Regular Article

Novel Polyfunctional Pyridines as Anticancer and Antioxidant Agents. Synthesis, Biological Evaluation and *in Silico* ADME-T Study

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Received September 23, 2016; accepted February 20, 2017

Two series of novel alkoxylated 2-oxo(imino)-3-pyridinecarbonitriles (structurally-relevant to some reported anticancer pyridines with phosphodiesterase 3A (PDE3A) inhibitory activity) were synthesized and evaluated for their *in vitro* differential tumor cell growth inhibitory potential against the breast MCF7, hepatocellular Hep-G2, colon CACO-2 cell lines, and a normal human foreskin fibroblast Hs27 cell line. Compounds 8, 16 and 19 displayed recognizable growth inhibitory ability and selectivity towards the breast MCF7 (LC_{50} 19.15, 17.34 and 14.70 μM, respectively) as compared with doxorubicin (LC_{50} 3.94 μM). Meanwhile, compounds 8, 15, 16, and 19 revealed a marginal inhibitory effect on the growth of the normal human foreskin fibroblast Hs27 cell line, beside a distinctive antioxidant potential in the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay. These four compounds were further assessed for their *in vitro* inhibition of PDE3A (a current antitumor therapeutic target), where 16 and 19 showed moderate to weak PDE3A inhibitory as compared with milrinone, the positive control. No clear straightforward liaison between the anticancer potential and PDE3A inhibitory activity could be deduced. Computations of the predicted pharmacokinetic properties, toxicity effects (ADME-T), drug-likeness and drug scores for the newly developed compounds showed non-violations of Lipinski's RO5 and Veber's criteria for good bioavailability, with a predicted high safety profile.

Key words pyridine; anticancer; antioxidant; phosphodiesterase-3A (PDE3A); pharmacokinetic property and effect (ADME-T)

The success of the conventional chemotherapeutic agents in the management of cancer was hampered by their inherent toxicity, harmful side effects together with the evolution of drug resistance by tumor cells. Therefore, along the past two decades there has been a noticeable movement towards the recognition of the biomolecular aspects of malignancy for the development of more safe and selective anticancer agents.[1,2]

Among the various cellular and molecular processes recently studied, the impairment of intracellular cyclic nucleotides cAMP and/or guanosine 5′-cyclic monophosphate (cGMP) production by excessive expression of phosphodiesterases (PDEs) has been linked to diverse cancer etiologies.[3] In other words, modulation of the levels of such cyclic nucleotides through inhibiting their breakdown by PDEs has received particular attention as a promising approach in tumor therapy.[4] Eleven families of PDEs (PDE1–11), including many isoforms, have been identified, which vary in their amino acid sequences, tissue distribution, substrate affinities, type of inhibitors and their bioregulatory pathways.[5] Thorough literature survey revealed that concomitant inhibition of certain PDE isoforms (*e.g.* PDE3, PDE4 and PDE5) led to elevation in the intracellular cAMP and/or cGMP levels which was reported to retrieve regular intracellular signaling, inhibit tumor cell growth, retard angiogenesis, induce apoptosis and cell cycle arrest in malignant rather than normal cells.[6–8] In particular, beside the documented cardiovascular contributions of the PDE3 isozymes (PDE3A and B),[9] overexpression of PDE3s has been accounted for the induction of several malignancies including osteosarcoma, squamous cell carcinoma, melanoma, and malignant salivary gland cells.[10,11] Moreover, PDE3 inhibition was reported to suppress cell proliferation of certain leukemia, breast, cervical and prostate cancer cell lines.[12–14] Subsequently, finding out novel specific PDE3 inhibitors became a prominent selective target for anticancer therapy, which offers an unprecedented opportunity to rescue healthy cells from the destructive harmful effects of conventional cytotoxic agents.

On the other hand, oxidative stress generated by the predominance of extravagant reactive oxygen species (ROS) that forcibly offence and destroy different body cells is known to lead to oxidative damage of DNA; a leading cause of carcinogenesis.[15] Moreover, ROS are known to provoke tumor growth and metastasis through induction of angiogenesis,[16] therefore, antioxidants capable of scavenging free radicals proved to contribute efficiently in the treatment of many types of cancer.[17]

As far as the chemotherapeutic potential is concerned, pyridine derivatives have come into view as heterocycles with versatile antimicrobial,[18] antitubercular[19] and anticancer[20–22] activities. Most notably, several functionalized pyridinecarbonitriles have received much interest for their peculiar antineoplastic[23–25] as well as antioxidant[26,27] activities. In this context, various 2-oxopyridine-3-carbonitriles and their 2-amino isosteres have revealed promising molecular-based anticancer activities being able to interfere with Pim-1 proto-oncogene, serine/threonine kinase (PIM1) Kinase and survivin protein which played articular roles in suppressing cancerous cell survival and proliferation, in addition to induction of apoptosis.[28,29] Several *in silico* molecular docking and *in vitro* testing studies have emphasized the importance of the pyridinecarbonitrile scaffold as novel PDE3 inhibitors and

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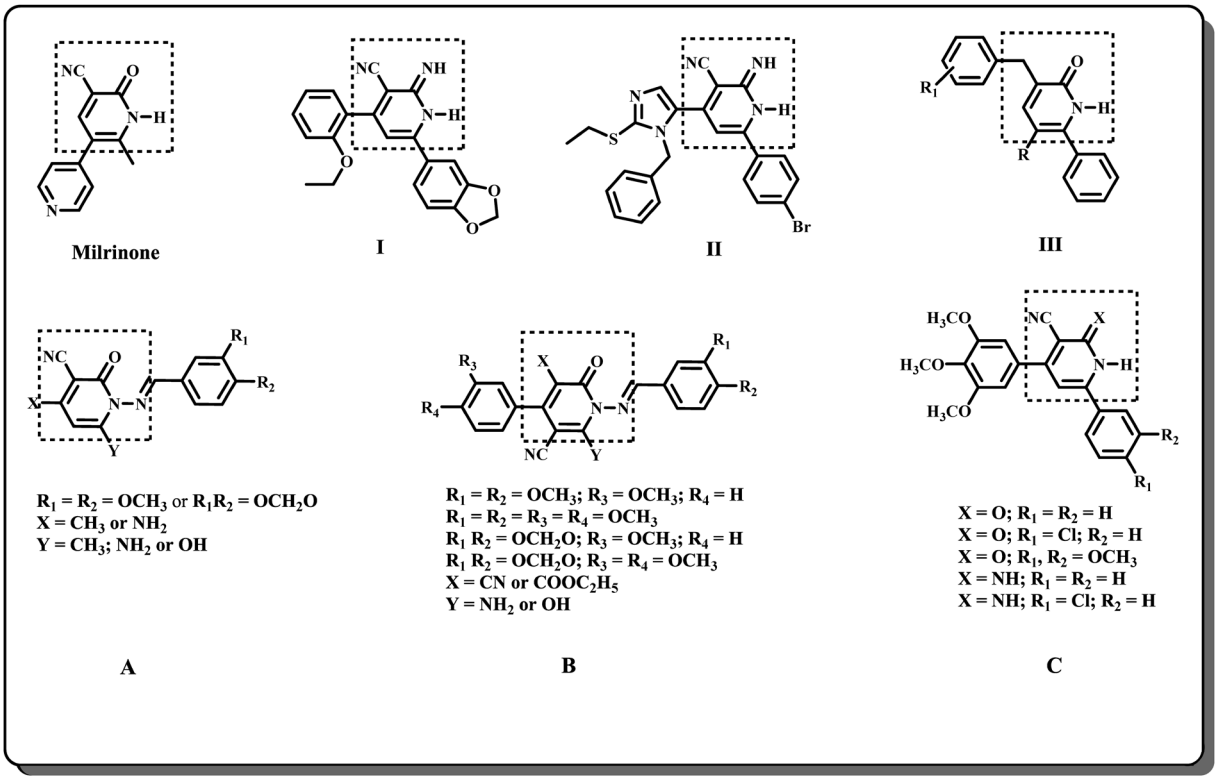


Fig. 1. 2-Oxo(imino)pyridine-3-carbonitrile-Based PDE3 Inhibitors

Milrinone, **I**[30], **II**[34], **III**[35] and the proposed structure modifications in the scaffold to obtain the target compounds **A**–**C**.

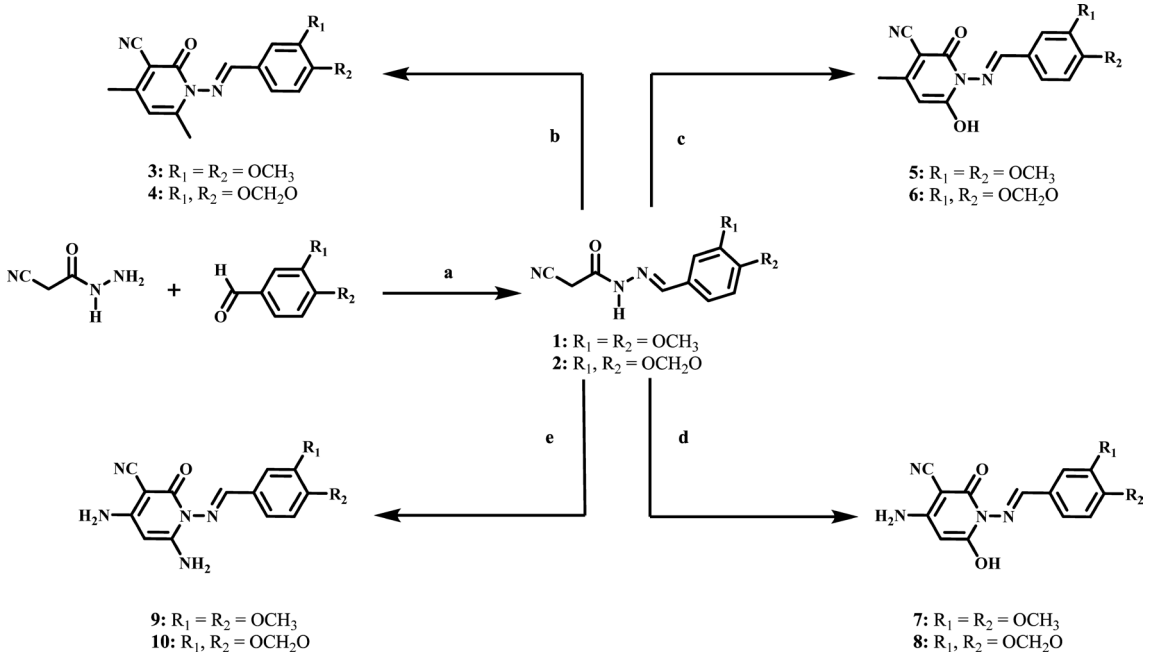
anticancer agents.[30–33] Among these, compound **I** (Fig. 1) stemmed as the most potent PDE3A inhibitor with significant cytotoxic potential.[30] Furthermore, another relevant series belonging to the previously-descibed skeleton with a C_4 2-alkylthio-1-benzyl-5-imidazolyl moiety as a basic counterpart was reported,[34] where *in silico*, *in vitro* PDE3A and anticancer studies suggested the existence of a clear correlation between their biological activities and PDE3A's molecular binding mode. Among which compound **II** exhibited the strongest PDE3A inhibition with IC_{50}=3.76 nM (Fig. 1). Additionally, a molecular study of a series of 2-pyridones (**III**; Fig. 1) with a substitution pattern relevant to the PDE3 inhibitor milrinone (Fig. 1), revealed a considerable affinity of five analogs to the PDE3A isozyme.[35] In previous publications, during our continuous efforts to find out novel pyridine-containing lead structures with diverse chemotherapeutic activities, the substituted 2-oxo(imino)pyridine-3-carbonitrile scaffold have emerged as a corner stone for building potential broad-spectrum antitumor and/or antimicrobial agents.[36–41]

In this investigation, we report the synthesis of some novel polyfunctionalized pyridines with the general structures **A**–**C** (Fig. 1). The targeted compounds were designed so as to preserve the 2-oxopyridine-3-carbonitrile scaffold, while introducing additional cyano and/or carboxylate functionalities as H-bond acceptors, and OH and/or NH_2 groups as H-bond donors. Alkoxylated aryl rings (either directly hocked to the pyridinecarbonitrile moiety at C_1, C_4 and/or C_6, or separated by an azavinyl linkage at N_1) were selected as basic counterparts owing to their reported augmentation of several chemotherapeutic activities through glorification of the overall lipophilicity of the molecules, beside their documented role in exerting antioxidant potential.[42–44] The selection of the azavinyl linkage based on literature reports revealing the remarkable contribution of this functionality in diverse anticancer activities.[45–47] Such substitution pattern was thought to be useful in assisting the interaction of the target molecules with different cellular targets through various intermolecular forces.

