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$Cu(OTf)_2$ catalyzed three component reaction: Efficient synthesis of spiro[indoline-3,4′-pyrano[3,2-*b*]pyran derivatives and their anticancer potency towards A549 human lung cancer cell lines

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ABSTRACT

$Cu(OTf)_2$ catalyzed efficient synthesis of spiropyrano[3,2-*b*]pyran-4(8*H*)-ones is accomplished via one-pot three component reaction between isatin, kojic acid and active methylenes. This synthetic protocol is operationally simple and affords product with good to excellent yields at a short reaction time. The synthesized compounds were evaluated for their tumor cell growth inhibitory activity against the human lung cancer cell line (A549) and found that 13 compounds exhibited moderate to good anticancer potency. Molecular docking studies were performed for all the synthesized compounds and the results showed that compound **4e** showed greater affinity for anaplastic lymphoma kinase (ALK) receptor.

Lung cancer is the most frequent cause of cancer-related death and accounts for more than a million deaths yearly worldwide with non-small cell lung cancer (NSCLC) accounting for 75–85% of lung cancer.[1] Molecular studies of lung cancer have provided new avenues for early diagnosis and therapeutic strategies, however, certain patients are still plagued by rapid disease recurrence and progression and there has been no significant improvement in their overall survival. Therefore, it remains a disease with poor prognosis and the primary cause of cancer-related deaths in both men and women despite recent advances made in drug development.[2] The development or presence of resistance to chemotherapeutic agents is a major obstacle to the effective treatment of lung cancer. Therefore, it seems necessary to identify and develop new molecular entities of improved efficacy and resistance to complement the present chemotherapeutic strategies.

5-Hydroxy-2-(hydroxymethyl)-4*H*-pyran-4-one commonly known as kojic acid is produced from carbohydrate sources in an aerobic process by various fungi and bacteria, such as *Aspergillus* and *Pencillium*.[3] The derivatives of kojic acid are common occurrence in a variety of natural and semi-synthetic products with diverse and compelling pharmacological activities.[4] Due to its γ-pyranone structure that contains an enolic hydroxyl group, kojic acid exhibits free radical scavenging and tyrosinase inhibiting activity.[5] On the other hand, spiro-oxindole system is the core structure of many bio-active natural alkaloids such as horsifiline, spirotryprostatine, alstonisine, welwitindolinone A, coerulescine, pteropodine and elacomine.[6] They are also present as sub-structure in pharmacological agents and act as modulators of muscarinic M_1 and 5-HT_2 receptors and potent non peptide inhibitors of p53-MDM2 interaction and microtubule assembly.[7] Recently, we have initiated a research program on the synthesis of a series of novel spiro-oxindoles[8] and/or screened them for their biocidal profile.[9] Inspired by the synthetic feasibility of spiro-oxindole[10] and kojic acid derivatives[11] it was thought worthwhile to fuse both these scaffolds as a single molecular entity in view of enhanced biological activity. We hypothesized such hybrid-heterocycles[12] might lead to an interesting class of compounds useful for the structure–activity relationship (SAR) studies. In 2004, Litvinov et al. have reported the amine catalyzed synthesis of similar compounds.[13] The scope described in this protocol was very limited to a specific substrate, which prompted us to investigate further this kind of transformation with particular emphasis on performing with a Lewis acid catalyst. Toward these ends, we herein report the synthesis of kojic acid tethered spiro-oxindoles via $Cu(OTf)_2$ catalyzed three component reaction and the anticancer potency of these compounds against A549 human lung cancer cell lines.

