.1 Hz, 1H, CH), 4.32 (d, *J*=2.2 Hz, 2H, NCH_2), 7.23 (s, 1H, pyridine-H5), 7.67– 8.11(m, 8H, Ar-H). MS m/z (%): 339 (M^+- 39, 100). Anal. Calcd for $C_{21}H_{12}Cl_2N_2O$ (379.25): C 66.51, H 3.19, Cl 18.70, N 7.39. Found: C 66.25, H 2.81, Cl 18.25, N 3.12.

3.1.8. 4,6-bis(4-Chlorophenyl)-1-(cyanomethyl)-2-oxo-1,2-dihydropyridine-3-carbonitrile (**10**)

47% yield; m.p. 165–167°C; IR (KBr, ν, cm^{-1}) 2222, 2216 (CN), 1677 (C=O); ^{1}H NMR ($CDCl_3$, δ ppm): 3.90(s, 2H, CH_2), 7.77– 8.29(m, 9H, 8Ar-H+pyridine-H5).MS m/z (%): 383 (M^++4, 6), 381 (M^++2, 31), 379 (M^+, 67). Anal. Calcd for $C_{20}H_{11}Cl_2N_3O$ (380.24): C 63.18, H 2.92, Cl 18.65, N 11.05. Found: C 62.89, H 2.66, Cl 18.41, N 10.91.

3.1.9. 1-Benzyl-4,6-bis(4-chlorophenyl)-2-oxo-1,2-dihydropyridine-3-carbonitrile (**11**)

42% yield; m.p. 209–211°C; IR (KBr, ν, cm^{-1}) 2217 (CN), 1680 (C=O); ^{1}H NMR (DMSO-d_6, δ ppm): 4.11 (s, 2H, CH_2), 7.36– 8.20(m, 14H, 13Ar-H+pyridine-H5).MS m/z (%): 434(M^++4, 3),

432 (M^++2, 15), 430 (M^+, 34). Anal. Calcd for $C_{25}H_{16}Cl_2N_2O$ (431.33): C 69.62, H 3.74, Cl 16.44, N 6.49. Found: C 69.49, H 3.52, Cl 16.16, N 6.22.

3.1.10. 1-Benzoyl-4,6-bis(4-chlorophenyl)-2-oxo-1,2-dihydropyridine-3-carbonitrile (**12**)

Yield 62%; m.p. 235–237°C; IR (KBr, ν, cm^{-1}): 2220 (CN), 1691, 1710 (2C=O); ^{1}H NMR ($CDCl_3$, δ ppm): 7.37–8.42(m, 14H, 13Ar-H, pyridine-H5); ^{13}C NMR ($CDCl_3$, δ ppm): 115.26 (CN), 125.21–142.40 (Ar-C), 169.67, 163.01 (C=O). MS m/z (%):448 (M^++4, 1), 446 (M^++2, 6), 444 (M^+, 13).Anal. calcd. For $C_{25}H_{14}Cl_2N_2O_2$ (445.31): C 67.19, H 3.17, Cl 15.92, N 6.29. Found: C 67.19, H 2.93, Cl 15.77, N 6.01.

3.1.11. 4,6-bis(4-Chlorophenyl)-1-(oxiran-2ylmethyl)-2-oxo-1,2-dihydropyridine-3-carbonitrile (**13**)

51% yield; m.p. 171–173°C; IR (KBr, ν, cm^{-1}): 2216 (CN), 1680; ^{1}H NMR (DMSO-d_6, δ ppm) δ 2.92 (dd, 1H, *J* = 1.50 Hz; *J* = 4.50 Hz, H-a'), 2.97 (dd, 1H, *J* = 4.20 Hz; *J* =4.55 Hz, H-a), 4.29–4.41 (m, 1H, H-b), 4.77 (dd, 1H, *J* = 2.17 Hz; *J* = 5.22 Hz, H-c'), 4.82 (dd, 1H, *J* = 2.27 Hz; *J* = 4.99 Hz, H-c), 7.24 (s, 1H, pyridine-H5), 7.66-8.33 (m, 8H, Ar-H). Anal. Calcd for $C_{21}H_{14}Cl_2N_2O_2$(397.26): C 63.49, H 3.55, Cl 17.85, N 7.05. Found: C 63.21, H 3.39, Cl 17.81, N 6.79.