The differential *in vitro* anticancer potential of the target compounds was investigated against a panel of three human tumor cell lines including the Caucasian breast adenocarcinoma MCF7, hepatocellular carcinoma Hep-G2, colon carcinoma CACO-2, and normal human foreskin fibroblast Hs27 cell lines. In a trial to search for a plausible mechanism behind the anticancer efficacy of the most active compounds, their antioxidant activity was determined using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, and the *in vitro* inhibitory activity against PDE3A isozyme was measured. Additionally, an *in silico* computation of the molecular properties, physicochemical profile, drug score and drug-likeness of the biologically active compounds was performed to predict their pharmacokinetic and toxicity properties (ADME-T), and to assess their suitability to serve as possible orally-active dug candidates.

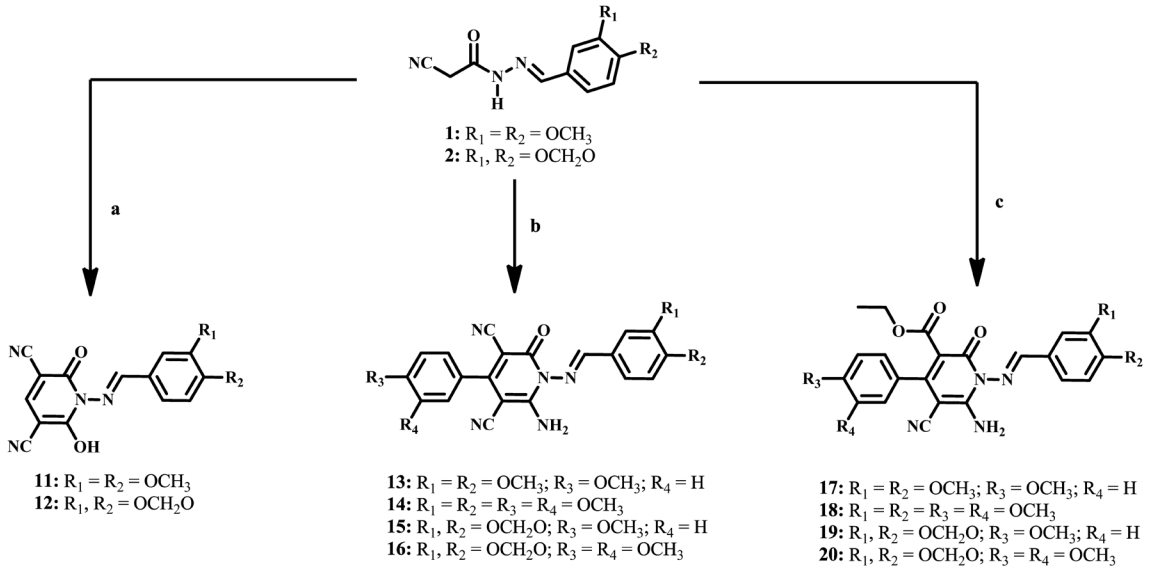
Results and Discussion

Chemistry The synthetic strategies adopted for the synthesis of the intermediates and target compounds are depicted in Charts 1, 2 and 3. In Chart 1, the key intermediates (*E*)-*N*′-(3,4-disubstituted benzylidene)-2-cyanoacetohydrazide **1** and **2** were prepared by reacting cyanoacetic acid hydrazide with the appropriate substituted benzaldehyde.[48] Refluxing **1** and **2** with acetyl acetone, ethyl acetoacetate, ethyl cyanoacetate or malononitrile in absolute ethanol containing catalytic amount of piperidine afforded the targeted (*E*)-1-((3,4-disubstituted benzylidene)amino)-4,6-



Reagents and conditions: a) Ethanol, r.t., 1h; b) Acetyl acetone, piperidine, ethanol, reflux, 5h.; c) Ethyl acetoacetate, piperidine, ethanol, reflux, 4h; d) Ethyl cyanoacetate, piperidine, ethanol, reflux, 3h; e) Malononitrile, piperidine, ethanol, reflux, 3h.

Chart 1. Synthesis of Compounds **1**–**10**



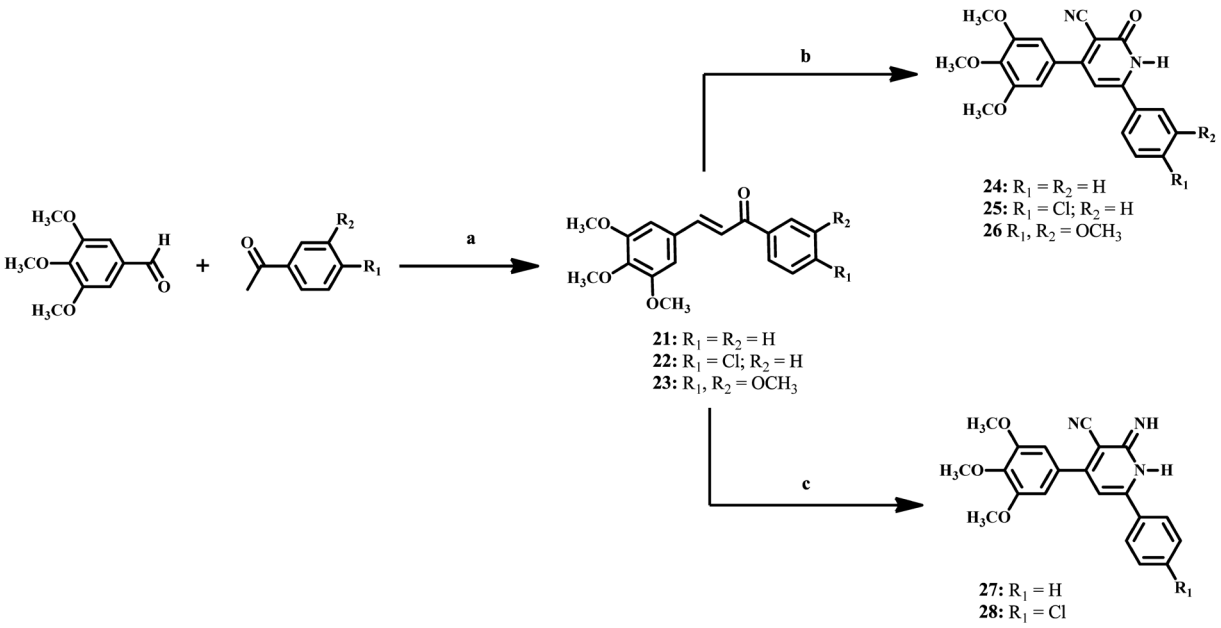
Reagents and conditions: a) Ethyl ethoxymethylenecyanoacetate, potassium carbonate, ethanol, reflux, 3h; b) 2-Arylidenemalononitrile, piperidine, ethanol, reflux, 3h.; c) 2-Arylidenecyanoacetae, sodium ethoxide, ethanol, reflux, 2h.

Chart 2. Synthesis of Compounds **11**–**20**

disubstituted-2-oxo-1,2-dihydropyridine-3-carbonitriles **3**–**8**.

In Chart 2, reacting **1** and **2** with ethyl ethoxymethylenecyanoacetate in the presence of anhydrous K_2CO_3 produced the respective (*E*)-1-((3,4-disubstituted benzylidene)amino)-6-hydroxy-2-oxo-1,2-dihydropyridine-3,5-dicarbonitriles **11** and **12**. On the other hand, cyclocondensation of **1** and **2** with the appropriate 2-arylidenemalononitrile in the presence of piperidine resulted in the formation of 2-oxo-1,2-dihydropyridine-3,5-dicarbonitrile derivatives **13**–**16**. Whereas, the ethyl carboxylate analogs **17**–**20** were obtained by refluxing **1** and **2** with 2-arylidenecyanoacetate in the presence of sodium ethoxide.

Shifting to Chart 3, (*E*)-1-aryl-3-(3,4,5-trimethoxyphenyl)-prop-2-en-1-ones **21**–**23** were served as key intermediates. They were prepared in excellent yields by condensing 3,4,5-trimethoxybenzaldehyde with the appropriate substituted acetophenone in the presence of potassium hydroxide according to a reported literature procedure.[49] Refluxing **21**–**23** with either ethyl cyanoacetate or malononitrile in the presence of ammonium acetate yielded the corresponding 2-oxo(imino)-1,2-dihydropyridines **24**–**28**.



Reagents and conditions: a) 6% ethanolic KOH, r.t., 24h; b) Ethyl cyanoacetate, ammonium acetate, ethanol, reflux, 12h; c) Malononitrile, ammonium acetate, ethanol, reflux, 24h.

Chart 3. Synthesis of Compounds **21**–**28**

Table 1. Cytotoxic Effects (LC_{50}; μM)[a] of the Active Compounds on Three Human Tumor and One Normal Non-transformed Cell Lines Using the MTT Assay

Cpd. No.	Human tumor cell lines			Human normal non-transformed cell line
	MCF7[b]	Hep-G2[c]	CACO-2[d]	Hs27[e]
6	116.73	—[f]	—	ND[g]
8	19.15	48.95	121.71	>500
10	41.04	109.33	226.73	ND
15	30.67	125.55	142.00	>500
16	17.34	45.78	111.63	>500
19	14.70	—	—	>500
25	35.42	111.64	—	ND
28	70.23	—	—	ND
Dox.[h]	3.94	3.11	22.81	ND

a) LC_{50}: Lethal concentration of the compound which causes death of 50% of cells in 24h (μM). *b*) MCF7 (Caucasian breast adenocarcinoma cell line). *c*) Hep-G2 (Human hepatocellular carcinoma cell line). *d*) CACO-2 (Human colon carcinoma cell line). *e*) Hs27 (normal non-transformed human foreskin fibroblast cell line). *f*) Totally inactive against this cell line. *g*) ND: not determined. *h*) Doxorubicin: positive control cytotoxic agent.

