Preparation of pyrimido[4,5-b][1,6]naphthyridin-4(1H)-one derivatives using a zeolite–nanogold catalyst and their in vitro evaluation as anticancer agent

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Abstract
Catalysis using supported gold nanoparticles has attracted significant research interest due to their unique properties and potential that is directly related to their particle size. An efficient one-pot, three-component procedure is developed for the preparation of pyrimido[4,5-b][1,6]naphthyridin-4(1H)-one derivatives (4a–h) by cyclocondensation of 6-amino-2-thioxo-2,3-dihydropyrimidin-4(1H)-one (1), aromatic aldehydes (2), and 1-benzylpiperidin-4-one (3) in the presence of zeolite-nano Au as a green catalyst in ethanol at 80 °C. The presented methodology has a number of advantages including a reusable catalyst, easy access, short reaction times, high yields, and an easy work-up. The nanogold catalyst is characterized by X-ray diffraction and transmission electron microscopy. The structures of the prepared compounds are established by elemental analyses and spectral data (infrared, mass spectrometry, 1H, and 13C NMR). While molecular docking studies show that products 4a and 4c have binding affinities with the active site of CDKs. A bio-evaluation assay revealed that some of the products exhibit strong to moderate effects against proliferation of Huh7 in an in vitro model of human liver cancer cells as confirmed by morphological alteration. Compounds 4c and 4a offer the lowest IC50 values at 22.5 and 39 µM, respectively.

Keywords
CDKs, nanocomposite, pyrimido[4,5-b][1,6]naphthyridin-4(1H)-ones, zeolite-nano Au

Introduction
1,6-Naphthyridines have received considerable attention because of their wide range of biological activities,1–3 including antitumor, anti-inflammatory, antimicrobial,4 and anticonvulsant. Both pyrimidine and 1,6-naphthyridine scaffolds have been shown to be important structural motifs in chemistry; therefore, the preparation of pyrimidonaphthyridine derivatives, in which these scaffolds are merged, might provide compounds that exhibit simultaneously the biological properties of each moiety.4,6

Gangjee and co-workers have described the construction of the pyrimidonaphthyridine skeleton via a multistep reaction.7 Previously, it was reported that pyrimido[4,5-b][1,6]naphthyridine moieties could be synthesized efficiently under microwave conditions.1 Hence, the continued development of diverse pyrimidonaphthyridine compounds is still in strong demand. Marjani and his team were able to synthesize pyrimidonaphthyridine derivatives in the presence of AgNPs under mild conditions.8 Some previously published protocols using acid,9–11 basic,12 or metal catalysts,12 or catalyst-free protocols under microwave13 or thermal conditions for the formation of analogs of the target moiety. Unfortunately, all previous methods produce very low yields; therefore, it was our aim to develop new
conditions based on using a nanocatalyst hoping to improve the yields. The use of a nanocatalytic system would allow the rapid, and selective chemical transformations coupled with the ease of catalyst separation and recovery.14 Using a nanosized catalyst (high surface area), the contact between the reactants and catalyst is increased dramatically (this phenomenon is close to homogeneous catalysis).15,16 The insolubility of the catalyst in the reaction solvent leads to a heterogeneous process, and hence, the catalyst can be separated easily from the reaction mixture (this phenomenon is close to heterogeneous catalysis).17-20 It is known that catalytic properties of metallic NPs are size- and shape-
dependent.21-25 It has been reported that gold nanoparticles are stabilized and well dispersed on various supports (metal oxides,26-31 carbon materials,32-40 metal-organic frameworks,41-43 zeolites,44 modified aluminum,34,44-47 ionic liquids,48 etc.) and have the ability to catalyze several reactions successfully.

Several reports have demonstrated the wide applications of zeolites as catalysts and adsorbents.49-53 These microporous materials are three-dimensional and crystalline hydrated aluminosilicates,54 and are highly rigid under dehydration.55 The important structural, physical, and chemical properties of zeolites, with tailored channels and cavities on the molecular scale, make them versatile and valuable for such broad applications as adsorbents and catalysts in industrial, agricultural, and environmental applications.56 Moreover, zeolite-nanogold possesses high thermal stability and plays the dual role of stabilizing the nanoparticles against sintering and their distinct pore structure can facilitate shape-selective catalysis. Zeolite nanoshell encapsulating gold nanoparticles has successfully been employed for cyclohexane oxidation, and these catalysts show better conversion with increased reusability.44

All these properties encouraged us to study the loading of zeolite with nanogold (Figure 1). For use as a catalyst in a simple route to synthesize pyrimido[4,5-b][1,6]naphthyridine via a one-pot reaction between 6-amino-2-thioxo-2,3-dihydropyrimidin-4(1H)-ones (1), aromatic aldehydes (2) and 1-benzylpiperidin-4-one (3) (Scheme 1). The structure and morphology of the catalyst were determined by X-ray diffraction (XRD) and transmission electron microscopy (TEM).