3.1.12. 1-(3-Chloro-2-hydroxypropyl)-4,6-bis(4-chlorophenyl)-2-oxo-1,2-dihydropyridine-3-carbonitrile (**14**)

47% yield; m.p. 113–115°C; (KBr, ν, cm^{-1}): 3424–3230 (OH), 2217 (CN), 1680(C=O). ^{1}H NMR (DMSO-d_6,δ ppm): 3.10 (dd, 1H, *J* = 5.81 Hz, *J* = 10.23 Hz, H-c`), 3.37 (dd, 1H, *J* = 5.15 Hz, J = 10.23 Hz, H-c ), 3.83 (dd, 1H, *J* = 5.43 Hz, *J* = 11.13 Hz, H-a`), 4.01 (dd, 1H, *J* = 5.64 Hz, *J* = 11.17 Hz, H-a), 5.66 (m, 1H, H-b), 5.87 (d, 1H, *J* = 5.23 Hz, OH), 7.38 (s, 1H, pyridine- H-5), 7.39–7.77 (m, 8H, Ar-H); ^{13}C NMR (DMSO): 43.01 (CH_2Cl), 67.39(CHOH), 69.34 (NCH_2), 115.41 (CN), 129.31–136.00(13Ar-C), 164.54(C=O). Anal. Calcd for $C_{21}H_{15}Cl_3N_2O_2$ (433.72): C 58.16, H 3.49, Cl 24.52, N 6.46. Found: C 57.81, H 3.15, Cl 24.35, N 6.20.

3.2. Anticancer

3.2.1. Materials and methods

3.2.1.1. Chemicals

Fetal bovine serum (FBS) and L-glutamine, were obtained from Gibco Invitrogen Company (Scotland, UK). Dulbecco's modified Eagle's (DMEM) medium was provided from Cambrex (New Jersey, USA). Dimethyl sulfoxide (DMSO), doxorubicin, penicillin, and streptomycin were obtained from Sigma Chemical Company (Saint Louis, MO, USA). The level of uPA protein was determined using Assay Max human urokinase (uPA) ELISA kit (Assaypro, USA).

3.2.1. 2. Cell lines and culturing

Anticancer activity screening for the tested compounds utilizing 3 different human tumor cell lines including breast cancer cell line MCF-7, liver cancer cell line HepG2, and lung carcinoma cell line A549 were obtained from the American Type Culture Collection (Rockville, MD, USA). The tumor cells were maintained in Dulbecco's modified Eagle's medium (DMEM)

supplemented with 10% heat inactivated fetal calf serum (GIBCO), penicillin (100 U/ml) and streptomycin (100 µg/ml) at 37 ^{o}C in humidified atmosphere containing 5% CO_2. Cells at a concentration of 0.50 x 10^6 were grown in a 25 cm^2 flask in 5 ml of complete culture medium.

3.2.2. In Vitro cytotoxicity assay

The cytotoxicity activity was measured *in vitro* using the Sulfo-Rhodamine-B stain (SRB) assay according to the previous reported standard procedure [32]. Cells were inoculated in 96-well microtiter plate (10^4 cells/ well) for 24 h before treatment with the tested compounds to allow attachment of cell to the wall of the plate. Test compounds were dissolved in DMSO at 1 mg/ml immediately before use and diluted to the appropriate volume just before addition to the cell culture. Different concentration of tested compounds and doxorubicin were added to the cells. Triplicate wells were prepared for each individual dose. Monolayer cells were incubated with the compounds for 48 h. at 37°C and in atmosphere of 5% CO_2. After 48 h cells were fixed, washed, and stained for 30 min with 0.4% (w/v) SRB dissolved in 1% acetic acid. Unbound dye was removed by four washes with 1% acetic acid, and attached stain was recovered with Tris-EDTA buffer. Color intensity was measured in an ELISA reader. The relation between surviving fraction and drug concentration is plotted to get the survival curve for each cell line after the specified time. The concentration required for 50% inhibition of cell viability (IC_{50}) was calculated and the results are given in Table 1. The results were compared to the antiproliferative effects of the reference control doxorubicin.