Biology

In Vitro 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT) Cytotoxicity Assay

The newly synthesized target compounds **3**–**20** and **24**–**28** were subjected to evaluation of their *in vitro* cytotoxic effect utilizing the standard MTT method[50,51] against a panel of three human tumor cell lines namely; Caucasian breast adenocarcinoma MCF7, hepatocellular carcinoma Hep-G2, colon carcinoma CACO-2, and a normal human foreskin fibroblast Hs27 cell line. The results are presented in Table 1 as LC_{50} (μM) which is the lethal concentration of the compound that causes death of 50% of the cells in 24h.

The obtained data revealed that, eight compounds namely; **6**, **8**, **10**, **15**, **16**, **19**, **25** and **28** were able to affect the viability of the tested tumor cell lines particularly the breast MCF7, whereas the rest of the investigated compounds were totally inactive. Moreover, the three tested human tumor cell lines exhibited a variable degree of sensitivity profiles towards the active compounds. For instance, the MCF7 cell line showed a recognizable sensitivity against compounds **8**, **16** and **19** (LC_{50} 19.15, 17.34 and 14.70 μM, respectively) which represented nearly 20–27% of the activity of doxorubicin (LC_{50} 3.94 μM). A moderate cytotoxic potential was displayed by compounds **10**, **15** and **25** (LC_{50} 41.04, 30.67 and 35.42 μM, respectively), whereas the rest of the active compounds showed weak efficiencies against the same cell line. On the other hand, the growth of Hep-G2 cell line was found to be mildly inhibited by five compounds (**8**, **10**, **15**, **16** and **25**) with LC_{50} range 45.78–125.55 μM, among which the analogs **8** and **16** were the most active (LC_{50} 48.95 and 45.78 μM, respectively). Regarding the CACO-2 cell line, it was proved to be the least sensitive tumor cell line as its growth was affected only by four analogs (**8**, **10**, **15** and **16**), with LC_{50} range 111.63–226.73 μM, representing nearly 10–20% of the activity of doxorubicin (LC_{50} 22.81 μM) against the same cell line. Taking both the growth inhibitory potential and the spectrum of cytotoxic activity as preference parameters, compounds **8** and **16** have stemmed as the most distinguished members in this study, with special effectiveness against the breast MCF7 tumor cell line, as compared with doxorubicin. Additionally, a marginal growth inhibitory effect on the Hs27 normal cell line (LC_{50} values >500 μM) was displayed by the analogs **8**, **15**, **16** and **19** (which showed distinctive growth inhibitory potential on the breast MCF7 tumor cell line), confirming their differential cytotoxicity towards tumor rather than normal cell lines.

Structure–Activity Correlation

A close examination of the structures of the active compounds revealed that they belong to two series: 1-((3,4-disubstituted benzylidene)amino)-4,5,6-trisubstituted-2-oxo-1,2-dihydropyridine-3-carbonitriles **3**–**20** (Charts 1, 2) and the

4,6-disubstituted-2-oxo(imino)-1,2-dihydropyridines **24**–**28** (Chart 3). In general, compounds belonging to the first series comprising the *N*-azavinylpyridine scaffold displayed better cytotoxic effects than the second one. Besides, the biological activity proved to be governed with the type and electronic nature of the substituents. For instance, compounds comprising the benzodioxole moiety (R_1, R_2=OCH_2O), displayed better cytotoxic effects when compared with the 3,4-dimethoxyphenyl congeners. In addition, the introduction of H-bond donors (*e.g.*, hydroxyl and amino groups) and H-bond acceptors (cyano and/or carboxylate functions), together with the pivotal 2-oxo and 3-carbonitrile groups on the pyridine ring, seems to modulate the targeted cytotoxic activity.

Among the first series, in spite of the inactivity shown by the 4,6-dimethyl derivatives (**3** and **4**), yet the 6-hydroxy analog **6** (R_1, R_2=OCH_2O) displayed a weak cytotoxic effect against MCF7 cell line. Introducing an additional amino group at the pyridine C_4 furnished compound **8** (R_1, R_2=OCH_2O), with a noticeable enhancement in both cytotoxic potency and spectrum as shown from its capability of inhibiting the growth of the three tested cell lines, with activity percentages lying between 6–20% of that of doxorubicin. This improvement might be ascribed to the increase in H-bond forming ability with certain tumor cell targets caused by both the hydroxyl and amino group. However, isosteric replacement of the 6-hydroxyl functionality with another amino group as shown in the 4,6-diamino derivative **10** (R_1, R_2=OCH_2O), resulted in about two-fold reduction in the cytotoxic potential against MCF7 and Hep-G2 cell lines, when compared with the analog **8**. This could be attributed to the reduction in the electronegativity of the amino group when compared with that of the hydroxyl one. On the other hand, while the 3,5-dicarbonitrile derivatives **11** and **12** were deprived of any cytotoxic efficacy, yet increasing the lipophilic character by introducing an alkoxylated phenyl substituent at the pyridine C_4, yielded two active compounds **15** (R_1, R_2=OCH_2O; R_3=OCH_3, R_4=H) and **16** (R_1, R_2=OCH_2O; R_3=R_4=OCH_3), among which the analog **16** displayed acceptable cytotoxic spectrum and potency profiles, showing 23 and 25% of the activity of doxorubicin against MCF7 and CACO-2 cell lines, respectively. Surprisingly, exchanging the essential C_3 carbonitrile functionality in **15** with a carboxylate as in **19** (R_1, R_2=OCH_2O; R_3=OCH_3; R_4=H), resulted in a two-fold improvement in the activity and selectivity towards the breast MCF7 cell line (14.7 *vs* 30.67 μM, respectively), however, with loss of activity against the other tested tumor cell lines.

In the second series (Chart 3), structure modifications involved the exchange of the benzylidineamino counterpart at position-1 with a lipophilic phenyl moiety at position-6, while conserving the essential 2-oxo (or 2-amino) and the 3-carbonitrile functionalities, together with the 4-substituted phenyl moiety. Such transformation furnished two mildly active compounds **25** and **28**, with an obvious reduction in both cytotoxic potential and spectrum (Table 1).

DPPH Radical Scavenging Antioxidant Assay

Compounds **6**, **8**, **10**, **15**, **16**, **19**, **25** and **28** which exhibited cytotoxic activity, were further evaluated for their antioxidant potential through their ability to scavenge the DPPH radical, according to a modified procedure from that described by Blois.[52] The antioxidant potential of the tested compounds was calculated as the percentage DPPH scavenging activity (SA%) in relation to a control and butylated hydroxyl toluene (BHT) was utilized as a reference standard antioxidant agent (Table 2).

Table 2. DPPH Radical-Scavenging Activity of the Cytotoxic-Active Compounds **6**, **8**, **10**, **15**, **16**, **19**, **25** and **28**

Compound	Concentration (M)	DPPH radical scavenging activity (% of control)[a]
Control[b]	—	100±0.05
BHT[c]	10^{-4}	20±0
	10^{-3}	36±1
6	10^{-4}	14±1
	10^{-3}	26±2
8	10^{-4}	17±1
	10^{-3}	31±2
10	10^{-4}	18±1
	10^{-3}	32±1
15	10^{-4}	15±0
	10^{-3}	28±2
16	10^{-4}	19±1
	10^{-3}	34±2
19	10^{-4}	12±2
	10^{-3}	23±1
25	10^{-4}	10±2
	10^{-3}	21±1
28	10^{-4}	9±0
	10^{-3}	19±2

a) Values are recorded as the mean of three independent experiments±S.D. *b*) Control: DPPH radical solution in methanol. *c*) BHT: Butylated hydroxyl toluene (reference standard antioxidant).

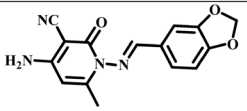
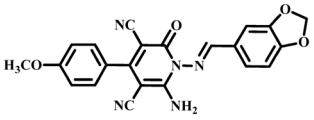
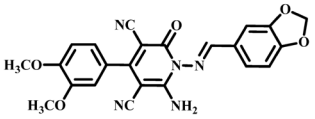
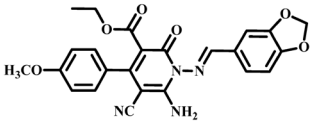
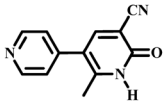
At the 10^{-3} M concentration level, the substituted 2-oxopyridine-3-carbonitriles **8**, **10** and **16** (SA 31, 32 and 34%, respectively) were nearly equiactive with BHT; the standard antioxidant utilized in this assay (SA 36%). Whereas, the analogs **6**, **15** and **19** displayed an appreciable antioxidant potential (SA 26, 28 and 23%, respectively). Nevertheless, the structure variants **25** and **28** displayed a relatively weak antioxidant activity (scavenging activity 21 and 19%), when compared with compounds **8**, **10** and **16**, the most active antioxidants in this study. In a similar pattern, the same order of DPPH radical scavenging potential was expressed at the 10^{-4} M concentration level, with a scavenging activity range 9–19%, when compared with BHT (scavenging activity 20%).

Determination of the *in Vitro* PDE3A Inhibitory Activity

The most active anticancer compounds **8**, **15**, **16** and **19** were assessed for their *in vitro* inhibition of PDE3A (a possible molecular target for the antitumor activity) using fluorescence polarization (FP). The basic principle for this assay is to cleave the fluorescently labeled c-AMP (substrate) into its respective nucleotide by PDE3A. Binding agent is then added to produce a change in FP that can be measured using a fluorescence reader. Milrinone was employed as a positive control and the IC_{50} values were determined by testing a range of 10 concentrations (0.01–100 μM), each in duplicate and the results were shown in Table 3.

The results revealed that all the investigated four compounds showed moderate to weak PDE3A inhibitory activity (IC_{50} range 34.78–85.33 μM), as compared with milrinone (IC_{50} 12.03 μM), the reference positive control utilized in this assay. Although compounds **8**, **16** and **19** displayed nearly equal LC_{50} values against the breast MCF7 cancer cell line (19.15,

Table 3. Anticancer Activity against the MCF7 Breast Cancer Cell Line[a] and *in Vitro* Inhibitory Effect on PDE3A Enzyme[b] of the Most Active Compounds **8**, **15**, **16** and **19**

Cpd no.	Structure	MCF 7 growth inhibition LC_{50} (μM)	PDE3A inhibition IC_{50} (μM)
8		19.15	85.33
15		30.67	77.26
16		17.34	34.78
19		14.7	49.15
Milrinone[c]		ND[d]	12.03

a) The most sensitive cell line to the tested compounds (see table 1). *b)* Employing c-AMP as a substrate. *c)* Reference positive control PDE3A inhibitor. *d)* ND: Not determined.

17.34 and 14.7 μM, respectively), yet their ability to inhibit the PDE3A enzyme is variable and not going in hand with the anticancer results. In particular, although compound **19** proved to be the most active anticancer agent against the MCF7 cell line (LC_{50} 14.7 μM), yet it did not express the highest PDE3A inhibitory activity (IC_{50} 49.15 μM) which represented about 25% of the activity of milrinone. On the contrary, the analog **16** with a relatively lesser anticancer potential (LC_{50} 17.34 μM), displayed an obviously higher PDE3A inhibitory potential (IC_{50} 34.78 μM; about 35% of the activity of milrinone).

