Multicomponent Reactions of Acetoacetanilide Derivatives with Aromatic Aldehydes and Cyanomethylene Reagents to Produce 4H-Pyrans and 1,4-Dihydropyridine Derivatives with Antitumor Activities

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The multi-component reaction of either acetoacetanilide derivative 1a or b with any of the aldehyde derivatives 2a–d and malononitrile 3 in the presence of triethylamine as a catalyst gave the 4H-pyrans 4a–g, respectively. Carrying the same reaction but using a catalytic amount of ammonium acetate gave the 1,4-dihydropyridine derivatives 5a–f, respectively. The use of ethyl cyanoacetate instead of malononitrile in the presence of a catalytic amount of triethylamine gave the 4H-pyrans 7a–d, respectively. Compound 8e was used to synthesize 1,4-dihydropyridine 9a–c and aryhydrazone 11a–e derivatives were synthesized from 4a and e. The anti-tumor evaluations of the newly synthesized products were tested against six human cancer and normal cell lines. The results showed that compounds 4a, b, f, 5d, f, 9 and 11a–d had optimal cytotoxic effect against cancer cell lines with 1IC50 < 550 nm. The toxicity of the most active compounds was further measured against shrimp larvae.

Key words 4H-pyrans, 1,4-dihydropyridine, cyanomethylene, cytotoxicity

Multicomponent reactions (MCRs) are one-pot reactions employing more than two starting materials, e.g., 3, 4, …, 7, where most of the atoms of the starting materials are incorporated in the final product.1) Several descriptive tags are regularly attached to MCRs they are atom economic, e.g. the majority if not all of the atoms of the starting materials are incorporated in the product; they are efficient, e.g. they efficiently yield the product since the product is formed in one-step instead of multiple sequential steps; they are convergent, e.g. several starting materials combine in one reaction to form the product; they exhibit a very high bond-forming-index (BFI), e.g. several non-hydrogen atom bonds are formed in one synthetic transformation.2,3) Therefore MCRs are often a useful alternative to sequential multistep synthesis.

In view of the increasing interest in the preparation of a large variety of heterocyclic compound libraries, the development of new synthetically valuable MCRs with several diversity points remains a challenge for both academic and industrial institutions.4) 4H-Pyrans and its derivatives are an important class of heterocyclic compounds possessing broad biological activities, such as anti-inflammatory,5) analgesic,6) antioxidant,7) antitubercular,8) antidepressant,9) sedative,10) antiamoebic11) and oral analgesic.12) From the aforementioned reports, it seems that the development of an efficient, rapid, and clean synthetic route towards focused libraries of such compounds is of great importance to both medicinal and synthetic chemists. The combination of multicomponent reactions (MCRs),13–15) which combines different types of MCRs in one pot process inheriting the high selectivity, efficiency, and atom economy of MCR, has gained more and more attention, since Dömling and Ugi reported the first MCR2 of a modified four-component reaction (4CR) and the Ugi 4CR.16) However, most of the reported MCR2 cases are limited in isonitrile based multicomponent reactions.17–20) Hence in this work, we report a one-pot, three-component reaction for the synthesis of 4H-pyrans and 1,4-dihydropyridine derivatives through the reaction of acetoacetanilide derivatives with cyanomethylene derivatives and aromatic aldehydes. All the synthesized compounds were characterized using IR (infrared), 1H-NMR, and 13C-NMR (nuclear magnetic resonance) spectrometry and were subjected to screening towards cancer cell lines.

Results and Discussion

Chemistry The reaction of either acetoacetanilide 1a or b with either benzaldehyde (2a), 4-chlorobenzaldehyde (2b), 3-nitrobenzaldehyde (2c) or furfural (2d) and malononitrile (3) in absolute ethanol containing a catalytic amount of triethylamine gave the 4H-pyrans 4a–g, respectively (Chart 1). The structures of 4a–g were established on the basis of analytical and spectral data. Thus, the 1H-NMR spectrum of 4a (as an example) showed a singlet at δ 1.91 for the methyl group, a singlet at δ 4.10 ppm (D2O exchangeable) indicating the NH2 group, a singlet at δ 5.91 ppm equivalent to the –CH=CH– group, a singlet at δ 7.10–7.52 ppm equivalent to the two phenyl groups and a singlet at δ 7.80 ppm for the NH group. Moreover, the 13C-NMR spectrum revealed δ 29.8 (CH3), 64.2 (4H-pyranc-4), 116.9 (CN), 119.0, 120.5, 120.9, 121.3, 121.7, 122.6, 124.0, 125.3, 130.2, 133.3, 136.9, 140.0 (2C6H5) and 164.3 (CO).

The formation of the 4H-pyrans 4a–g can be explained by the possible mechanism presented in Chart 2. The reaction can occur via the initial formation of the intermediate acrylonitrile derivative A followed by its nucleophilic attack of the anion of the acetoacetanilide derivatives to produce the intermediate B. The final products D were formed via the initial cyclization and subsequent tautomerization of the cyclic intermediate C.22)

To survey the scope of this reaction for the synthesis of 4H-pyran and 1,4-dihydropyridine derivatives, different catalyst was used. Thus, the multicomponent reaction of acetoactanilide-

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lide 1a or b with either 2a, b, c or d and 3 in absolute ethanol containing a catalytic amount of ammonium acetate gave the 1,4-dihydropyridine derivatives 5a–g, respectively (Chart 3). The IR spectrum of 5a exhibits absorption band at 3339 and 3246 (NH, NH₂), 3061 (CH-aromatic), 2921 (CH₃), 2207 (CN) and 1700 (C=O) cm⁻¹. The ¹H-NMR of compound 4a revealed the presence of the 4H-pyran H-4 at δ 5.91 ppm. The presence of the pyridine H-4 singlet at δ 4.99 ppm, the aromatic protons in the range δ 7.24–7.61 ppm and a singlet at δ 8.69 ppm (D₂O exchangeable) assigning for the NH group are consistent with the proposed structure of compound 5a.