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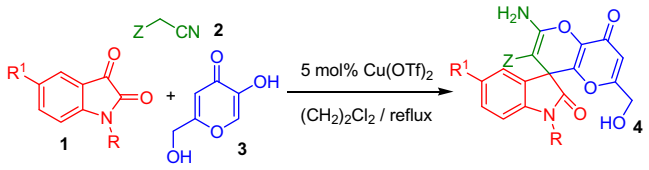
Our study began with isatin **1a**, malononitrile **2a** and kojic acid **3**. The choice of the catalyst played a crucial role and the use of late transition metal catalysts was at the center of our study. In the course of our work on the applications of copper and zinc catalysts in various heterocycle syntheses,[14] we thought they will be catalytically effective for the preparation of targeted spiro-compounds. Knowledge gained from our previous work allowed us to arrive quickly at optimal reaction conditions (Table 1); in fact $Cu(OTf)_2$ at a loading of 5 mol % in refluxing dichloroethane emerged as the reaction conditions of choice, giving the product **4a** in an 93% yield (entry 6). The use of anhydrous CuCl, $CuCl_2$, $CuBr_2$, $ZnCl_2$ and $Zn(NTf)_2$ (entries 1–5) was much less effective in this transformation. Finally, $Zn(OTf)_2$ resulted in product formation in moderate yield (entry 7). Since 5 mol % of $Cu(OTf)_2$ was optimal for the preferred transformation, we set out to investigate the scope of other substrates (Scheme 1, Table 2).[15]

Gratifyingly, isatins with electron-rich and electron-poor substituent underwent the desired reaction in good to excellent yield. As seen from Table 2, there was a wide tolerance for several isatins varying in C5 and N-substitution. Other reaction partners such as malonitrile, ethyl cyanoacetate and methyl cyanoacetate performed with equal aplomb. It is pertinent to note that, substrates possessing alkynes did not undergo isomerization to the corresponding allenyl product.[16] Interestingly, this copper-catalyzed transformation is not limited to mono-systems. As illustrated in Scheme 2, we have been able to apply this chemistry to the synthesis of complex bis-spiroxindole system. Thus, treatment of bis-isatin (1.0 mmoL) with malononitrile (2.0 mmoL) and kojic acid (2.0 mmoL) under our standard reaction conditions produced the corresponding product in 68% yield. The structures of all the products were confirmed by spectral data (FTIR, ^{1}H NMR, ^{13}C NMR and MS) and elemental analyses. For an illustrative example, the IR spectrum of compound **4k** showed broad peaks at 3400–3300 cm^{-1} revealed the presence of $-NH_2$, –NH and –OH functionalities. The stretching bands at 1732 and 1643 cm^{-1} correspond to ester and amide carbonyl groups, respectively. The ^{1}H NMR spectrum of **4k** recorded in DMSO-d_6, showed fifteen protons. The broad signals at δ_H 8.16 ppm and δ_H 10.75 ppm were assigned to $-NH_2$ and –NH protons (D_2O exchangeable), respectively. In ^{13}C NMR spectrum, a less intense peak at δ_C 51.4 ppm indicates the presence of a spiro-carbon and a peak at δ_C 177.1 ppm corresponds to an amide carbonyl carbon. Also, DEPT-135 and 2D chemical shift correlation experiments were used to support this observation. MS data was acquired in the positive ionization FIA mode, exhibited two isotopic peaks at m/z = 417 $[^{35}M_{Cl}+H]^+$, 419 $[^{37}M_{Cl}+H]^+$ in 3:1 ratio. Finally, single crystal X-ray diffraction studies unequivocally confirmed the structure of the product **4k** (Fig. 1).[17]

A tentative mechanistic description for this Cu-catalyzed three component reaction was proposed (Scheme 3). Mechanistically, the formation of isatylidene malononitrile **I** from isatin **1** and malononitrile **2** involves typical Knoevenagel condensation and can be taken for granted at this juncture. The nitrophilic $Cu(OTf)_2$ coordinates with the cyano group of **I** leading to intermediate **II**. Subsequent C-alkylation of kojic acid **3** with electron deficient C=C double bond leads to intermediate **III** and **IV**. Thorpe-Ziegler type reaction of intermediate **IV** (nucleophilic attack of enolic hydroxyl to the copper-activated cyano group)[18] results in the cyclized oxonium intermediate **V**. The later upon deprotonation leads to iminium intermediate **VI** and finally proto-decupration of intermediate **VI** affords the spiroxindole product **4**.



Scheme 1.