Since the PDE3A and the tumor growth inhibitory activities are not going in hand, such enzymatic inhibition would be considered as a possible (but not the principle) mechanism of action behind their anticancer potential, therefore other phosphodiesterases and/or different targets might participate in the anticancer activity. This finding could be reinforced by the inactivity of the positive control milrinone to inhibit the growth of some tumor cell line despite its inhibition of PDE3 as reported by Abadi *et al.*[30]

In Silico Evaluation of the ADME-T and Drug-Likeness

Drug-likeness is a term that describes an integrated equilibrium between multiple molecular properties and structure features that define whether a particular compound is comparable to already known drugs. Among the common principles applied to evaluate of the drug-like properties of a compound, stemmed prominently the Lipinski's rule of 5 (RO5)[53] and Veber's criteria.[54] These properties comprise hydrophobicity, electronic distribution, hydrogen-bonding capability, molecule size and flexibility that would affect the behavior of a molecule in a living system including bioavailability, transport properties, affinity to proteins, reactivity, toxicity and metabolic stability.

In this context, a computational study for the biologically active compounds (**5**–**10**, **12**–**17**, **19**, **20**, **24**–**26** and **28**) utilizing the Molinspiration online property calculation toolkit,[55] was carried out to determine the Lipinski's molecular properties and the number of rotatable bonds (nROTB), together with the topographical polar surface area (TPSA; a sum of polar atoms' surfaces: a descriptor for drug absorption, penetrability and bioavailability), the percentage of absorption (ABS%) calculated as (ABS%=109−0.345×TPSA)[56] and the molecular volume (a determinant of the transport characteristics).

The results presented in Table 4 revealed that all the tested compounds comply with Lipinski's rule of 5, where Log*P* values ranged between 0.45–4.15 (<5), molecular weight (MW) range 297–490 (<500), HBA range 5–10 (≤10) and HBD range 1–4 (<5), suggesting that these compounds would not be expected to cause problems with oral bioavailability. The only exception was noted for compound **20** which showed one violation to the RO5 (HBD >10). Moreover, all the tested compounds showed nROTB values of 2–9 (<10) indicating acceptable molecular flexibility with consequent expected good permeability and oral bioavailability. Additionally, all the evaluated compounds showed TPSA range 84.36–138.19 Å^2 (<140 Å^2), indicating good permeability and transport of the compounds in the cellular plasma membrane; except for the analogs **14**, **16** and **20** which were not so far from the ideal value (PSA 144.91, 144.91 and 147.43 Å^2, respectively). Furthermore, all the tested compounds exhibited a considerable % ABS range 58.14–79.9%, which is a designation of good

Table 4. *In Silico* ADME-T Calculations, Lipinski's Parameters Drug-Likeness, and Drug Score[a, b] of the Synthesized Compounds

Cpd.	Lipinski's parameters					nROTB[h]	TPSA[i]	% ABS[j]	Volume[k]	Log S[l]	Drug-likeness	Drug-score
	Log P[c]	MW[d]	HBA[e]	HBD[f]	Violations[g]							
5	1.72	313.31	7	1	0	4	96.86	75.58	275.59	−4.12	2.72	0.77
6	1.96	297.27	7	1	0	2	95.15	76.17	248.43	−4.80	0.93	0.62
7	0.75	314.30	8	3	0	4	121.1	67.22	270.32	−4.00	1.33	0.73
8	0.99	298.26	8	3	0	2	122.88	66.61	243.16	−4.68	0.47	0.52
9	0.45	313.32	8	4	0	4	128.68	64.61	273.59	−4.08	1.23	0.72
10	0.70	297.27	8	4	0	2	128.68	64.61	246.43	−4.75	−0.56	0.51
12	1.27	308.25	8	1	0	2	120.65	67.38	248.73	−4.94	0.88	0.61
13	2.50	429.44	9	2	0	6	135.68	62.19	376.12	−5.32	2.99	0.35
14	2.10	459.46	10	2	0	7	144.91	59.01	401.66	−5.34	2.59	0.33
15	2.75	413.39	9	2	0	4	135.68	62.19	348.95	−6.00	1.26	0.29
16	2.34	443.42	10	2	0	5	144.91	59.01	374.5	−6.01	1.84	0.29
17	2.96	476.49	10	2	0	9	138.19	61.32	420.59	−5.07	−5.72	0.17
19	3.20	460.45	10	2	0	7	138.19	61.32	393.43	−5.75	−7.46	0.16
20	2.79	490.47	11	2	1	8	147.43	58.14	418.97	−5.77	−6.92	0.15
24	3.31	362.38	6	1	0	5	84.36	79.90	324.33	−3.47	2.44	0.47
25	3.99	396.83	5	1	0	5	84.36	79.90	337.86	−4.21	3.03	0.41
26	2.96	422.44	8	1	0	7	102.82	73.53	375.42	−3.51	3.99	0.45
28	4.15	395.85	6	2	0	5	90.41	77.81	341.02	−3.39	1.60	0.43

a) Molinspiration chemoinformatics property calculator (2014). *b*) OSIRIS property explorer (2014). *c*) Partition coefficient. *d*) Molecular weight. *e*) Number of H-Bond acceptors (O and N atoms). *f*) Number of H-Bond donors (OH and NH groups). *g*) number of Rule of 5 violations. *h*) number of rotatable bonds. *i*) Topological polar surface area. *j*) Absorption %. *k*) Molecular volume. *l*) Aqueous solubility prediction.

bioavailability upon oral administration. It is to be noted that TPSA is inversely proportional to %ABS *e.g.* the analog **25** possesses the maximum absorption (79.9%) whereas its corresponding TPSA was least among the series (84.36 Å^2).

On the other hand, the OSIRIS Property Explorer (2014: Version 2) software[57] was utilized to calculate the aqueous solubility (Log S) of the tested compounds (being significantly affecting absorption as well as distribution characteristics), where they gave moderate Log S values ranging between −3.39 and −6.01 mol/L. Finally, the same OSIRIS software was employed to determine the overall and drug score values which is an expression representing integration of drug-likeness, several physicochemical parameters and toxicity probabilities in one numerical value that can be utilized to foresee compound's ability to act as a drug candidate. A positive value for drug-likeness and drug score indicates that the compound contains fragments that are often present in most of currently used drugs. The results revealed that about 80% of the evaluated compounds gave positive values for drug-likeness lying between 0.47 and 3.99, excluding four compounds (**10**, **17**, **19** and **20**) which displayed negative values of drug-likeness (between −7.46 and −0.56). Additionally, all the evaluated compounds (including those compounds with negative drug-likeness scores) have displayed positive values ranged from 0.15 to 0.77 in the drug score calculation (Table 4). According to OSIRIS findings, prediction of the expected toxicity risks pointed out that none of the twenty three investigated compounds would exert tumorigenic, mutagenic, irritant or reproductive toxicity.

Conclusion

The main objective of this research work was to synthesize two series of novel alkoxylated 2-oxo(imino)-3-pyridinecarbonitriles supported with different pharmacophores, to be evaluated for their *in vitro* differential anticancer potential against a panel of three human tumor cell lines. Eight compounds (**6**, **8**, **10**, **15**, **16**, **19**, **25** and **28**) displayed growth inhibitory capability towards the tested cell lines, among which **8**, **16** and **19** displayed recognizable growth inhibitory ability and selectivity towards the breast MCF7 (LC_{50} 19.15, 17.34 and 14.70 μM, respectively) as compared with doxorubicin (LC_{50} 3.94 μM). Meanwhile, compounds **8**, **15**, **16**, and **19** revealed a marginal inhibitory effect on the growth of the normal human foreskin fibroblast Hs27 cell line, beside a distinctive antioxidant potential in the DPPH assay. These four compounds were further assessed for their *in vitro* inhibition of PDE3A (a current antitumor therapeutic target), where **16** and **19** showed moderate to weak PDE3A inhibitory (IC_{50} 34.78 and 49.15 μM, respectively) as compared with milrinone (IC_{50} 12.03 μM), the positive control. In general, the antioxidant potential showed a considerable alignment with the cytotoxic experimental data suggesting a possible role of free radical scavenging in the antitumor effect of the active compounds. On the other hand, the PDE3A and the tumor cell growth inhibitory activities are not in parallel, indicating that inhibition of PDE3A would not be the principle mechanism, but other molecular targets might participate in the anticancer activity. Collectively, the observed *in vitro* cytotoxic and antioxidant potentials of the active compounds were engaged with the compounds comprising the *N*-azavinylpyridine scaffold, and were modulated by the H-bond forming functionalities and/or substitution of the pyridine ring with benzodioxole moieties. These findings are concordant with previous studies that reported the important roles displayed by the lipophilicity and the excellent bioavailability of the benzodioxole fragment in relevant biological activities.[58–61] Moreover, *in silico* computation of the predicted ADME-T of the newly developed compounds showed non-violations of Lipinski's RO5 and Veber's criteria, suggesting their liability to act as new orally-active drug candidates with a predicted high safety profile. Finally, the selective anticancer

potential of the active compounds against the breast MCF7 tumor cell line and their reliable antioxidant activity, together with their *in vitro* PDE3A inhibitory activity, make such type of polyfunctional pyridines the appropriate matrix for future derivatization and optimization hoping to determine the scope and limitations of their bioactivities, and to find out more active and selective lead pyridine derivatives as anticancer agents with possible antioxidant and/or PDE3A inhibitory activities.

Experimental

Chemistry Melting points (mps) were determined in open glass capillaries on a Gallenkamp melting point apparatus and were uncorrected. The IR spectra were recorded on Shimadzu FT-IR 8400S infrared spectrophotometer using the KBr pellet technique. ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Bruker WM-600 FT NMR spectrometer using tetramethylsilane (TMS) as an internal standard and dimethyl sulfoxide (DMSO)-d_6 as a solvent (Chemical shifts in δ, ppm). Splitting patterns were designated as follows: s: singlet; d: doublet; t triplet; q: quartet; m: multiplet. Mass spectra were recorded on Agilent LC-MS 6120 single quad. Elemental analyses were performed on a 2400 PerkinElmer, Inc. Series 2 analyser and the found values were within ±0.4% of the theoretical values. Follow up of the reactions and checking the homogeneity of the compounds were made by TLC on silica gel-protected aluminium sheets (Type 60 F254, Merck) and the spots were detected by exposure to UV-lamp at λ 254. The synthesis of (*E*)-*N*′-(3,4-disubstituted benzylidene)-2-cyanoacetohydrazide **1**, **2**[48] and (*E*)-1-aryl-3-(3,4,5-trimethoxyphenyl)prop-2-en-1-one **21**–**23**[49] were performed according to reported literature procedures.

General Procedure for the Synthesis of (*E*)-1-((3,4-Dimethoxybenzylidene)amino)-4,6-dimethyl-2-oxo-1,2-dihydropyridine-3-carbonitrile (3) and (*E*)-1-((Benzo[1,3]-dioxol-5-ylmethylene)amino)-4,6-dimethyl-2-oxo-1,2-dihydropyridine-3-carbonitrile (4) A solution of **1** or **2** (2 mmol) and acetylacetone (0.20 g, 2 mmol) in absolute ethanol (10 mL) containing four drops of piperidine, was heated under reflux for 5 h. After cooling to room temperature, the reaction mixture was poured onto crushed ice containing few drops of dilute hydrochloric acid. The separated solid product was filtered, washed with water, dried and recrystallized from dioxane.