With the optimized conditions established above, we decided to probe the generality of this multicomponent reaction using different cyanomethylene reagents. Thus, the reaction of acetoacetanilide 1a or b with either 2a, b or d and ethyl cyanoacetate (6) in absolute ethanol containing a catalytic amount of triethylamine gave the 4H-pyran derivatives 7a–d (Chart 4).

To explore the scope and limitations of the products of the multicomponent reactions we have examined the reactivity of one of the 4H-pyran derivatives 4a–g towards chemical reagents. For such purpose, the high yield of compound 4e encouraged us to do further reactions. Thus, the reaction of compound 4e with either of aniline (8a), 4-chloroaniline (8b) or p-toluidine (8c) gave the N-arylpyridine derivatives 9a–c, respectively (Chart 5). Analytical and spectral data of the latter products were consistent with their respective structures. The methyl group present in compounds 4a and e showed interesting reactivity towards electrophilic reagents. Thus either compound 4a or e reacted with the either benzenediazonium chloride (10a), 4-chlorobenzenediazonium chloride (10b) or 4-methylbenzenediazonium chloride (10c) to afford the corresponding arylhydrazone derivatives 11a–e (Chart 5).

The newly synthesized products were screened towards six cancer cell lines where some of them showed high cytotoxicity.

**In Vitro Cytotoxicity**

The heterocyclic compounds, prepared in this study, were evaluated according to standard protocols for their *in vitro* cytotoxicity against six human cancer cell lines including cells derived from human gastric cancer (NUGC), human colon cancer (DLD1), human liver cancer (HA22T and HEPG2), human breast cancer (MCF) and nasopharyngeal carcinoma (HONE1) as well as a normal fibroblast cells (WI38). The reference compound used is the CHS-828 (Fig. 1) which is a pyridylecyano guanidine anti-tumor agent. All of the IC₅₀ values (the sample concentration that produces 50% reduction in cell growth) in nanomolar (nM) are listed in Table 1. Some heterocyclic compounds was observed with significant cytotoxicity against most of the cancer cell lines tested (IC₅₀=10–1000 nM). All the synthesized compounds were tested for their cytotoxicity against normal fibroblast cells. The results obtained showed that normal fibroblasts cells (WI38) were affected to a much lesser extent (IC₅₀>10000 nM).

**Structure–Activity Relationship**

From Table 1, it is convenient to notice that compounds 4b and f have higher cytotoxicity than 4a and e–e and g. Such high cytotoxicity of both compounds is attributed to the presence of Cl atom at position 4 of one of aryl moieties. However, compound 4e showed high potency against breast cancer MCF which is equivalent to the cytotoxic effect of the standard CHS 828 (IC₅₀=18 nM). Considering the 1,4-dihydropyridine
derivatives 5a–f, each one of these derivative revealed selective activity against certain cancer cell lines. Compound 5a showed selective higher activity against liver cancer HEPG2 (IC50 = 22 nM) than 5b–f. The introduction of 3-nito group in 5d exhibited remarkable increase in the activity against colon cancer DLD1 than 5a–c and f. Moreover compound 5f exhibited high cytotoxicity effect (IC50 = 38 nM) against gastric cancer (NUGC) compared to the standard CHS 828 (IC50 = 25 nM). The remarkable activity of 5f is due to the presence of the furan moiety. Comparing compound 4f with 5d, it is obvious that 4H-pyran ring present in 4f showed lower growth inhibitory effect than 1,4-dihydropyridine ring in 5d. Comparing the activities of compounds 4a, d and f with 7a–c, it is observed that compounds 4a, d and f showed higher cytotoxicity compared to compounds 7a–c. Higher cytotoxicity of 4a, d and f is due to the presence of CN group attached to the 4H-pyran ring while in case of compounds 7a–c, the COOEt group lowered their cytotoxicity. The high activity of 9b compared to 9a and c as well as 5c is due to the presence of three aryl moieties and Cl atom on one of aryl groups. Considering the 4H-pyran derivatives 11a–e, compounds 11a–d showed high growth inhibitory effects. This may be attributed to the presence of arylhydrazonomethyl group attached to 4H-pyran ring. Moreover, 11d substituted with 4-chloro group showed the highest cytotoxicity among the five compounds with remarkable activity against the five human cancer cell lines namely: NUGC, DLD1, HA22T, HEPG2 and HONE1 with
IC₅₀'s 32, 50, 27, 221 and 228 nM, respectively.

Toxicity Bioactive compounds are often toxic to shrimp larvae. Thus, in order to monitor these chemicals' in vivo lethality to shrimp larvae (Artemia salina), Brine-Shrimp Lethality Assay was used. Results were analysed with LC₅₀ program to determine LC₅₀ values and 95% confidence intervals. Results are given in Table 2 for the compounds which exhibited optimal cytotoxic effect against cancer cell lines which are the compounds 4a, b, f, 5d, f, 9 and 11a–d. The shrimp lethality assay is considered as a useful tool for preliminary assessment of toxicity, and it has been used for the detection of fungal toxins, plant extract toxicity, heavy metals, cyanobacteria toxins, pesticides, and cytotoxicity testing of dental materials, natural and synthetic organic compounds. It has also been shown that A. salina toxicity test results have a correlation with rodent and human acute oral toxicity data. Generally, a good correlation was obtained between A. salina toxicity test and the rodent data. Likewise, the predictive screening potential of the aquatic invertebrate tests for acute oral toxicity in man, including A. salina toxicity test, was slightly better than the rat test for test compounds.