Results and discussion

**Physicochemical characterization of the nanocomposite**

Low-angle XRD patterns corresponding to the prepared zeolite-Au nanocomposite are shown in Figure 2. The sum of the reflection intensities at 2θ of 15.5°, 24.0°, 28.0°, 32.0°, and 63.0° corresponding to the (450), (309), (207), and (202) planes of a cubic crystal system is also shown in Figure 2 for the prepared nanogold indicating the formation of a cubic crystal of zeolite-nano Au. The intensities of the peaks are relatively high being an indication of high crystallinity. The formed zeolite is a mixture of sodium alumina silicate and sodium aluminum oxide silicate, which is confirmed from standard data for zeolites.

![Figure 1. TEM micrograph of zeolite-doped AuNp used as a catalyst for the preparation of pyrimido[4,5-b][1,6]naphthyridines.](Image)

The method used to prepare the nanocomposite in this work produced a uniform dispersion of small particles, around 4–6 nm gold nanoparticles on zeolite, as shown in the TEM image in Figure 3. The formation of small nanoparticles may help the incorporation of gold nanoparticles within the zeolite framework as also indicated by XRD measurements.

**Chemistry**

The uracil nucleobase has different tautomeric forms in equilibrium, which is strongly dependent on the interaction of these molecules with their environment. Knowledge regarding this tautomerization in different environments can provide insight into the influence of solvent/catalyst effects on molecular stability. In this work, we have reported the one-pot, three-component condensation reaction of 6-amino-2-thiouracil (1) with aromatic aldehydes (2) and piperidinone (3) in ethanol using a zeolite-nanogold catalyst to form fused pyrimido-naphthyridinones (4a–h) at reflux temperature (Scheme 1).

From Table 1, it is very clear that the percentage of the product 4a is greater in ethanol in the presence of zeolite-gold as a nanocatalyst (entry 4), while in the presence of zeolite, only a poor yield was obtained (entry 6). We noticed that in ethanol and using catalysts such as nano ZnO, ZnO, or L-proline, the yields were higher (entries 1–3) compared to that obtained with CH3OH/piperidine (entry 5). The acidic medium, as acetic acid (entry 7), gives a yield less than that in a basic medium.

The formation of the pyrimidonaphthyridine product (4) is consistent with a Knoevenagel condensation, followed by a Michael addition and cyclization, but the details of this process have not been investigated. Compounds (4a–h) were characterized by spectral and analytical methods. For the compound 4a we find that its 1HNMR shows the presence of a methine at δ = 5.33 (s) and a signal at δ = 6.37 (brs) for other signals due to NH the NH groups of the uracil
moiety occurred at $\delta = 11.62$ (s), and $12.04$ (br), while signals due to the piperidinone ring moiety occurred at $\delta = 2.35$ (t), $2.51$ (t), $2.89$ (s), $-\text{NCH}_2$ $\delta = 3.60$ and signals for phenyl moieties appeared at $\delta = 6.78$–$7.96$ (Figure 4). Full spectral and analytical data are given in section “Experimental.” The different substituents on the aryl groups lightly influenced the yields (Table 2).

**Molecular docking**

Genetic alteration of one or more components of the INK4 CDK4,6/cyclin D-retinoblastoma pathway is found in more than half of all human cancers. Therefore, CDK4 is an attractive target for the development of a novel anticancer agent. Docking studies of compounds 4a and 4c into the active site of human CDK4, 6/cyclin-D are conducted. Compound 4a was docked into the binding pocket of 2WGF; the theoretical binding mode between 4a and 2WGF is shown in Figure 5. The NH group of thiouracil in compound 4a formed interactions with the Met B213 residues (bond length: 2.17 Å), and there were arene–cation interactions between Arg P214 and added unsubstituted phenyl group.

In order to increase the activity of 4a, two methoxy groups were added to the aromatic ring to give 4c. The theoretical binding mode between 4c and 2WGF is shown in Figure 6. Compound 4c adopted in the pocket of the 2WGF/CDK4/cyclin-D. The two methoxy groups on the aromatic ring of 4c bind at the 2WGF pocket. Detailed analysis showed that the two methoxy-substituted ring of 4c formed arene–cation interactions with the residues Arg B214, Arg P390, and Met B212. It was shown that Asp P306 (bond length: 2.07 Å) formed a hydrogen bond with the NH of thiouracil, and an arene–cation interaction occurred between Arg A78 and the phenyl group of 1-benzylpiperidin-4-one.