(*E*)-1-((3,4-Dimethoxybenzylidene)amino)-4,6-dimethyl-2-oxo-1,2-dihydropyridine-3-carbonitrile (**3**)

Yield: 76%. mp: 150–152°C. ^{1}H-NMR (600 MHz, [D_6]DMSO) δ: 2.01 (s, 3H, CH_3), 2.38 (s, 3H, CH_3), 3.71, 3.82 (2s, 6H, 2 OCH_3), 5.69 (s, 1H, pyridine-C_5–H), 6.92–7.23 (m, 3H, dimethoxyphenyl-H), 8.02 (s, 1H, N=CH). ^{13}C-NMR (600 MHz, [D_6]DMSO) δ: 19.9 (CH_3), 27.4 (CH_3), 56.9 (OCH_3), 57.9 (OCH_3), 118.8 (CN), 106.8, 110.8, 114.0, 123.5, 129.2, 132.4, 135.2, 137.2, 152.5, 157.2 (Ar C), 164.0 (C=N), 168.9 (CO). IR (KBr) cm^{-1}: 2227 (CN), 1687 (C=O). MS *m*/*z* (%): 311 (28) [*M*]$^+$. *Anal.* Calcd for $C_{17}H_{17}N_3O_3$: C, 65.58; H, 5.50; N, 13.50. Found: C, 65.43; H, 5.32; N, 13.61.

(*E*)-1-((Benzo[1,3]dioxol-5-ylmethylene)amino)-4,6-dimethyl-2-oxo-1,2-dihydropyridine-3-carbonitrile (**4**)

Yield: 82%. mp: 190–192°C. ^{1}H-NMR (600 MHz, [D_6]DMSO) δ: 2.09 (s, 3H, CH_3), 2.39 (s, 3H, CH_3), 5.82 (s, 1H, pyridine-C_5–H), 6.13 (s, 2H, O–CH_2–O), 7.09–7.34 (m, 3H, benzodioxole-H), 7.80 (s, 1H, N=CH). ^{13}C-NMR (600 MHz, [D_6]DMSO) δ: 18.9 (CH_3), 25.9 (CH_3), 89.9 (O–CH_2–O), 119.8 (CN), 104.8, 110.5, 114.0, 123.5, 128.9, 132.4, 135.2, 136.8, 150.5, 156.9 (Ar C), 165.0 (C=N), 168.6 (CO). IR (KBr) cm^{-1}: 2223 (CN), 1675 (C=O). MS *m*/*z* (%): 295 (21) [*M*]$^+$. *Anal.* Calcd for $C_{16}H_{13}N_3O_3$: C, 65.08; H, 4.44; N, 14.23. Found: C, 65.19; H, 4.17; N, 13.97.

General Procedure for the Synthesis of (*E*)-1-((3,4-Dimethoxybenzylidene)amino)-6-hydroxy-4-methyl-2-oxo-1,2-dihydropyridine-3-carbonitrile (5) and (*E*)-1-((Benzo[1,3]-dioxol-5-ylmethylene)amino)-6-hydroxy-4-methyl-2-oxo-1,2-dihydropyridine-3-carbonitrile (6) To a solution of **1** or **2** (2 mmol) in absolute ethanol (10 mL) containing four drops of piperidine, ethyl acetoacetate (0.26 g, 2 mmol) was added. The reaction mixture was heated under reflux for 4 h then allowed to attain room temperature. Working up of the reaction mixture was carried out as described under **3** and **4**. The separated solid product was filtered, washed with water, dried and recrystallized from ethanol.

(*E*)-1-((3,4-Dimethoxybenzylidene)amino)-6-hydroxy-4-methyl-2-oxo-1,2-dihydropyridine-3-carbonitrile (**5**)

Yield: 68%. mp: 160–161°C. ^{1}H-NMR (600 MHz, [D_6]DMSO) δ: 2.18 (s, 3H, CH_3), 3.76, 3.81 (2s, 6H, 2 OCH_3), 5.68 (s, 1H, pyridine-C_5-H), 6.92–7.15 (m, 3H, dimethoxyphenyl–H), 8.01 (s, 1H, N=CH). ^{13}C-NMR (600 MHz, [D_6]DMSO) δ: 19.3 (CH_3), 57.8 (OCH_3), 58.6 (OCH_3), 118.9 (CN), 104.1, 111.6, 115.2, 122.8, 127.8, 133.4, 135.6, 137.2, 155.6, 157.2 (Ar C), 164.8 (C=N), 168.5 (CO). IR (KBr) cm^{-1}: 3430 (OH), 2220 (CN), 1685 (C=O). MS *m*/*z* (%): 314 (48) [*M*+H]$^+$. *Anal.* Calcd for $C_{16}H_{15}N_3O_4$: C, 61.34; H, 4.83; N, 13.41. Found: C, 61.15; H, 4.65; N, 13.15.

(*E*)-1-((Benzo[1,3]dioxol-5-ylmethylene)amino)-6-hydroxy-4-methyl-2-oxo-1,2-dihydropyridine-3-carbonitrile (**6**)

Yield: 65%. mp: 240–242°C. ^{1}H-NMR (600 MHz, [D_6]DMSO) δ: 2.39 (s, 3H, CH_3), 5.79 (s, 1H, pyridine-C_5–H), 6.08 (s, 2H, O–CH_2–O), 7.09–7.34 (m, 3H, benzodioxole–H), 8.06 (s, 1H, N=CH), 11.39 (s, 1H, OH, D_2O exchangeable). ^{13}C-NMR (600 MHz, [D_6]DMSO) δ: 19.5 (CH_3), 89.7 (O–CH_2–O), 119.6 (CN), 105.2, 110.5, 114.0, 123.5, 128.9, 132.4, 135.2, 136.8, 154.9, 156.1 (Ar C), 165.5 (C=N), 167.0 (CO). IR (KBr) cm^{-1}: 3343 (OH), 2226 (CN), 1673 (C=O). MS *m*/*z* (%): 298 (33) [*M*+H]$^+$. *Anal.* Calcd for $C_{15}H_{11}N_3O_4$: C, 60.61; H, 3.73; N, 14.14. Found: C, 60.43; H, 3.89, N; 14.10.

General Procedure for the Synthesis of (*E*)-4-Amino-1-((3,4-dimethoxybenzylidene)amino)-6-hydroxy-2-oxo-1,2-dihydropyridine-3-carbonitrile (7) and (*E*)-4-Amino-1-((benzo[1,3]dioxol-5-ylmethylene)amino)-6-hydroxy-2-oxo-1,2-dihydropyridine-3-carbonitrile (8) To a solution of **1** or **2** (2 mmol) in absolute ethanol (10 mL) containing four drops of piperidine, ethyl cyanoacetate (0.23 g, 2 mmol) was added. The reaction mixture was heated under reflux for 3 h then allowed to attain room temperature. Working up of the reaction mixture was carried out as described under **3** and **4**. The separated solid product was filtered, washed with water, dried and recrystallized from the proper solvent.

(*E*)-4-Amino-1-((3,4-dimethoxybenzylidene)amino)-6-hydroxy-2-oxo-1,2-dihydropyridine-3-carbonitrile (**7**)

Yield: 70%. mp: 192–194°C (MeOH). ^{1}H-NMR (600 MHz, [D_6]DMSO) δ: 3.73, 3.79 (2s, 6H, 2 OCH_3), 5.66 (s, 1H, pyridine-C_5–H), 6.73–7.02 (m, 3H, dimethoxyphenyl–H), 7.92 (s, 1H, N=CH), 8.39 (br s, 2H, NH_2, D_2O exchangeable).

11.68 (brs, 1H, OH, D_2O exchangeable). ^{13}C-NMR (600 MHz, [D_6]DMSO) δ: 57.9 (OCH_3), 59.9 (OCH_3), 118.8 (CN), 106.8, 110.5, 114.0, 123.5, 129.2, 132.4, 135.2, 137.2, 152.2, 157.2 (Ar C), 165.0 (C=N), 169.2 (CO). IR (KBr) cm^{-1}: 3465–3239 (OH, NH_2), 2225 (CN), 1670 (C=O). MS *m/z* (%): 315 (34) $[M+H]^+$. *Anal.* Calcd for $C_{15}H_{14}N_4O_4$: C, 57.32; H, 4.49; N, 17.83. Found: C, 57.14; H, 4.61; N, 17.59.

(*E*)-4-Amino-1-((benzo[1,3]dioxol-5-ylmethylene)amino)-6-hydroxy-2-oxo-1,2-dihydropyridine-3-carbonitrile (**8**)

Yield: 58%. mp: 171–173°C (EtOH). ^{1}H-NMR (600 MHz, [D_6]DMSO) δ: 5.98 (s, 1H, pyridine-C_5–H), 6.05 (s, 2H, O–CH_2–O), 6.94–7.16 (m, 3H, benzodioxole-H), 7.91 (s, 1H, N=CH), 11.62 (s, 1H, OH, D_2O exchangeable). ^{13}C-NMR (600 MHz, [D_6]DMSO) δ: 88.9 (O–CH_2–O), 119.2 (CN), 104.9, 111.5, 115.2, 123.5, 128.3, 132.6, 135.7, 137.5, 153.4, 156.8 (Ar C), 164.3 (C=N), 168.3 (CO). IR (KBr) cm^{-1}: 3451–3241 (OH, NH_2), 2212 (CN), 1682 (C=O). MS *m/z* (%): 299 (28) $[M+H]^+$. *Anal.* Calcd for $C_{14}H_{10}N_4O_4$: C, 56.38; H, 3.38; N, 18.78. Found: C, 56.56; H, 3.34; N, 18.91.

General Procedure for the Synthesis of (*E*)-4,6-Diamino-1-((3,4-dimethoxybenzylidene)amino)-2-oxo-1,2-dihydropyridine-3-carbonitrile (9) and (*E*)-4,6-Diamino-1-((benzo[1,3]dioxol-5-ylmethylene)amino)-2-oxo-1,2-dihydropyridine-3-carbonitrile (10) To a solution of **1** or **2** (2 mmol) in absolute ethanol (10 mL) containing four drops of piperidine, malononitrile (0.13 g, 2 mmol) was added. The reaction mixture was heated under reflux for 3 h then allowed to attain room temperature. Working up of the reaction mixture was carried out as described under **3** and **4**. The separated solid product was filtered, washed with water, dried and recrystallized from the proper solvent.

(*E*)-4,6-Diamino-1-((3,4-dimethoxybenzylidene)amino)-2-oxo-1,2-dihydropyridine-3-carbonitrile (**9**)

Yield: 78%. mp: 252–254°C (MeOH). ^{1}H-NMR (600 MHz, [D_6]DMSO) δ: 3.79, 3.84 (2s, 6H, 2 OCH_3), 5.65 (s, 1H, pyridine-C_5–H), 7.02–7.13 (m, 3H, dimethoxyphenyl–H), 8.15 (s, 1H, N=CH), 8.39, 9.81 (2 brs, 4H, 2 NH_2, D_2O exchangeable). ^{13}C-NMR (600 MHz, [D_6]DMSO) δ: 57.6 (OCH_3), 59.8 (OCH_3), 119.5 (CN), 105.9, 110.8, 117.8, 128.2, 129.7, 131.5, 132.9, 133.4, 154.2, 156.2 (Ar C), 165.4 (C=N), 169.7 (CO). IR (KBr) cm^{-1}: 3387–3258 (NH_2), 2220 (CN), 1678 (C=O). MS *m/z* (%): 313 (38) $[M]^+$. *Anal.* Calcd for $C_{15}H_{15}N_5O_3$: C, 57.50; H, 4.83; N, 22.35. Found: C, 57.82; H, 4.79; N, 22.46.