In order to prevent the toxicity results from possible false effects originated from solubility of compounds and dimethyl sulfoxides (DMSO's) possible toxicity effect, compounds were prepared by dissolving in DMSO in the suggested DMSO volume ranges. It is clear from Table 2 that compounds 4f and 11a–d showed non toxicity against the tested organisms. On the other hand, compounds 5f and 9b are very toxic, in addition, compounds 4a, b and 5d are harmful.

Conclusion

In summary, we have developed a convenient synthetic approach for novel 4H-pyran and 1,4-dihydropyridine derivatives. The region selective attack by different reagents on the active center moiety in acetocetanilide derivatives led to the diversity of the produced systems. Most of the newly synthesized compounds were found to be promising anti-proliferative agents. Results showed that compounds 4a, f, 5d, f, 9 and 11a–d are the most active compounds towards the tumor cell lines. In addition, compounds 4f and 11a–d showed non toxicity against shrimp larvae.

Experimental

Chemistry All melting points were uncorrected on a Stuart apparatus melting point apparatus. IR spectra (KBr, cm⁻¹)
followed by few drops of Et₃N. The whole reaction mixture nonitrile (0.66 g, 1.0 mmol) was added to the previous mixture of benzaldehyde (1 g, 1.0 mmol), 4-chlorobenzaldehyde (1.4 g, acetoacetanilide (1.91 g, 1.0 mmol), or 2-methylpyran-3-carboxylic Acid Arylamide (4a–g) was heated under reflux for 3 h then was allowed to cool and was filtered and washed with water. The crude product was pure by recrystallization from ethanol.

6-Amino-5-cyano-2-methyl-4-phenyl-4H-pyr-3-carboxylic Acid Phenylamide (4a)

Pale yellow crystals, yield 70%; mp 202°C; IR (KBr) cm⁻¹: 3437, 3340 (NH, NH₂), 3214 (CH-aromatic), 2936 (CH₃), 2210 (CN), 1680(C=O). ¹H-NMR (300 MHz, DMSO-d₆) δ: 2.04 (s, 3H, CH₃), 3.94 (4H-pyr-an), 7.18-7.71 (m, 9H, C₆H₅, C₆H₄), 7.82 (s, 1H, D₂O exchangeable, NH). ¹³C-NMR (75 MHz, DMSO-d₆): 29.8 (CH₃), 64.2 (4H-pyr-an-C), 116.9 (CN), 119.0, 120.5, 120.9, 121.3, 121.7, 122.6, 124.0, 125.3, 130.2, 133.3, 136.9, 140.0 (C₂H₅, 4H-pyr-an-C), 164.3 (CO). Anal. Calcd for C₂H₁₇N₂O₂ (331.37): C, 72.49; H, 4.09; N, 11.39. Found: C, 72.19; H, 4.09; N, 11.39.

General Procedure for the Synthesis of 4-Aryl-4H-pyran-3-carboxylic Acid Arylamide (4a–g)

To a solution of either acetocetanilide (1.77 g, 1.0 mmol), or 2-methylace-toacetanilide (1.91 g, 1.0 mmol), in 1,4-dioxane (40 mL), either acetoacetanilide (1.77 g, 1.0 mmol), or 2-methylpyran-3-carboxylic Acid Phenylamide (4a–g) was added. Malononitrile (0.66 g, 1.0 mmol) was added to the previous mixture followed by few drops of Et₂N. The whole reaction mixture was heated under reflux for 3 h then was allowed to cool and poured into ice cold water with stirring. The separated solid was filtered and washed with water. The crude product was pure by recrystallization from ethanol.

were determined on a Shimadzu IR 435 spectrophotometer ¹H- and ¹³C-NMR spectra were recorded on a Mercury-300BB (300 MHz) (Cairo University) in DMSO-d₆ as solvent using TMS [Si(CH₃)₄] as internal standard and chemical shifts are expressed as δ ppm. Elemental analyses were obtained from the Microanalytical Data Center at Cairo University, Egypt and were performed on Vario El III Elemental CHNS analyzer.