**Cytotoxicity assay**

Previously, it has been reported that the type of cancer cell plays a crucial role in antitumor activity of tested compounds. Upon screening our compounds against proliferation of Huh7, an in vitro model of human liver cancer cells, our results showed that the tested compounds showed different anti-proliferative activities against Huh7, with IC$_{50}$ values ranging from 22.5 to 87 µM. Compounds 4c and 4a were the most cytotoxic inhibiting
proliferation of Huh7 with IC50 values of 22.5 and 39 µM, respectively, followed by compounds 4b and 4d (IC50 values 41 and 53 µM, respectively), while compound 4h did not reveal any significant toxic effect on Huh7 (Figure 7).

Conclusion

Novel of pyrimido[4,5-b][1,6]naphthyridin-4(1H)-one (4a–h) is prepared using a zeolite–nanogold catalyst, molecular docking of compounds 4a and 4c into the active site of human CDK4, 6/cyclin-D. Their in vitro evaluation as anti-cancer agent shows that compounds 4c and 4a were the most cytotoxic inhibiting proliferation of Huh7.

Experimental

Synthesis of nano Au-zeolite

The growth and ripening of gold nanoparticles were assembled on the surface and cavities of zeolite networks. Zeolite powder (Sigma-Aldrich, USA) was activated by annealing for 5 h at 200°C in vacuum oven to get rid of humidity and activate the zeolite networks. 50 mL (0.01 M) gold chloride and HAuCl4 (Sigma-Aldrich) solution were mixed with 1 g activated zeolite under stirring at 60°C for 3 h forming yellowish solution. Heat up the solution till boiling and then add 1 mL of 1% trisodium citrate (Sigma-Aldrich) solution, then left to stir for 5 min till characteristic pink color

Table 2. Yields of compounds.

| Entry | Ar      | Product | Yield (%)a |
|-------|---------|---------|------------|
| 1     | C6H5    | 4a      | 89         |
| 2     | 4-MeOC6H4 | 4b      | 75         |
| 3     | 3,4-(MeO)2C6H3 | 4c | 89         |
| 4     | 3,4,5-(MeO)3C6H2 | 4d | 90         |
| 5     | 4-FC6H4 | 4e      | 75         |
| 6     | 2,4-F2C6H3 | 4f | 90         |
| 7     | 4-F3CC6H4 | 4g | 87         |
| 8     | 4-O2NC6H4 | 4h | 90         |

aComparative value to uracil.

Figure 4. NMR of synthesized compound 4a.

Figure 5. Compound 4a was docked into the binding pocket of 2WGF.

Figure 6. Compound 4c was docked into the binding pocket of 2WGF.
of gold nanoparticles formed. Centrifuge the solution at 10K r/min for 30 min for precipitating Au-zeolite nanocomposite. The obtained nanocomposite was dried in vacuum oven at 70° for 8 h and stored in desiccator for further characterization.57

Characterization of nano Au-zeolite

The particle size and the morphology of the prepared catalyst were characterized by high TEM (Philips, The Netherlands). The measuring mode of the sample in the TEM instrument depends on its suspension in water followed by ultrasonication for 600 s in ultra 8050-H Clifton. It was then applied in the TEM instrument on 100 mesh copper grade coated with carbon. Powder XRD patterns were recorded with a PANalytical X’Pert PRO diffractometer using a Cu Kα radiation source for the investigation of the crystalline structure and phase.

Molecular docking

All molecular modeling calculations and docking studies were performed using Molecular Operating Environment (MOE), version 2009.10, Chemical Computing Group. The program was used via the Windows XP operating system installed on an Intel Pentium IV PC with a 2.9 MHz processor and 512RAM. The prepared compounds were built using the MOE builder interface and subjected to energy minimization using MOPAC. The produced model was subjected to a systematic conformational search, where all items were set as default with a root mean square (RMS) gradient of 0.01 kcal/mol and an RMS distance of 0.1 Å.