(*E*)-4,6-Diamino-1-((benzo[1,3]dioxol-5-ylmethylene)-amino)-2-oxo-1,2-dihydropyridine-3-carbonitrile (**10**)

Yield: 85%. mp: 238–239°C (EtOH). ^{1}H-NMR (600 MHz, [D_6]DMSO) δ: 5.64 (s, 1H, pyridine-C_5–H), 6.14 (s, 2H, O–CH_2–O), 6.98 (d, J=8.1 Hz, 1H, benzodioxole-C_7–H), 7.06–7.09 (m, 2H, benzodioxole-$C_{4,6}$–H), 8.11 (s, 1H, N=CH), 8.40 (brs, 2H, NH_2, D_2O exchangeable). ^{13}C-NMR (600 MHz, [D_6]DMSO) δ: 89.7 (O–CH_2–O), 120.3 (CN), 106.5, 111.9, 116.8, 127.5, 128.9, 132.5, 132.9, 133.4, 153.1, 157.2 (Ar C), 165.8 (C=N), 170.1 (CO). IR (KBr) cm^{-1}: 3366–3250 (NH_2), 2213 (CN), 1667 (C=O). MS *m/z* (%): 297 (29) $[M]^+$. *Anal.* Calcd for $C_{14}H_{11}N_5O_3$: C, 56.56; H, 3.73; N, 23.56. Found: C, 56.38; H, 3.71; N, 23.59.

General Procedure for the Synthesis of (*E*)-1-((3,4-Dimethoxybenzylidene)amino)-6-hydroxy-2-oxo-1,2-dihydropyridine-3,5-dicarbonitrile (11) and (*E*)-1-((Benzo[1,3]-dioxol-5-ylmethylene)amino)-6-hydroxy-2-oxo-1,2-dihydropyridine-3,5-dicarbonitrile (12) A mixture of **1** or **2** (2 mmol), ethyl ethoxymethylenecyanoacetate (0.34 g, 2 mmol) and anhydrous potassium carbonate (0.28 g, 2 mmol) in absolute ethanol (10 mL), was heated under reflux for 3 h. The solvent was evaporated under reduced pressure and the obtained residue was dissolved in water then acidified with dilute hydrochloric acid to pH 3–4. The precipitate thus formed was filtered, washed with water, dried and recrystallized from dioxane.

(*E*)-1-((3,4-Dimethoxybenzylidene)amino)-6-hydroxy-2-oxo-1,2-dihydropyridine-3,5-dicarbonitrile (**11**)

Yield: 48%. mp: 260–262°C. ^{1}H-NMR (600 MHz, [D_6]DMSO) δ: 3.89 (s, 6H, 2 OCH_3), 6.75–7.32 (m, 3H, dimethoxyphenyl–H), 7.79 (s, 1H, pyridine-C_4–H), 8.27 (s, 1H, N=CH), 10.84 (brs, 1H, OH, D_2O exchangeable). ^{13}C-NMR (600 MHz, [D_6]DMSO) δ: 56.1 (OCH_3), 57.2 (OCH_3), 116.4 (CN), 118.3 (CN), 102.9, 106.5, 111.9, 127.9, 128.9, 132.5, 132.9, 133.4, 152.2, 156.4 (Ar C), 165.8 (C=N), 169.8 (CO). IR (KBr) cm^{-1}: 3400 (OH), 2210 (CN), 1664 (C=O). MS *m/z* (%): 325 (17) $[M+H]^+$. *Anal.* Calcd for $C_{16}H_{12}N_4O_4$: C, 59.26; H, 3.73; N, 17.28. Found: C, 58.98; H, 3.85; N, 17.13.

(*E*)-1-((Benzo[1,3]dioxol-5-ylmethylene)amino)-6-hydroxy-2-oxo-1,2-dihydropyridine-3,5-dicarbonitrile (**12**)

Yield: 53%. mp: 181–183°C. ^{1}H-NMR (600 MHz, [D_6]DMSO) δ: 6.11 (s, 2H, O–CH_2–O), 6.72–7.32 (m, 3H, benzodioxole–H), 7.87 (s, 1H, pyridine-C_4-H), 8.07 (s, 1H, N=CH), 10.87 (brs, 1H, OH, D_2O exchangeable). ^{13}C-NMR (600 MHz, [D_6]DMSO) δ: 87.4 (O–CH_2–O), 117.1 (CN), 118.6 (CN), 103.9, 106.2, 110.8, 127.8, 128.5, 132.2, 132.9, 133.5, 153.1, 155.9 (Ar C), 164.9 (C=N), 170.2 (CO). IR (KBr) cm^{-1}: 3465 (OH), 2216 (CN), 1678 (C=O). MS *m/z* (%): 309 (12) $[M+H]^+$. *Anal.* Calcd for $C_{15}H_8N_4O_4$: C, 58.45; H, 2.62; N, 18.18. Found: C, 58.73; H, 2.65; N, 18.02.

General Procedure for the Synthesis of (*E*)-6-Amino-4-aryl-1-((3,4-dimethoxybenzylidene)amino)-2-oxo-1,2-dihydropyridine-3,5-dicarbonitriles (13, 14) and (*E*)-6-Amino-4-aryl-1-((benzo[1,3]dioxol-5-ylmethylene)amino)-2-oxo-1,2-dihydropyridine-3,5-dicarbonitriles (15, 16) To a solution of **1** or **2** (2 mmol) in absolute ethanol (10 mL), were added the appropriate 2-arylidenemalononitrile (2 mmol) and four drops of piperidine. The reaction mixture was heated under reflux for 3 h. After cooling, the separated product was filtered, washed with ethanol, dried, and recrystallized from the proper solvent.

(*E*)-6-Amino-1-((3,4-dimethoxybenzylidene)amino)-4-(4-methoxyphenyl)-2-oxo-1,2-dihydropyridine-3,5-dicarbonitrile (**13**)

Yield: 58%. mp: 257–259°C (DMF). ^{1}H-NMR (600 MHz, [D_6]DMSO) δ: 3.84, 3.85, 3.87 (3s, 9H, 3 OCH_3), 6.99–7.71 (m, 7H, Ar-H), 8.36 (brs, 2H, NH_2, D_2O exchangeable), 8.77 (s, 1H, N=CH). ^{13}C-NMR (600 MHz, [D_6]DMSO) δ: 57.3 (OCH_3), 58.4 (OCH_3), 63.1 (OCH_3), 119.6 (CN), 120.4 (CN), 104.2, 109.9, 111.9, 123.1, 128.2, 128.9, 131.7, 132.9, 137.2, 138.5, 139.1, 139.7, 139.7, 140.3, 141.5, 153.5 (Ar C), 167.4 (C=N), 170.2 (CO). IR (KBr) cm^{-1}: 3388–3239 (NH_2), 2221 (CN), 1663 (C=O). MS *m/z* (%): 430 (18) $[M+H]^+$. *Anal.* Calcd for $C_{23}H_{19}N_5O_4$: C, 64.33; H, 4.46; N, 16.31. Found: C, 64.21; H, 4.32; N, 16.54.

(*E*)-6-Amino-1-((3,4-dimethoxybenzylidene)amino)-4-(3,4-dimethoxyphenyl)-2-oxo-1,2-dihydropyridine-3,5-dicarbonitrile (**14**)

Yield: 66%. mp: 245–247°C (dioxane). ^{1}H-NMR (600 MHz,

[D_6]DMSO) δ: 3.80, 3.82, 3.85, 3.87 (4s, 12H, 4 OCH_3), 7.00–7.19 (m, 4H, Ar-H), 7.48 (d, J=8.4 Hz, 1H, dimethoxybenzylidene-C_6–H), 7.71 (s, 1H, dimethoxybenzylidene-C_2–H), 8.35 (brs, 2H, NH_2, D_2O exchangeable), 8.76 (s, 1H, N=CH). ^{13}C-NMR (600 MHz, [D_6]DMSO) δ: 57.1 (OCH_3), 58.2 (OCH_3), 62.4 (OCH_3), 64.0 (OCH_3), 119.9 (CN), 120.9 (CN), 104.6, 109.9, 111.8, 122.3, 127.4, 128.7, 131.2, 132.9, 137.5, 138.3, 139.4, 139.6, 139.7, 139.9, 140.1, 152.5 (Ar C), 168.9 (C=N), 170.7 (CO). IR (KBr) cm^{-1}: 3370–3232 (NH_2), 2230 (CN), 1675 (C=O). MS m/z (%): 459 (15) $[M]^+$. *Anal.* Calcd for $C_{24}H_{21}N_5O_5$: C, 62.74; H, 4.61; N, 15.24. Found: C, 62.85; H, 4.66; N, 15.18.

(*E*)-6-Amino-1-((benzo[1,3]dioxol-5-ylmethylene)-amino)-4-(4-methoxyphenyl)-2-oxo-1,2-dihydropyridine-3,5-dicarbonitrile (**15**)

Yield: 58%. mp: 265–267°C (DMF). ^{1}H-NMR (600 MHz, [D_6]DMSO) δ: 3.85 (s, 3H, OCH_3), 6.17 (s, 2H, O–CH_2–O), 6.98–7.99 (m, 7H, Ar-H), 8.37 (brs, 2H, NH_2, D_2O exchangeable), 8.80 (s, 1H, N=CH). ^{13}C-NMR (600 MHz, [D_6]DMSO) δ: 62.0 (OCH_3), 88.9 (O–CH_2–O), 118.7 (CN), 119.6 (CN), 103.9, 110.1, 112.4, 122.3, 127.4, 129.2, 131.4, 132.8, 137.5, 138.3, 139.1, 139.7, 140.1, 140.9, 141.6, 152.9 (Ar C), 169.1 (C=N), 170.8 (CO). IR (KBr) cm^{-1}: 3367–3240 (NH_2), 2219 (CN), 1670 (C=O). MS m/z (%): 413 (11) $[M]^+$. *Anal.* Calcd for $C_{22}H_{15}N_5O_4$: C, 63.92; H, 3.66; N, 16.94. Found: C, 64.13; H, 3.78; N, 16.81.

(*E*)-6-Amino-1-((benzo[1,3]dioxol-5-ylmethylene)amino)-4-(3,4-dimethoxyphenyl)-2-oxo-1,2-dihydropyridine-3,5-dicarbonitrile (**16**)

Yield: 53%. mp: 260–262°C (DMF). ^{1}H-NMR (600 MHz, [D_6]DMSO) δ: 3.81, 3.87 (2s, 6H, 2 OCH_3), 6.17 (s, 2H, O–CH_2–O), 6.98–7.72 (m, 6H, Ar-H), 8.37 (brs, 2H, NH_2, D_2O exchangeable), 8.79 (s, 1H, N=CH). ^{13}C-NMR (600 MHz, [D_6]DMSO) δ: 62.6 (OCH_3), 63.9 (OCH_3), 87.8 (O–CH_2–O), 119.2 (CN), 120.4 (CN), 104.3, 111.1, 113.3, 122.3, 127.5, 128.5, 131.2, 133.4, 136.6, 138.1, 138.9, 139.6, 140.2, 140.9, 141.8, 153.5 (Ar C), 164.7 (CN), 168.4 (C=N), 169.9 (CO). IR (KBr) cm^{-1}: 3380–3245 (NH_2), 2225 (CN), 1665 (C=O). MS m/z (%): 443 (10) $[M]^+$. *Anal.* Calcd for $C_{23}H_{17}N_5O_5$: C, 62.30; H, 3.86; N, 15.79. Found: C, 62.54; H, 3.94; N, 16.00.