### Table 1. Cytotoxicity of the Newly Synthesized Products against a Variety of Cancer Cell Lines[a] [IC₅₀ (in µg)]

| Compound | NUGC | DLD1 | HA22T | HEPG2 | HONE1 | MCF | WI38 |
|----------|------|------|-------|-------|-------|-----|------|
| 4a       | 389  | 1220 | 1480  | 125   | 1620  | 36  | na   |
| 4b       | 228  | 569  | 213   | 1112  | 2052  | 2011 | 632  |
| 4c       | 2265 | 2139 | 2257  | 2177  | 2250  | 18   | 262  |
| 4d       | 1126 | 2168 | 1312  | 1232  | 1824  | 2330 | 549  |
| 4e       | 2377 | 1389 | 2076  | 1890  | 2170  | 779  | na   |
| 4f       | 880  | 760  | 1089  | 239   | 128   | 320  | na   |
| 4g       | 1128 | 1892 | 2377  | 1328  | 1290  | 2673 | 360  |
| 5a       | 1288 | 2187 | 2530  | 22    | 2135  | 1729 | 650  |
| 5b       | 1156 | 1280 | 1650  | 1226  | 699   | 821  | 910  |
| 5c       | 2211 | 1070 | 1288  | 1302  | 2179  | 1229 | 489  |
| 5d       | 48   | 59   | 122   | 2334  | 3289  | 480  | na   |
| 5e       | 1622 | 396  | 274   | 2120  | 670   | 1186 | 490  |
| 5f       | 38   | 163  | 120   | 3744  | 441   | 1264 | 860  |
| 7a       | 1232 | 1166 | 2225  | 2216  | 326   | 1286 | na   |
| 7b       | 1280 | 2419 | 2160  | 1284  | 2130  | 2073 | 872  |
| 7c       | 2101 | 2458 | 2258  | 350   | 2180  | 1140 | 428  |
| 7d       | 1562 | 770  | 528   | 238   | 139   | 2389 | na   |
| 9a       | 1760 | 1429 | 1267  | 2309  | 1876  | 2690 | na   |
| 9b       | 84   | 167  | 219   | 2023  | 1210  | 1142 | na   |
| 9c       | 2210 | 2433 | 1650  | 2560  | 1544  | 2457 | 520  |
| 11a      | 48   | 55   | 128   | 128   | 248   | 128  | 838  |
| 11b      | 135  | 158  | 278   | 279   | 206   | 668  | 829  |
| 11c      | 122  | 3210 | 59    | 1245  | 1140  | 1130 | na   |
| 11d      | 32   | 50   | 27    | 221   | 228   | 2055 | 780  |
| 11e      | 3138 | 2366 | 2228  | 2130  | 1584  | 326  | 650  |
| CHS 828  | 25   | 2315 | 2067  | 1245  | 15    | 18   | na   |

[a] NUGC, gastric cancer; DLD1, colon cancer; HA22T, liver cancer; HEPG2, liver cancer; HONE1, nasopharyngeal carcinoma; HR, gastric cancer; MCF, breast cancer; WI38, normal fibroblast cells. The sample concentration produces a 50% reduction in cell growth.
121.9, 122.4, 123.9, 128.2, 129.3, 129.9, 131.2, 132.3, 133.8, 139.7, 141.2, 142.4 (C₆H₅, C₆H₄, 4H-pyranC), 164.0 (CO). Anal. Calcd for C₂₃H₂₃N₅O₄ (376.37): C, 63.82; H, 4.28; N, 14.89. Found: C, 63.21; H, 4.09; N, 14.92.

6-Amino-5-cyano-2-methyl-4-phenyl-4-pyran-3-carboxylic Acid Phenylamide (4d)

Red crystals, yield 81%; mp 246°C; IR (KBr) cm⁻¹: 3434, 3284 (NH, NH₂), 3141 (CH-aromatic), 2926 (CH₃), 2177 (CN). 1H-NMR (300 MHz, DMSO-d₆) δ: 1.91 (s, 3H, CH₃), 2.17 (s, 3H, CH₃), 2.27 (s, 3H, CH₃), 3.76 (s, 2H, D₂O exchangeable, NH₂), 3.99 (s, 2H, D₂O exchangeable, NH₂), 4.12 (s, 2H, D₂O exchangeable, NH₂), 5.92 (s, 1H, CH, 4H-pyran), 7.16–7.62 (m, 8H, C₆H₅), furan CH), 7.80 (s, 1H, D₂O exchangeable, NH). 13C-NMR (75 MHz, DMSO-d₆): 29.4, 37.9 (2CH₃), 64.6 (4CH₂-pyranC), 164.5 (CO). Anal. Calcd for C₂₃H₂₃N₅O₄ (376.37): C, 63.82; H, 4.28; N, 14.89. Found: C, 63.21; H, 4.09; N, 14.92.

6-Amino-5-cyano-4-furan-2-yl-2-methyl-4H-pyran-3-carboxylic Acid o-Tolylamide (4e)

White crystals, yield 85%; mp 246°C; IR (KBr) cm⁻¹: 3434, 3284 (NH, NH₂), 3141 (CH-aromatic), 2926 (CH₃), 2177 (CN), 1690 (C=O). 1H-NMR (300 MHz, DMSO-d₆) δ: 1.91 (s, 3H, CH₃), 2.27 (s, 3H, CH₃), 3.76 (s, 2H, D₂O exchangeable, NH₂), 3.99 (s, 2H, D₂O exchangeable, NH₂), 4.12 (s, 2H, D₂O exchangeable, NH₂), 5.92 (s, 1H, CH, 4H-pyran), 6.94–7.81 (m, 8H, C₂H₅), 8.22 (s, 1H, D₂O exchangeable, NH). 13C-NMR (75 MHz, DMSO-d₆): 29.4, 37.9 (2CH₃), 64.6 (4H-pyranC), 164.5 (CO). Anal. Calcd for C₂₃H₂₃N₅O₄ (376.37): C, 63.82; H, 4.28; N, 14.89. Found: C, 63.21; H, 4.09; N, 14.92.

6-Amino-4-(4-chlorophenyl)-5-cyano-2-methyl-4H-pyran-3-carboxylic Acid o-Tolylamide (4f)

White crystals, yield 75%; mp 214°C; IR (KBr) cm⁻¹: 3437, 3343 (NH, NH₂), 3061 (CH-aromatic), 2925 (CH₃), 2179 (CN), 1690 (C=O). 1H-NMR (300 MHz, DMSO-d₆) δ: 1.91 (s, 3H, CH₃), 2.27 (s, 3H, CH₃), 4.14 (s, 2H, D₂O exchangeable, NH₂), 5.92 (s, 1H, CH, 4H-pyran), 6.94–7.81 (m, 8H, C₂H₅), 8.22 (s, 1H, D₂O exchangeable, NH). 13C-NMR (75 MHz, DMSO-d₆): 29.4, 37.9 (2CH₃), 64.6 (4H-pyranC), 164.5 (CO). Anal. Calcd for C₂₃H₂₃N₅O₄ (376.37): C, 63.82; H, 4.28; N, 14.89. Found: C, 63.21; H, 4.09; N, 14.92.