3.2.3. Determination the level of uPA protein expression

The level of uPA protein expression was determined using AssayMax human urokinase (uPA) ELISA kit (Assaypro, USA) according to manufacturer's instructions. The prepared compounds as well as standard drug, doxorubicin were incubated for 48 h with MCF7, HepG2 and A549 cells at concentration of 1/10 of the IC_{50} values of each compound which shown in Table 1.

After 48 h from compounds treatment, medium was collected and centrifuged at 2000 xg for 10 min to remove cellular debris. Add 50 µl of the cell extract per well and incubate for 2 h. Wells were washed with 200 µl of wash buffer then add 50 µl of biotinylated uPA antibody to each well and incubate for 1 h at 25ºC. After washing, plates were incubated with 50 µl of streptavidin-peroxidase conjugate per well and incubate for 30 minutes then wash the microplate as described above. Add 50 µl of chromogen substrate per well and incubate for about 10 min or till the optimal blue color density develops. Add 50 µl of stop solution to each well. The color will change from blue to yellow. Read the absorbance on a microplate reader at a wavelength of 450 nm immediately and the concentrations of uPA in the samples were determined and the percentage of uPA inhibition for each compound was calculated as compared with control cancer cells (DMSO treated).

3.2.4. Statistical analysis

The results are reported as Mean ± Standard error (S.E.) for at least three times experiments. Statistical differences were analyzed according to followed by one way ANOVA test followed by student's *t* test wherein the differences were considered to be significant at $p < 0.05$.

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Highlights

► Novel *N*-nicotinonitrile derivatives **3-14** have been synthesized. ► The anticancer evaluation of all prepared compounds has been assessed against breast MCF-7 cancer, liver HepG2 cancer and lung A549 carcinoma cell lines. ► Compounds **11** and **12** caused down-regulation of urokinase activity and revealed promising activity compared to doxorubicin.