Table 1
Screening of Lewis acids[ab]

Entry	Catalyst	Time (min)	Yield[c] (%)
1	CuCl	30	–
2	$CuCl_2$	30	10
3	$CuBr_2$	30	27
4	$ZnCl_2$	30	30
5	$Zn(NTf)_2$	30	22
6	**$Cu(OTf)_2$**	**30**	**93**
7	$Zn(OTf)_2$	30	52

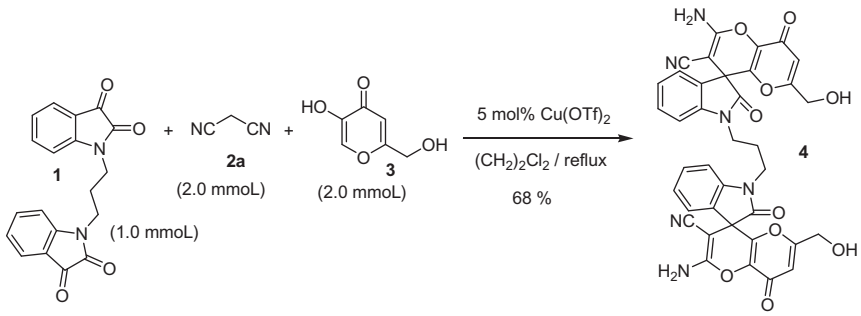
[a] Yields of **4a** using different Lewis acids.
[b] All reactions were carried out using 1.0 mmoL of isatin **1a**, malononitrile **2a** and kojic acid **3**, catalyst (5 mol %) and dichloroethane (2 mL).
[c] Isolated yield.

Table 2
$Cu(OTf)_2$ catalyzed synthesis of spiroxindoles **4a**–**4s**

Entry	R	R^1	Z	Time (min)	Product[a]	Yield[b] (%)
1	H	H	CN	30	**4a**	93
2	H	F	CN	20	**4b**	91
3	H	Cl	CN	20	**4c**	89
4	H	Br	CN	25	**4d**	90
5	Me	H	CN	40	**4e**	84
6	Allyl	H	CN	45	**4f**	80
7	Propargyl	H	CN	45	**4g**	82
8	Bn	H	CN	45	**4h**	85
9	H	H	COOEt	35	**4i**	89
10	H	F	COOEt	25	**4j**	87
11	H	Cl	COOEt	25	**4k**	86
12	H	Br	COOEt	30	**4l**	90
13	H	Me	COOEt	45	**4m**	85
14	Me	H	COOEt	45	**4n**	81
15	Allyl	H	COOEt	45	**4o**	77
16	H	H	COOMe	30	**4p**	87
17	H	NO_2	COOMe	25	**4q**	91
18	H	Me	COOMe	30	**4r**	88
19	Bn	H	COOMe	35	**4s**	81

[a] All products were characterized by IR, NMR and mass.
[b] Isolated yield.

Cytotoxicity of all the synthesized compounds against human lung cancer cell lines (A549) was determined by MTT assay. Growth of lung cancer cells was measured by the ability of living cells to reduce the yellow MTT to purple formazan products.[19] A549 cancer cell lines were cultivated at 37 °C in an atmosphere of 5% CO_2, 95% air and 100% relative humidity in Dulbecco's modified Eagle's minimal medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and 2.0 mM L-glutamine along with antibiotics (about 100 IU/mL of penicillin, 100 μg/mL of streptomycin) with the pH adjusted to 7.2. Cells (5×10^5) were trypsinized for the passage into the well plate and were seeded in 96 well plates containing medium with different concentrations such as 12.5, 25, 50, 100, 150 and 200 μg/mL and were allowed to adhere to the surface of well plates. After various durations of cultivation, the solution in the medium was removed. An aliquot of 100 μL of medium containing 1 mg/mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) was loaded to the plate. Incubation at 37 °C for 4 h allowed reduction of MTT by mitochondrial dehydrogenase to an insoluble formazan product. Well contents were removed and the formazan product was solubilised by addition of 100 μL of DMSO, which led to the formation of purple colour. The amount of formazan product is directly proportional to the number of living cells. Absorbance of each well was read on ELISA reader at 540 nm. From the absorbance % of inhibition



Scheme 2.