6-Amino-5-cyano-2-methyl-4-phenyl-4H-pyran-3-carboxylic Acid o-Tolylamide (4g)

Pale yellow crystals, yield 70%; mp 213°C; IR (KBr) cm⁻¹: 3437, 3346 (NH, NH₂), 3070 (CH-aromatic), 2941 (CH₃), 2182 (CN), 1706 (C=O). 1H-NMR (300 MHz, DMSO-d₆) δ: 1.91 (s, 3H, CH₃), 2.17 (s, 3H, CH₃), 4.30 (s, 2H, D₂O exchangeable, NH₂), 6.00 (s, 1H, CH, 4H-pyran), 6.98–8.23 (m, 8H, C₂H₅), 8.30 (s, 1H, D₂O exchangeable, NH). 13C-NMR (75 MHz, DMSO-d₆): 28.4, 38.6 (2CH₃), 64.5 (4H-pyranC), 166.7

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Table 2. Toxicity of Newly Synthesized Compounds Which Exhibited Optimal Cytotoxic Effect against Cancer Cell Lines

| Compound | Conc. (µg/mL) | Mortality(a) | Toxicity | LC₅₀ | Upper 95% lim | Lower 95% lim |
|----------|--------------|--------------|----------|------|---------------|---------------|
| 4a       | 10           | 0            | Harmful  | 403.26 | 110.23        | 88.25         |
|          | 100          | 5            |          |      |               |               |
|          | 1000         | 10           |          |      |               |               |
| 4b       | 10           | 1            | Harmful  | 82.28  |               |               |
|          | 100          | 3            |          |      |               |               |
|          | 1000         | 10           |          |      |               |               |
| 4f       | 0            | 0            | Non toxic| 979.19 |               |               |
|          | 100          | 0            |          |      |               |               |
|          | 1000         | 2            |          |      |               |               |
| 5d       | 10           | 0            | Harmful  | 20.7   | 216.29        | 162.51        |
|          | 100          | 6            |          |      |               |               |
|          | 1000         | 8            |          |      |               |               |
| 5f       | 10           | 2            | Very toxic| 12.8   |               |               |
|          | 100          | 8            |          |      |               |               |
|          | 1000         | 10           |          |      |               |               |
| 9b       | 10           | 1            | Very toxic| 16.22  |               |               |
|          | 100          | 6            |          |      |               |               |
|          | 1000         | 10           |          |      |               |               |
| 11a      | 10           | 0            | Non toxic| 981.41 |               |               |
|          | 100          | 0            |          |      |               |               |
|          | 1000         | 2            |          |      |               |               |
| 11b      | 10           | 0            | Non toxic| 999.09 |               |               |
|          | 100          | 0            |          |      |               |               |
|          | 1000         | 5            |          |      |               |               |
| 11c      | 10           | 0            | Non toxic| 991.05 |               |               |
|          | 100          | 0            |          |      |               |               |
|          | 1000         | 3            |          |      |               |               |
| 11d      | 10           | 0            | Non toxic| 892.41 |               |               |
|          | 100          | 1            |          |      |               |               |
|          | 1000         | 4            |          |      |               |               |

(a) Ten organisms (A. salina) tested for each concentration.
(CN), 118.8, 119.8, 121.7, 122.9, 123.7, 125.9, 126.3, 130.9, 131.2, 131.8, 132.3, 138.7, 139.2, 140.7, 142.3, 144.5 (2C=H, 4H-pyranC), 164.2 (CO). Anal. Calcd for C12H12N2O (390.39): C, 64.61; H, 4.65; N, 14.35. Found: C, 63.92; H, 4.75; N, 13.92.

**General Procedure for the Synthesis of 4-Aryl-1,4-dihydro-pyridine-3-carboxylic Acid Arylamide (5a–f)**

To a solution of either acetoacetanilide (1.77 g, 1.0 mmol) or 2-methylacetoacetanilide (1.91 g, 1.0 mmol) in absolute ethanol (15 mL), either of benzaldehyde (1 g, 1.0 mmol), 4-chlorobenzaldehyde (1.4 g, 1.0 mmol), 3-nitrobenzaldehyde (1.5 g, 1.0 mmol) or furfural (1 g, 1.0 mmol) was added. Ammonium acetate (0.77 g, 1.0 mmol) and malononitrile (0.66 g, 1.0 mmol) were added and the mixture heated under reflux for 2 h. The reaction mixture was allowed to cool and poured into ice cold water with stirring. The separated solid was filtered and washed with water. The crude product was purified by recrystallization from ethanol.