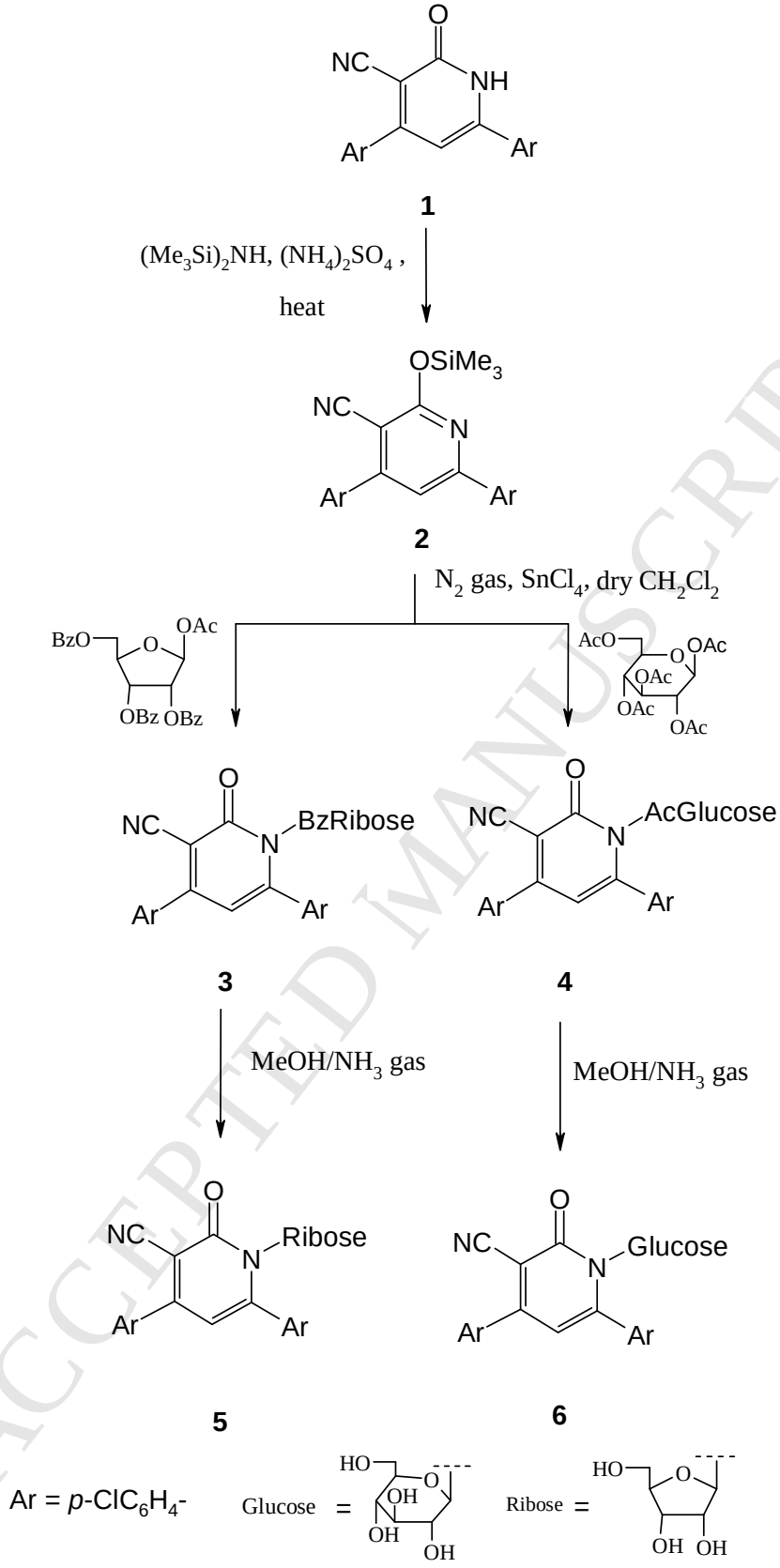
Table 1. *In vitro cytotoxicity activity and the percent inhibition of uPA of the synthesized compounds on different cell lines.*

Compound	IC_{50} (µg/ml)			% inhibition of uPA [a]		
	MCF-7	HepG2	A549	MCF-7	HepG2	A549
Doxorubicin	2.80±0.24	3.90±0.37	4.11±0.40	91±3.70	88±6.36	82±6.35
DMSO	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
1	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
3	5.90±0.56	8.00±0.92	N.A.	66±5.44	60±5.09	N.A.
4	6.30±0.60	7.25±0.35	N.A.	54±4.93	48±4.82	N.A.
5	5.70±0.72	6.90±0.65	N.A.	60±2.84	56±1.98	N.A.
6	14.18±1.36	16.10±1.40	N.A.	9±0.47	6±0.45	N.A.
7	19.70±1.90	22.30±2.32	N.A.	3±0.45	2±0.27	N.A.
8	19.00±1.85	20.00±1.92	N.A.	3±0.45	5±046	N.A.
9	16.70±1.50	18.30±1.75	N.A.	7±0.94	5±0.46	N.A.
10	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
11	3.65±0.29	4.20±0.44	N.A.	86±3.09	83±4.65	N.A.
12	2.60±0.27	3.75±0.44	N.A.	90±5.76	87±6.45	N.A.
13	5.00±0.63	6.20±0.80	N.A.	77±4.28	63±4.65	N.A.
14	4.60±0.48	4.80±0.47	N.A.	82±6.95	67±4.78	N.A.