Biological assay

Cell culture. The Huh7, in vitro model of human liver cancer cell line, was obtained from ATCC (USA). Cells were cultured in DMEM media (Gibco, USA) supplemented with 10% fetal bovine serum (Gibco), antibiotics (2% penicillin-streptomycin (100 IU/mL)), and 0.5% fungi zone (Gibco). The cells were maintained in monolayer culture at 37°C under a humidified atmosphere of 5% CO2. The cells were sub-cultured by trypsinization (0.025% trypsin and 0.0025% EDTA; Gibco), and maintained in the Tissue Culture Laboratory at the Virology & Immunology Unit, Cancer Biology Department, National Cancer Institute, Cairo University, Egypt, with cryogenic banking of low-passage cells to maintain uniformity of cell properties through the study. Cell numbers and viabilities were monitored by standard Trypan blue dye exclusion procedures.58,59

Treatment of cells and colorimetric MTT assay. For investigation of the cellular toxicity of all the synthesized compounds against proliferation of Huh7 cells, 8 × 10^3 cells/well were plated in a 96 tissue culture plate with 10%
DMEM. After 24 h, five different twofold dilutions of compounds 4a–h (100, 50, 25, 12.5, and 6.25 µg/mL) were tested against proliferation of Huh7 cells and the plate was sealed and kept under standard conditions in a CO2 incubator at 37 °C for 48 h. After the incubation period, the plate was investigated for morphological changes of the cells under an inverted microscope and photos were captured (see Figure 8). MTT solution at a concentration of 5 mg/mL PBS was added to all the wells, which were then wrapped with aluminum foil and incubated for 3–4 h at 37 °C. The medium was then removed and 100 µL at DMSO was added to all the wells which were then shaken for 10 min to dissolve the created formazan crystals in the wells. The MTT formazan product was identified via measuring the absorbance using an enzyme-linked immunosorbent assay (ELISA) plate reader (BioTek Model: ELX 800, USA), where positive and negative controls were run in the plate. Negative control cells with media only (untreated cells) were set as 100% viable, while the positive control cells were subjected to osmotic pressure using distilled water to give zero viability and were used to subtract the background from all optical density values. The ELISA plate reader measured the absorbance at 570 and 620 nm as a reference wavelength. The cells were monitored by phase-contrast microscopy at 40× magnification for any morphological changes. The viability of the cells (%) in relation to the control wells with untreated cells was calculated using the following equation

\[
\text{Cell viability} = \frac{A_{\text{test}}}{A_{\text{control}}} \times 100
\]

where \( A_{\text{test}} \) is the absorbance of the test sample and \( A_{\text{control}} \) is the absorbance of the control sample. The results were the average of three wells and 100% viability was determined from the negative control, that is, untreated cells.

For each compound concentration, five wells were used (five replicate wells were prepared for each individual dose). The average was calculated. Data are expressed as the percentage of relative viability compared with the untreated cells. The cytotoxicity dose was calculated as a dose induced \( \approx 100\% \) relative on viability.

**Chemistry.** Melting points were measured on a Gallenkamp electrothermal melting point apparatus and are uncorrected. Infrared (IR) spectra were recorded as KBr disks using a Shimadzu FTIR Prestige 21 spectrophotometer. 1H and 13C NMR spectra were recorded in DMSO-d6 at 300 MHz on a Varian Mercury NMR spectrometer using TMS as the internal standard. Chemical shifts (δ) are reported in parts per million (ppm), and J values are given in hertz. The mass spectra were recorded on a GCeMS-QP1000 EX mass spectrometer at 70 eV. Elemental analyses were carried out at the Micro-analytical Centre of Cairo University, Giza, Egypt.

**General procedure for the synthesis of 7-benzyl-5-aryl-2-thioxo-2,3,5,6,7,8,9,10-octahydropyrimido[4,5-b][1,6]naphthyridin-4(1H)-ones (4)**

6-Amino-2-thioxo-2,3-dihydropyrimidin-4(1H)-one (1) (0.3 mmol), 1-benzylpiperidin-4-one (0.3 mmol) (3), and aromatic aldehyde (2) (0.3 mmol) were refluxed in ethanol (15 mL)/zeolite-nano Au for 2 h. The mixture was filtrated to isolate the catalyst and the solution was allowed to cool. The resulting precipitate was washed with ethanol and dried. Recrystallization from acetic acid gave crystals of the desired compound.