6-Amino-5-cyano-2-methyl-4-phenyl-1,4-dihydro-pyridine-3-carboxylic Acid Phenylamide (5a)

Yellow crystals, yield 65%; mp 302°C; IR (KBr) cm⁻¹: 3339, 3246 (NH, NH₂), 3061 (CH-aromatic), 2921 (CH₃), 2207 (CN), 1700 (C=O). ¹H-NMR (300 MHz, DMSO-d₆): 1.91 (s, 3H, CH₃), 3.54 (s, 2H, D₂O exchangeable, NH₂), 4.99 (s, 1H, CH, 4H-pyridine), 7.24–7.61 (m, 10H, C₆H₅), 8.69 (s, 1H, D₂O exchangeable, NH), 13C-NMR (75 MHz, DMSO-d₆): 8.64 (1H, 4H-pyridine), 7.64–7.67 (m, 9H, C₆H₅, C₆H₄), 8.63 (s, 1H, D₂O exchangeable, NH). ¹³C-NMR (75 MHz, DMSO-d₆): 50.80 (D₂O exchangeable, NH₂), 13C-NMR (75 MHz, DMSO-d₆): 28.9 (C₃H₆), 64.3 (1,4-dihydropyridineC-4), 116.5 (CN), 118.8, 120.6, 122.3, 123.6, 125.0, 125.2, 126.8, 130.8, 132.1, 132.3, 138.7, 142.2 (C₆H₅), 144.1 (C₆H₄), 146.4 (CO). Anal. Calcd for C₂₁H₁₈N₄O₂ (334.37): C, 66.78; H, 4.87; N, 16.96. Found: C, 70.09; H, 5.28; N, 16.96.

6-Amino-4-(4-chlorophenyl)-5-cyano-2-methyl-1,4-dihydro-pyridine-3-carboxylic Acid Phenylamide (5b)

Yellow crystals, yield 68%; mp 343°C; IR (KBr) cm⁻¹: 3325, 3287 (NH, NH₂), 3062 (CH-aromatic), 2920 (CH₃), 2210 (CN), 1688 (C=O). ¹H-NMR (300 MHz, DMSO-d₆): 1.90 (s, 3H, CH₃), 3.54 (s, 2H, D₂O exchangeable, NH₂), 5.00 (s, 1H, CH, 4H-pyridine), 6.94–7.47 (m, 9H, C₆H₅, C₆H₄), 8.63 (s, 1H, D₂O exchangeable, NH). ¹³C-NMR (75 MHz, DMSO-d₆): 28.9 (C₃H₆), 64.3 (1,4-dihydropyridineC-4), 116.5 (CN), 118.8, 120.6, 122.3, 123.6, 125.0, 125.2, 126.8, 130.8, 132.1, 132.3, 138.7, 142.2 (C₆H₅), 144.1 (C₆H₄), 146.4 (CO). Anal. Calcd for C₁₉H₁₈N₄O (363.48): C, 65.84; H, 4.70; N, 15.36. Found: C, 65.21; H, 5.91; N, 14.11.

6-Amino-5-cyano-2-methyl-4-phenyl-1,4-dihydro-pyridine-3-carboxylic Acid O-Tolylamide (5c)

Orange crystals, yield 72%; mp 377°C; IR (KBr) cm⁻¹: 3455, 3329 (NH, NH₂), 3063 (CH-aromatic), 2978 (CH₃), 2200 (CN), 1681 (C=O). ¹H-NMR (300 MHz, DMSO-d₆): 1.92 (s, 3H, CH₃), 2.21 (s, 3H, CH₃), 3.31 (s, 2H, D₂O exchangeable, NH₂), 4.99 (s, 1H, CH, 4H-pyridine), 6.90–7.49 (m, 9H, C₆H₅, C₆H₄), 7.59 (s, 1H, D₂O exchangeable, NH), 8.39 (s, 1H, D₂O exchangeable, NH). ¹³C-NMR (75 MHz, DMSO-d₆): 28.6 (C₃H₆), 64.8 (1,4-dihydropyridineC-4), 116.5 (CN), 119.6, 120.8, 122.6, 122.9, 124.6, 125.8, 128.3, 129.4, 132.7, 135.2, 137.4, 138.4, 142.8, 143.9 (C₆H₅, C₆H₄, 1,4-dihydropyridineC), 164.2 (CO). Anal. Calcd for C₂₁H₂₁N₂O₂ (344.41): C, 73.23; H, 5.85; N, 16.27. Found: C, 73.83; H, 4.99; N, 14.93.

6-Amino-4-(4-chlorophenyl)-5-cyano-2-methyl-1,4-dihydro-pyridine-3-carboxylic Acid O-Tolylamide (5d)

White crystals, yield 60%; mp 191°C; IR (KBr) cm⁻¹: 3332, 3236 (NH, NH₂), 3058 (CH-aromatic), 2984 (CH₃), 1763, 1673 (C=O). ¹H-NMR (300 MHz, DMSO-d₆): 1.31 (t, J=7.2, 3H, CH₂ CH₃), 2.20 (s, 3H, CH₃), 3.54 (s, 2H, D₂O exchangeable, NH₂), 4.32 (q, J=7.2 Hz, 2H, CH₂ CH₃), 4.80 (s, 1H, CH, 4H-pyran), 7.02–8.06 (m, 10H, 2CH₃), 8.40 (s, 1H, D₂O exchangeable, NH). ¹³C-NMR (75 MHz, DMSO-d₆): 16.2 (OCH₂ CH₃), 28.8 (CH₃), 56.3 (OCH₂ CH₃), 64.4 (4H-py-
ranC-4), 119.8, 121.6, 122.6, 124.0, 124.8, 125.3, 127.4, 131.8, 133.8, 133.9, 134.7, 139.8 (2C, H, 4H-pyranC), 164.8, 166.3 (2C). Anal. Calcd for C_{32}H_{58}NO_{12} (738.42): C, 69.83; H, 5.86; N, 7.40. Found: C, 71.69; H, 5.39; N, 8.52.