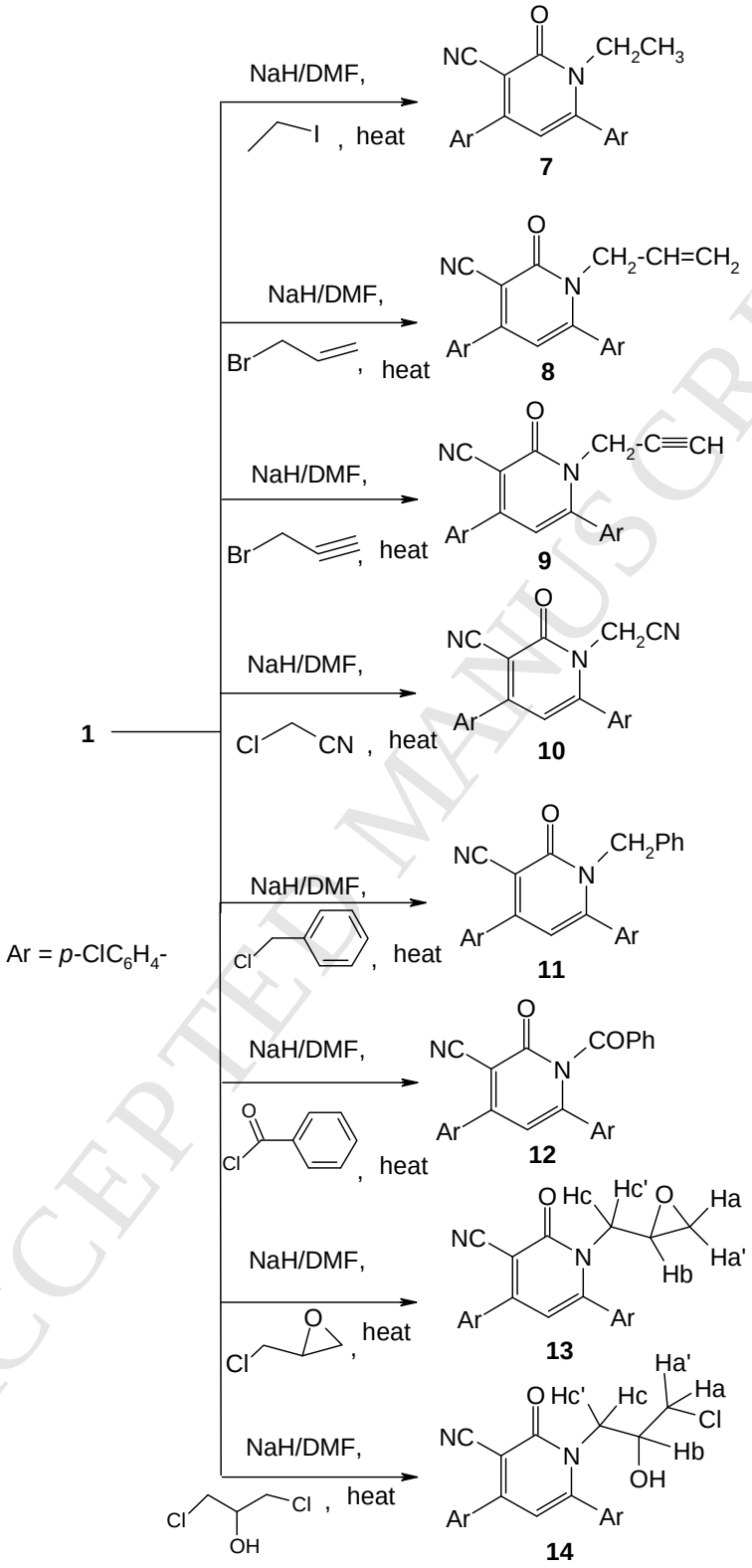
Data were expressed as Mean ± Standard error (S.E.) of three independent experiments.

[a]The percentage changes as compared with control untreated cancer cells (DMSO treated).

N.A. is no activity



Scheme 1. Synthesis of compounds **2-6**



Scheme 2. Synthesis of compounds **7-14**