General Procedure for the Synthesis of (*E*)-Ethyl 6-Amino-4-aryl-5-cyano-1-((3,4-dimethoxybenzylidene)-amino)-2-oxo-1,2-dihydropyridine-3-carboxylates (17, 18) and (*E*)-Ethyl 6-Amino-4-aryl-1-((benzo[1,3]dioxol-5-ylmethylene)amino)-5-cyano-2-oxo-1,2-dihydropyridine-3-carboxylates (19, 20) To a solution of **1** or **2** (2 mmol) in absolute ethanol (10 mL) containing sodium metal (0.046 g, 2 mmol), was added the appropriate 2-arylidenecyanoacetate (2 mmol). The reaction mixture was heated under reflux for 2 h, then the solvent was evaporated under reduced pressure. The obtained residue was dissolved in water and neutralized with dilute hydrochloric acid to pH 7. The precipitate thus formed was filtered, washed with water, dried and recrystallized from the proper solvent.

(*E*)-Ethyl 6-Amino-5-cyano-1-((3,4-dimethoxybenzylidene)-amino)-4-(4-methoxyphenyl)-2-oxo-1,2-dihydropyridine-3-carboxylate (**17**)

Yield: 41%. mp: 183–185°C (EtOH). ^{1}H-NMR (600 MHz, [D_6]DMSO) δ: 1.24 (t, J=6.9 Hz, 3H, ester–CH_3), 3.80 (s, 3H, OCH_3), 3.82 (s, 6H, 2 OCH_3), 4.26 (q, J=6.9 Hz, 2H, ester–CH_2), 6.75–7.56 (m, 7H, Ar-H), 7.79 (s, 2H, NH_2, D_2O exchangeable), 8.26 (s, 1H, N=CH). ^{13}C-NMR (600 MHz, [D_6]DMSO) δ: 15.9 (CH_3), 57.9 (OCH_3), 59.2 (OCH_3), 65.7 (OCH_3), 80.1 (CH_2–O), 87.3 (O–CH_2–O), 118.7 (CN), 104.2, 110.3, 120.3, 122.7, 127.9, 128.7, 132.4, 133.2, 134.6, 137.4, 138.7, 138.9, 139.6, 139.9, 141.2, 152.1 (Ar C), 164.2 (C=N), 168.2 (CO), 170.7 (CO). IR (KBr) cm^{-1}: 3360–3274 (NH_2), 2227 (CN), 1730 (C=O ester), 1675 (C=O). MS m/z (%): 477 (18) $[M+H]^+$. *Anal.* Calcd for $C_{25}H_{24}N_4O_6$: C, 63.02; H, 5.08; N, 11.76. Found: C, 63.21; H, 5.16; N, 11.56.

(*E*)-Ethyl 6-Amino-5-cyano-1-((3,4-dimethoxybenzylidene)-amino)-4-(3,4-dimethoxyphenyl)-2-oxo-1,2-dihydropyridine-3-carboxylate (**18**)

Yield: 65%. mp: 165–167°C (MeOH). ^{1}H-NMR (600 MHz, [D_6]DMSO) δ: 1.24 (t, J=6.9 Hz, 3H, ester–CH_3), 3.81 (s, 6H, 2 OCH_3), 3.84 (s, 6H, 2 OCH_3), 4.26 (q, J=6.9 Hz, 2H, ester–CH_2), 6.79–7.57 (m, 6H, Ar-H), 7.99 (s, 2H, NH_2, D_2O exchangeable), 8.32 (s, 1H, N=CH). ^{13}C-NMR (600 MHz, [D_6]DMSO) δ: 16.1 (CH_3), 58.2 (OCH_3), 59.4 (OCH_3), 64.9 (OCH_3), 66.2 (OCH_3), 80.4 (CH_2-O), 87.8 (O–CH_2–O), 119.2 (CN), 103.9, 110.5, 121.1, 123.3, 127.0, 128.9, 132.6, 133.5, 134.7, 137.4, 138.2, 138.7, 139.6, 140.2, 142.9, 153.5 (Ar C), 165.1 (C=N), 169.1 (CO), 171.3 (CO). IR (KBr) cm^{-1}: 3357–3271 (NH_2), 2215 (CN), 1722 (C=O ester), 1663 (C=O). MS m/z (%): 507 (21) $[M+H]^+$. *Anal.* Calcd for $C_{26}H_{26}N_4O_7$: C, 61.65; H, 5.17; N, 11.06. Found: C, 61.39; H, 5.12; N, 10.86.

(*E*)-Ethyl 6-Amino-1-((benzo[1,3]dioxol-5-ylmethylene)-amino)-5-cyano-4-(4-methoxyphenyl)-2-oxo-1,2-dihydropyridine-3-carboxylate (**19**)

Yield: 62%. mp: 208–209°C (EtOH). ^{1}H-NMR (600 MHz, [D_6]DMSO) δ: 0.95 (t, J=6.9 Hz, 3H, ester–CH_3), 3.81 (s, 3H, OCH_3), 3.91 (q, J=6.9 Hz, 2H, ester–CH_2), 6.09 (s, 2H, O–CH_2–O), 6.71–7.74 (m, 7H, Ar-H), 8.12 (s, 1H, N=CH). ^{13}C-NMR (600 MHz, [D_6]DMSO) δ: 15.8 (CH_3), 65.9 (OCH_3), 79.5 (CH_2-O), 87.4 (O–CH_2–O), 118.7 (CN), 103.9, 110.1, 120.4, 123.1, 127.4, 128.6, 131.3, 132.9, 134.6, 137.2, 138.1, 138.9, 139.6, 139.9, 141.3, 152.3 (Ar C), 169.3 (C=O), 171.4 (CO). IR (KBr) cm^{-1}: 3355–3266 (NH_2), 2221 (CN), 1725 (C=O ester), 1670 (C=O). MS m/z (%): 461 (23) $[M+H]^+$. *Anal.* Calcd for $C_{24}H_{20}N_4O_6$: C, 62.60; H, 4.38; N, 12.17. Found: C, 62.83; H, 4.37; N, 12.18.

(*E*)-Ethyl 6-Amino-1-((benzo[1,3]dioxol-5-ylmethylene)-amino)-5-cyano-4-(3,4-dimethoxyphenyl)-2-oxo-1,2-dihydropyridine-3-carboxylate (**20**)

Yield: 67%. mp: 182–184°C (MeOH). ^{1}H-NMR (600 MHz, [D_6]DMSO) δ: 1.23 (t, J=6.9 Hz, 3H, ester–CH_3), 3.87 (s, 3H, OCH_3), 4.21 (q, J=6.9 Hz, 2H, ester–CH_2), 6.22 (s, 2H, O–CH_2–O), 6.94–7.56 (m, 6H, Ar-H), 8.00 (s, 2H, NH_2, D_2O exchangeable), 8.34 (s, 1H, N=CH). ^{13}C-NMR (600 MHz, [D_6]DMSO) δ: 15.2 (CH_3), 66.2 (OCH_3), 68.2 (OCH_3), 79.2 (CH_2-O), 87.1 (O–CH_2–O), 118.4 (CN), 104.6, 109.8, 120.1, 122.2, 127.4, 128.7, 131.2, 132.9, 134.4, 137.4, 138.4, 138.6, 139.7, 139.9, 140.4, 150.9 (Ar C), 169.9 (C=O), 171.7 (CO). IR (KBr) cm^{-1}: 3357–3271 (NH_2), 2219 (CN), 1727 (C=O ester), 1668 (C=O). MS m/z (%): 491 (19) $[M+H]^+$. *Anal.* Calcd for $C_{25}H_{22}N_4O_7$: C, 61.22; H, 4.52; N, 11.42. Found: C, 61.51; H, 4.61; N, 11.26.

General Procedure for the Synthesis of 6-Aryl-2-oxo-4-(3,4,5-trimethoxyphenyl)-1,2-dihydropyridine-3-carbonitriles (24–26) A mixture of chalcone **21**–**23** (2.5 mmole), ammonium acetate (1.54 g, 20 mmole) and ethyl cyanoacetate (0.28 g, 0.28 mL, 2.5 mmole) in absolute ethanol

(50 mL) was heated under reflux for 12 h during which yellow crystals started to form. After cooling, the separated solid was filtered, washed with ethanol, dried and recrystallized from the proper solvent.

2-Oxo-6-phenyl-4-(3,4,5-trimethoxyphenyl)-1,2-dihydropyridine-3-carbonitrile (**24**)

Yield: 90%. mp: 274–276°C (dioxane). ^{1}H-NMR (600 MHz, [D$_6$]DMSO) δ: 3.75 (s, 3H, OCH$_3$), 3.87 (s, 6H, 2 OCH$_3$), 6.89 (s, 1H, pyridine–C$_5$–H), 7.06 (s, 2H, trimethoxyphenyl–C$_{2,6}$–H), 7.52–7.56 (m, 3H, phenyl–C$_{3,4,5}$–H), 7.90 (d, J=7.2 Hz, 2H, phenyl–C$_{2,6}$–H), 12.74 (s, 1H, NH, D$_2$O exchangeable). ^{13}C-NMR (600 MHz, [D$_6$]DMSO) δ: 56.2 (OCH$_3$), 57.3 (OCH$_3$), 60.4 (OCH$_3$), 120.3 (CN), 101.3, 103.9, 112.5, 128.3, 129.5, 132.3, 137.6, 149.2, 153.4, 157.1, 163.2, 164.7, 168.9, 170.1 (Ar C), 176.8 (CO). IR (KBr) cm^{-1}: 3348 (NH), 2217 (CN), 1688 (C=O). MS m/z (%): 362 (48) [M]$^+$. *Anal.* Calcd for C$_{21}$H$_{18}$N$_2$O$_4$: C, 69.60; H, 5.01; N, 7.73. Found: C, 69.73; H, 5.16; N, 7.79.

6-(4-Chlorophenyl)-2-oxo-4-(3,4,5-trimethoxyphenyl)-1,2-dihydropyridine-3-carbonitrile (**25**)

Yield: 93%. mp: 280–281°C (DMF). ^{1}H-NMR (600 MHz, [D$_6$]DMSO) δ: 3.74 (s, 3H, OCH$_3$), 3.86 (s, 6H, 2 OCH$_3$), 6.95 (s, 1H, pyridine–C$_5$–H), 7.06 (s, 2H, trimethoxyphenyl–C$_{2,6}$–H), 7.61 (d, J=8.4 Hz, 2H, p-chlorophenyl–C$_{2,6}$–H), 7.94 (d, J=8.4 Hz, 2H, p-chlorophenyl–C$_{3,5}$–H), 12.78 (s, 1H, NH, D$_2$O exchangeable). ^{13}C-NMR (600 MHz, [D$_6$]DMSO) δ: 56.1 (OCH$_3$), 57.4 (OCH$_3$), 60.2 (OCH$_3$), 121.4 (CN), 101.9, 103.7, 112.0, 128.7, 129.3, 132.3, 137.1, 148.9, 153.2, 157.4, 163.2, 164.4, 169.3, 170.3 (Ar C), 177.2 (CO). IR (KBr) cm^{-1}: 3291 (NH), 2210 (CN), 1671 (C=O). MS m/z (%): 397 (41) [M]$^+$, 399 (14) (M+2]$^+$. *Anal.* Calcd for C$_{21}$H$_{17}$ClN$_2$O$_4$: C, 63.56; H, 4.32; N, 7.06. Found: C, 63.47; H, 4.30; N, 6.99.