2-Amino-4-furan-2-yl-6-methyl-5-phenylcarbamoyl-4H-pyrane-3-carboxylic Acid Ethyl Ester (7b)

Red crystals, yield 62%; mp 220°C; IR (KBr) cm⁻¹: 3426, 3282 (NH, NH), 3064 (CH-aromatic), 2971 (CH₃), 2933 (CH₂CH₃), 2876 (CH₃), 1725, 1661 (CO). Anal. Calcd for C_{27}H_{23}ClN₄O (454.95): C, 71.28; H, 5.10; N, 12.31. Found: C, 71.73; H, 4.90; N, 12.04.

6-Amino-5-cyano-2-methyl-4-phenyl-1,4-dihydropyridine-3-carboxylic Acid o-Tolylamide (9b)

Yellow crystals, yield 60%; mp 184°C; IR (KBr) cm⁻¹: 3349, 3342 (NH, NH), 3033 (CH-aromatic), 2177 (CN), 1691 (C=O). 1H-NMR (300 MHz, DMSO-d₆): δ 1.38 (t, J = 7.2 Hz, 3H, CH₂CH₃), 2.00 (s, 3H, CH₃), 2.08 (s, 3H, CH₃), 2.89 (s, 2H, D₂O exchangeable, NH). 13C-NMR (75 MHz, DMSO-d₆): 16.2 (OCH₂CH₃), 28.6 (CH₃), 56.4 (OCH₂CH₃), 64.6 (4H-pyran-C-4), 120.7, 121.5, 122.3, 125.2, 126.8, 129.6, 129.8, 132.6, 134.9, 147.2, 148.8, 154.4 (C₆H₅), 4H-pyran-C). Anal. Calcd for C_{22}H_{20}N₂O₆ (382.51): C, 77.12; H, 5.75; N, 13.32. Found: C, 76.44; H, 5.41; N, 13.52.

General Procedure for the Synthesis of 1,4-Diaryl-1,4-dihydropyridine-3-carboxylic Acid o-Tolylamide (9a–c)

A mixture of equimolar amounts of aniline (1 g, 10 mmol), 4-chloroaniline (1.3 g, 10 mmol), or p-toluidine (1.1 g, 10 mmol) in N,N-dimethylformamide (DMF) (15 mL), compound 4e (3.45 g, 10 mmol) was added. The reaction mixture was heated under reflux for 5 h. The solid products formed upon pouring onto ice/water mixture and collected by filtration and crystallized from ethanol.

6-Amino-5-cyano-4-phenyl-1,4-dihydropyridine-3-carboxylic Acid o-Tolylamide (9a)

Yellow crystals, yield 60%; mp 184°C; IR (KBr) cm⁻¹: 3451, 3343 (NH, NH), 3058 (CH-aromatic), 2945 (CH₃), 2719 (CN), 1715 (C=O). 1H-NMR (300 MHz, DMSO-d₆): δ 1.89 (s, 3H, CH₃), 2.20 (s, 3H, CH₃), 4.27 (s, 2H, D₂O exchangeable, NH), 5.80 (s, 1H, CH, 4H-pyridine), 6.94–7.46 (m, 15H, 2C₆H₅, C₆H₅ and NH). 13C-NMR (75 MHz, DMSO-d₆): 28.1 (CH₃), 32.8 (CH₄), 64.5 (1,4-dihydropyridine-C₄), 116.5 (CN), 122.5, 129.2, 129.5, 130.8, 130.9, 132.3, 135.2, 136.0, 136.2, 137.0, 138.0, 139.4, 142.6 (2C, C₆H₅, C₆H₅-dihydridipryline), 166.6 (CO). Anal. Calcd for C_{27}H_{29}N₂O₄ (420.51): C, 77.12; H, 5.75; N, 13.32. Found: C, 76.44; H, 5.41; N, 13.52.

General Procedure for the Synthesis of 2-(Arylhydrazonomethyl)-4H-pyrane-3-carboxylic Acid Arylamide (11a–e)

To a cold solution (0–5°C) of either 4a (3.31 g, 1.0 mmol) or 4e (3.45 g, 1.0 mmol) in absolute ethanol (20 mL) containing sodium hydroxide (1.00 g), an equimolar amount of either diazotized aniline, diazotized p-chloroaniline or diazotized p-toluidine [prepared by the addition of sodium nitrite solution (0.70 g, 1.0 mmol in 5 mL water) to a cold solution (0–5°C) of either diazotized aromatic amine (1.0 mmol) in concentrated hydrochloric acid (12 mL) with continuous stirring] was gradually added while stirring. The solid products formed upon cooling in an ice-bath were collected by filtration, washed with water and crystallized from 1,4-dioxane.

5-Amino-6-cyano-4-phenyl-2-(phenylhydrazonomethyl)-4H-pyran-3-carboxylic Acid Phenylamide (11a)

Orange crystals, yield 78%; mp 180°C; IR (KBr) cm⁻¹: 3439, 3342 (NH, NH), 3033 (CH-aromatic), 2177 (CN), 1691 (C=O). 1H-NMR (300 MHz, DMSO-d₆): δ 4.10 (s, 2H, D₂O exchangeable, NH), 5.90 (s, 1H, CH, 4H-pyridine), 6.94–7.46 (m, 16H, 3C₆H₅, CH=N), 10.58 (b, 1H, D₂O exchangeable, NH), 12.36 (s, 1H, D₂O exchangeable, NH). 13C-NMR (75 MHz, DMSO-d₆): 64.5 (4H-pyran-C₄), 116.6 (CN), 120.8, 121.7, 123.6, 124.8, 125.3, 126.8, 127.8, 131.6, 133.4, 134.6,
133.8, 135.0, 135.3, 136.8, 137.3, 139.2, 139.6, 140.6 (2C 6H5, (CN), 119.3, 120.9, 122.7, 123.5, 125.9, 127.0, 129.2, 132.8, -Tolylamide (C26H20ClN5O2 (469.92): C, 66.45; H, 4.29; N, 14.90. Found: C, 1H, D 2O exchangeable, NH). 13C-NMR (75 MHz, DMSO-