**7-Benzyl-5-phenyl-2-thioxo-2,3,5,6,7,8,9,10-octahydropyrimido[4,5-b][1,6]naphthyridin-4(1H)-one (4a)**

- Colorless powder (89%); m.p. 297–298 °C. IR (KBr) (ν/cm−1): 3399, 3181, 1607, 1550. 1H NMR (300 MHz, DMSO-d6): 2.34 (t, \( J = 6 \) Hz, 2H, CH2), 2.51 (t, \( J = 6 \) Hz, 2H, CH2), 3.34 (s, 2H, CH2), 3.61 (s, 2H, CH2), 5.33 (s, 1H, CH), 6.37 (brs, 1H, NH), 6.78-7.96 (m, 10H, H-Ar), 11.62 (br, 1H, NH), 12.04 (br, 1H, NH). 13C NMR (75 MHz, DMSO-d6): 21.7, 31.2, 41.1, 52.7, 61.1, 78.6, 90.7, 125.7, 126.9, 127.5, 128.3, 128.7, 129.0, 129.1, 129.7, 138.4, 153.9, 162.1, 175.0. MS (EI, 70 eV): m/z (%): 403 (M+, 25), 402 (M+, 100). Anal. calcd for C23H22N4OS (402.15): C, 68.63; H, 5.51; N, 13.92; found: C, 68.92; H, 5.60; N, 13.99.

**7-Benzyl-5-(4-methoxyphenyl)-2-thioxo-2,3,5,6,7,8,9,10-octahydropyrimido[4,5-b][1,6]naphthyridin-4(1H)-one (4c)**

- Image: Figure 8. Cell morphology of Huh7: (a) untreated cell control, (b) Huh7 treated with a high concentration of 4c, and (c) Huh7 treated with a lower concentration of 4c. Images were taken at a magnification power of 100×.
7-Benzyl-2-thioxo-5-[4-(trifluoromethyl)phenyl]-2,3,5,6,7,8,9,10-octahydropyrimido[4,5-b][1,6]napthyridin-4(1H)-one (4g). Colorless powder (87%); m.p. 288–290°C. IR (KBr) (v\(_{\text{max}}/\text{cm}^{-1}\)): 3500, 3320, 1605, 1560. \(^1\)H NMR (300 MHz, DMSO-d\(_6\)): \(\delta\) (ppm) 2.05 (t, \(J = 6\) Hz, 2H, CH\(_2\)), 5.42 (s, 2H, CH\(_2\)), 5.63 (t, \(J = 6\) Hz, 2H, CH\(_2\)), 5.87 (s, 2H, CH\(_2\)), 6.77 (brs, 1H, NH), 7.35–8.14 (m, 9H, H-Ar), 8.46 (s, 1H, NH), 10.84 (brs, 2H, NH). 13C NMR (75 MHz, DMSO-d\(_6\)): \(\delta\) (ppm) 21.7, 10.41, 52.7, 56.3, 61.1, 78.6, 90.9, 104.8, 107.2, 125.7, 126.9, 128.7, 129.1, 134.5, 136.0, 152.9, 154.2, 163.4, 173.2. MS (EI, 70 eV): m/z (%): 492 (M\(^+\)), 100). Anal. calecd for C\(_{24}\)H\(_{24}\)N\(_4\)O\(_2\)S (432.16): C, 66.62; H, 5.53; N, 12.99.

7-Benzyl-2-thioxo-5-(4,5-dimethoxyphenyl)-2-thio-2,3,5,6,7,8,9,10-octahydropyrimido[4,5-b][1,6]napthyridin-4(1H)-one (4e). Colorless powder (89%); m.p. 218–220°C. IR (KBr) (v\(_{\text{max}}/\text{cm}^{-1}\)): 3351, 3181, 1623, 1545. \(^1\)H NMR (300 MHz, DMSO-d\(_6\)): \(\delta\) (ppm) 2.07 (t, \(J = 6\) Hz, 2H, CH\(_2\)), 2.50 (t, \(J = 6\) Hz, 2H, CH\(_2\)), 2.53 (t, \(J = 6\) Hz, 2H, CH\(_2\)), 2.67 (t, \(J = 6\) Hz, 2H, CH\(_2\)), 3.15 (s, 2H, CH\(_2\)), 3.44 (s, 2H, CH\(_2\)), 3.74 (s, 3H, OCH\(_3\)), 3.80 (s, 3H, OCH\(_3\)), 5.30 (s, 2H, CH\(_2\)), 6.57 (brs, 1H, NH), 6.59-7.40 (m, 8H, H-Ar), 8.51 (s, 2H, NH), 11.75 (s, 1H, NH). MS (EI, 70 eV): m/z (%): 463 (M\(^+\) + 4, 100). Anal. calecd for C\(_{24}\)H\(_{24}\)N\(_4\)O\(_2\)S (462.17): C, 64.91; H, 5.67; N, 12.11; found: C, 64.72; H, 5.63; N, 12.07.

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