6-(3,4-Dimethoxyphenyl)-2-oxo-4-(3,4,5-trimethoxyphenyl)-1,2-dihydropyridine-3-carbonitrile (**26**)

Yield: 87%. mp: 250–252°C (DMF). ^{1}H-NMR (600 MHz, [D$_6$]DMSO) δ: 3.74 (s, 3H, OCH$_3$), 3.87 (s, 2H, 4 OCH$_3$), 6.88 (s, 1H, pyridine–C$_5$–H), 7.04 (s, 2H, trimethoxyphenyl-C$_{2,6}$–H), 7.10 (d, J=8.4 Hz, 1H, dimethoxyphenyl-C$_5$–H),7.48 (s, 1H, dimethoxyphenyl-C$_2$–H), 7.54 (d, J=8.4 Hz, 1H, dimethoxyphenyl-C$_6$–H), 12.62 (s, 1H, NH, D$_2$O exchangeable). ^{13}C-NMR (600 MHz, [D$_6$]DMSO) δ: 56.2 (OCH$_3$), 57.3 (OCH$_3$), 60.4 (OCH$_3$), 60.4 (OCH$_3$), 60.4 (OCH$_3$), 120.3 (CN), 101.3, 103.9, 112.5, 128.3, 129.5, 132.3, 137.6, 149.2, 153.4, 157.1, 163.2, 164.7, 168.9, 170.1 (Ar C), 176.8 (CO). IR (KBr) cm^{-1}: 3324 (NH), 2215 (CN), 1676 (C=O). MS m/z (%): 422 (39) [M]$^+$. *Anal.* Calcd for C$_{23}$H$_{22}$N$_2$O$_6$: C, 65.39; H, 5.25; N, 6.63. Found: C, 65.42; H, 5.29; N, 6.62.

General Procedure for the Synthesis of 6-Aryl-2-imino-4-(3,4,5-trimethoxyphenyl)- 1,2-dihydropyridine-3-carbonitriles (27, 28) A mixture of chalcone **21**, **22** (2.5 mmole), ammonium acetate (1.54 g, 20 mmole) and malononitrile (0.33 g, 2.5 mmol) in absolute ethanol (50 mL) was heated under reflux for 24 h then left to cool to room temperature. The separated solid was filtered, washed with water, dried and recrystallized from the proper solvent.

2-Imino-6-phenyl-4-(3,4,5-trimethoxyphenyl)-1,2-dihydropyridine-3-carbonitrile (**27**)

Yield: 82%. mp: 175–177°C (dioxane). ^{1}H-NMR (600 MHz, [D$_6$]DMSO) δ: 3.83 (s, 3H, OCH$_3$), 3.93 (s, 6H, 2 OCH$_3$), 7.20 (s, 1H, pyridine–C$_5$–H), 7.51 (s, 2H, trimethoxyphenyl–C$_{2,6}$–H), 7.52–7.86 (m, 5H, phenyl–H). ^{13}C-NMR (600 MHz, [D$_6$]DMSO) δ: 56.2 (OCH$_3$), 57.4 (OCH$_3$), 60.1 (OCH$_3$), 121.3 (CN), 102.3, 103.7, 112.1, 128.5, 129.1, 133.2, 137.4, 148.7, 153.1, 157.3, 163.1, 164.2, 169.0, 169.7 (Ar C), 176.8 (C=N). IR (KBr) cm^{-1}: 3266 (NH), 2212 (CN). MS m/z (%): 361 (36) [M]$^+$. *Anal.* Calcd for C$_{21}$H$_{19}$N$_3$O$_3$: C, 69.79; H, 5.30; N, 11.63. Found: C, 69.58; H, 5.41; N, 11.61.

6-(4-Chlorophenyl)-2-imino-4-(3,4,5-trimethoxyphenyl)-1,2-dihydropyridine-3-carbonitrile (**28**)

Yield: 91%. mp: 206–208°C (DMF). ^{1}H-NMR (600 MHz, [D$_6$]DMSO) δ: 3.80 (s, 3H, OCH$_3$), 3.82 (s, 6H, 2OCH$_3$), 6.98 (s, 1H, pyridine-C$_5$–H), 7.03 (s, 2H, trimethoxyphenyl-C$_{2,6}$–H), 7.59 (d, J=8.4 Hz, 2H, p-chlorophenyl-C$_{2,6}$–H), 7.83 (d, J=8.4 Hz, 2H, p-chlorophenyl-C$_{3,5}$–H). ^{13}C-NMR (600 MHz, [D$_6$]DMSO) δ: 56.1 (OCH$_3$), 57.4 (OCH$_3$), 60.2 (OCH$_3$), 121.4 (CN), 101.9, 103.7, 112.0, 128.7, 129.3, 132.3, 137.1, 148.9, 153.2, 157.4, 163.2, 164.4, 169.3, 170.3 (Ar C), 177.2 (C=N). IR (KBr) cm^{-1}: 3372 (NH), 2219 (CN). MS m/z (%): 396 (29) [M]$^+$, 398 (10) [M+2]$^+$. *Anal.* Calcd for C$_{21}$H$_{18}$ClN$_3$O$_3$: C, 63.72; H, 4.58; N, 10.62. Found: C, 63.88; H, 4.62; N, 10.81.

Biology

In Vitro MTT Cytotoxicity Assay

The synthesized compounds were investigated for their *in vitro* cytotoxic effect *via* the standard method MTT[50,51] against a panel of three human tumor cell lines (ATCC®) namely; Caucasian breast adenocarcinoma MCF7 (HTB-22™), hepatocellular carcinoma HepG2 (HB-8065™), colon carcinoma CACO-2 (HTB-37™), and normal human foreskin fibroblast Hs27 normal cell line (ATCC® CRL-1634™). The procedures were done in a sterile area using a Laminar flow cabinet biosafety class II level (Baker, SG403INT, Stanford, ME, U.S.A.). Cells were batch cultured for 10 d, then seeded at concentration of 10×10^3 cells/well in fresh complete growth medium in 96-well microtiter plastic plates at 37°C for 24h under 5% CO_2 using a water jacketed carbon dioxide incubator (Sheldon, TC2323, Cornelius, OR, U.S.A.). Media was aspirated, fresh medium (without serum) was added and cells were incubated either alone (negative control) or with different concentrations of the test compounds. DMSO was employed as a vehicle for dissolution of the tested compounds and its final concentration on the cells was less than 0.2%. Cells were suspended in RPMI 1640 medium (for HepG2 and CACO-2 cell lines) and Dulbecco's modified Eagle's medium (DMEM) (for MCF7 cell line), 1% antibiotic-antimycotic mixture (10000 IU/mL penicillin potassium, 10000 μg/mL streptomycin sulphate and 25 μg/mL amphotericin B), and 1% L-glutamine in 96-well flat bottom microplate at 37°C under 5% CO_2. After 24 h of incubation, the medium was aspirated, 40 μL of MTT salt (2.5 μg/mL) were added to each well and incubated for further 4 h at 37°C under 5% CO_2. To stop the reaction and dissolve the formed crystals, 200 μL of 10% sodium dodecyl sulphate (SDS) in deionized water was added to each well and incubated overnight at 37°C. The absorbance was then measured using a microplate multi-well reader (Bio-Rad Laboratories Inc., model 3350, Hercules, CA, U.S.A.) at λ 595 nm and a reference wavelength of λ 620 nm. A statistical significance was tested between samples and negative control (cells with vehicle) using independent t-test by SPSS 11 program. The results are presented in Table 1 as LC_{50} (μM) which is the lethal concentration of the compound which causes death of 50% of the cells in 24 h.

DPPH Radical Scavenging Antioxidant Assay

Compounds **6**, **8**, **10**, **15**, **16**, **19**, **25** and **28** which exhibited prominent antitumor activity, were tested for their ability to show antioxidant effect through scavenging of the DPPH radical, according to a modified procedure from that described by Blois.[52] Two concentrations of the tested compounds (10^{-3} and 10^{-4} M) were mixed with a methanolic solution of DPPH (0.1 mL of 1 mM) at room temperature, so that the total volume of the reaction mixture is 3 mL. The mixture was shaken vigorously and allowed to stand in the dark for 30 min at room temperature. Thereafter, the absorbance (A) of the obtained solution was measured spectrophotometrically in the visible region at λ_{max} 517 nm. The same procedure was performed for a control which is DPPH radical solution in methanol alone. BHT was utilized as a reference standard antioxidant in this experiment. Each experiment was carried out in triplicate. The capability of the tested compounds to scavenge DPPH radical was calculated according to the following equation:

$$\text{DPPH scavenging effect (\%)} = (A_C - A_S)/A_C \times 100$$

where A_C is the absorbance of control (DPPH radical solution in methanol), and A_S represents the value of the absorbance of the sample (solution of DPPH radical and tested compound in methanol). The obtained data are presented in Table 2.

PDE3A Inhibitory Activity

The *in vitro* PDE3A inhibitory activity of the most active anticancer compounds (**8**, **15**, **16** and **19**) and milrinone (reference standard), was evaluated using PDE3A Assay Kit (Catalogue No. 60330) supplied by BPS Biosciences, San Diego, CA, U.S.A. This kit was designed for identification of PDE3A inhibitors using FP. The assay was conducted following the manufacturer's instructions. The enzymatic reactions were performed in 96-well microtiter plates using a 50 μL mixture containing PDE assay buffer, 20 μL PDE3A (20 pg/μL) and 5 μL inhibitor. Twenty five microliters of substrate solution containing 200 nM FAM-cAMP was then added and the plates were incubated at room temperature for 60 min. The binding reactions were conducted by adding 100 μL of binding agent (1 : 100 dilution in binding agent diluent). After incubation at room temperature for 60 min, FP was measured at an excitation of 485 nm and an emission of 528 nm using a Biotek Synergy™ 2 microplate reader. Compounds were tested in a range of 10 concentrations, each in duplicate. The IC_{50} values were calculated from dose response curve obtained by plotting the percentage of enzyme inhibition *versus* the concentration using PrismTM 4 software (GraphPad).

Acknowledgements This paper was funded by the Deanship of Scientific Research (DSR), King Abdulaziz University, Jeddah, Kingdom Saudi Arabia, under Grant number (166-224-D1435). The authors, therefore, acknowledge with thanks DSR technical and financial support. Sincere gratitude is conveyed to the staff members of the City for Scientific Research and Technology Applications, Alexandria, Egypt, for carrying out the cytotoxic screening of the newly synthesized compounds.

Conflict of Interest The authors declare no conflict of interest.

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