-CH=CH2, 5.80 (s, 1H, CH, 4

169.9 (C

139.8, 140.9, 141.2, (2C 6H5, C 6H4, 4

pyranC-4), 116.5 (CN), 119.6, 121.4, 122.2, 123.8, 124.2, 127.4, 133.8, 135.0, 135.3, 136.3, 137.3, 139.2, 139.6, 140.6 (2CH2, C6H4, 4H-pyranC), 164.6 (CO), 166.9 (C=N). Anal. Calcd for C26H21N5O2 (449.50): C, 66.45; H, 4.29; N, 14.90. Found: C, 65.84; H, 4.51; N, 14.73.

5-Amino-4-cyano-4-phenyl-2-(phenylhydrazonomethyl)-4H-pyran-3-carboxylic Acid O-Tolylamide (11f)

Yellow crystals, yield 82%; mp 95°C; IR (KB) cm⁻¹: 3454, 3343 (NH), 3205 (CH-aromatic), 2181 (CN), 1677 (C=O). H¹-NMR (300 MHz, DMSO-d₆) δ: 4.66 (s, 2H, D₂O exchangeable, NH₂), 7.80 (s, 1H, CH, 4H-pyran), 6.80 (s, 1H, CH=N), 6.94–7.46 (m, 14H, 2C 6H5, C 6H4), 10.58 (s, 1H, D₂O exchangeable, NH), 12.37 (s, 1H, 1H, O exchangeable, NH). ¹³C-NMR (75 MHz, DMSO-d₆): 64.2 (4H-pyran-C), 116.8 (CN), 120.8, 120.8, 124.7, 124.9, 126.2, 127.4, 129.8, 129.5, 132.6, 134.2, 135.8, 136.4, 136.7, 137.4, 138.1, 138.5, 139.5, 140.3, 141.6, (C6H5, 2C6H4, 4H-pyran-C), 164.2 (CO), 169.9 (C=N). Anal. Calcd for C₂₇H₂₃N₅O₂ (513.50): C, 71.71; H, 4.86; N, 16.08. Found: C, 71.49; H, 5.03; N, 16.58.

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Conflict of Interest

The authors declare no conflict of interest.

In Vitro Cytotoxic Assay

Fetal bovine serum (FBS) and l-glutamine, were purchased from Gibco Invitrogen Co. (Scotland, U.K.). RPMI-1640 medium was purchased from Cambrex (NJ, U.S.A.). DMSO, doxorubicin, penicillin, streptomycin and sulfonamide B (SRB) were purchased from Sigma Chemical Co. (Saint Louis, MO, U.S.A.).

Cell Cultures

The cell cultures were obtained from the European Collection of cell Cultures (ECACC, Salisbury, U.K.) and human gastric cancer (NUGC and HR), human colon cancer (DLD1), human liver cancer (HA22T and HEP2G), human breast cancer (MCF), nasopharyngeal carcinoma (HONE1) and normal fibroblasts (WI38) were kindly provided by the National Cancer Institute (NCI, Cairo, Egypt). They grow as monolayer and routinely maintained in RPMI-1640 medium supplemented with 5% heat inactivated FBS, 2µM glutamine and antibiotics (penicillin 100 U/mL, streptomycin 100 g/mL), at 37°C in a humidified atmosphere containing 5% CO₂. Exponentially growing cells were obtained by plating 1.5×10⁶ cells/mL for the seven human cancer cell lines including cells derived from 0.75×10⁶ cells/mL followed by 24h of incubation. The effect of the vehicle solvent (DMSO) on the growth of these cell lines was evaluated in all the experiments by exposing untreated control cells to the maximum concentration (0.5%) of DMSO used in each assay.

Toxicity

All toxicity tests were 96-h static renewal tests and water quality measurements (dissolved oxygen, pH, temperature, salinity) were taken in the control containers each day. Tests were run in a Revcos Environmental Chamber at 25°C, 20% salinity, and a 16-h light:8-h dark cycle. Amedia change was made every 24h. Larvae used for all tests were one to two days old and exposed in 600-mL glass beakers containing 400 mL of media with 10 larvae/beaker and three replicates/concentration. Larvae were fed newly hatched Artemia after daily media change. For the individual pesticide toxicity tests, the following concentrations were used. Larval nominal atrazine concentrations were 625, 1250, 2500, 5000, 10000 mg/L and control. Larval nominal imidacloprid concentrations were 100, 200, 400, 600, 800 mg/L and control. For imidacloprid, adult shrimp toxicity tests were also run to complete the grass shrimp toxicity profile. Adult shrimp (acclimated for two weeks before testing) were exposed in 4-L wide mouth glass jars containing 2-L of media and 10 shrimp/jar with two replicates/concentration, and were run under conditions as described above for larvae.Adults were not fed during the test. Adult nominalimidacloprid concentrations were 226, 328, 475.6, 689.6, 1000 mg/L and control.

Conflict of Interest

The authors declare no conflict of interest.
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