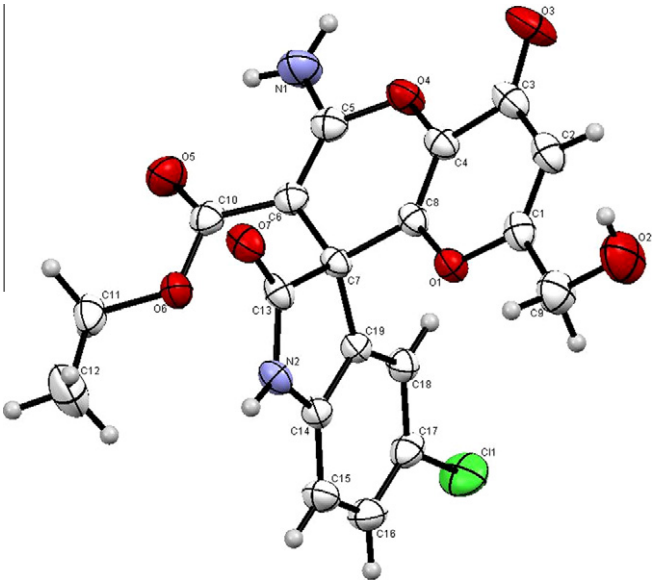


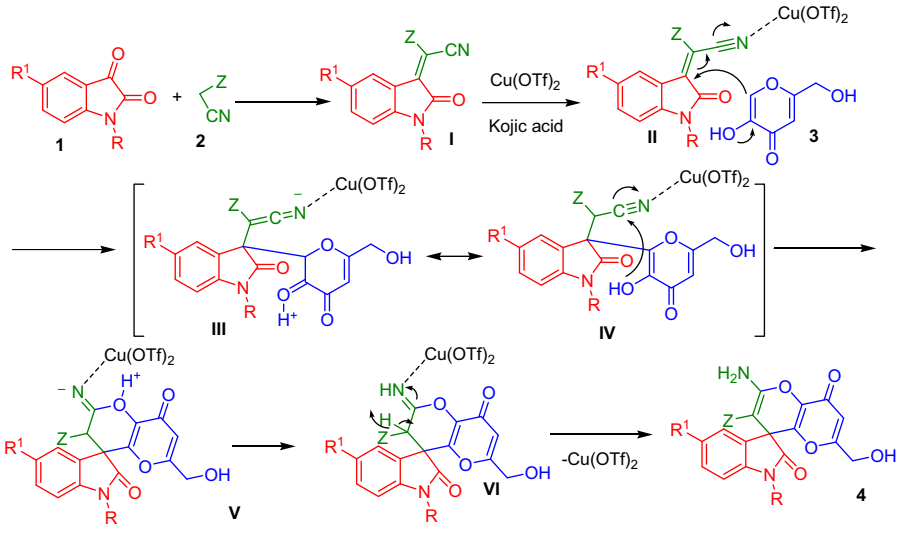
Figure 1. ORTEP diagram of compound **4k**.

Table 3
IC_{50} and FEB values of compounds **4a–s**

Entry	Compounds	IC_{50} (μM)	FEB[a] (kcal/mol)
1	**4a**	52.8	−4.68
2	**4b**	>200	−5.12
3	**4c**	51.4	−5.19
4	**4d**	>200	−4.91
5	**4e**	51.1	−5.99
6	**4f**	59.4	−4.84
7	**4g**	>200	−5.12
8	**4h**	>200	−4.18
9	**4i**	>200	−5.18
10	**4j**	>200	−4.09
11	**4k**	53.1	−4.50
12	**4l**	52.8	−5.17
13	**4m**	52.9	−4.48
14	**4n**	55.8	−4.96
15	**4o**	54.6	−4.59
−16	**4p**	56.2	−5.23
17	**4q**	55.9	−5.01
18	**4r**	62.8	−5.04
19	**4s**	55.1	−4.97
20	Crizotinib[b]	–	−7.58

[a] Free energy of binding.
[b] Reference drug.

was calculated by using the formula, % of inhibition = $A_c - A_t / A_c \times 100$, where A_c is the mean absorbance of control and A_t is that of test. From the results nonlinear regression graph was plotted between % cell growth inhibition and $\log_{10}$ concentration (μM). The half maximal inhibitory concentration (IC_{50} value) was determined averaged from three replicate experiments using SPSS 11.5 software. Analysis of the screening data (Table 3) revealed that compounds **4e** (IC_{50} = 51.1 μM) and **4c** (IC_{50} = 51.4 μM) possessing



Scheme 3.

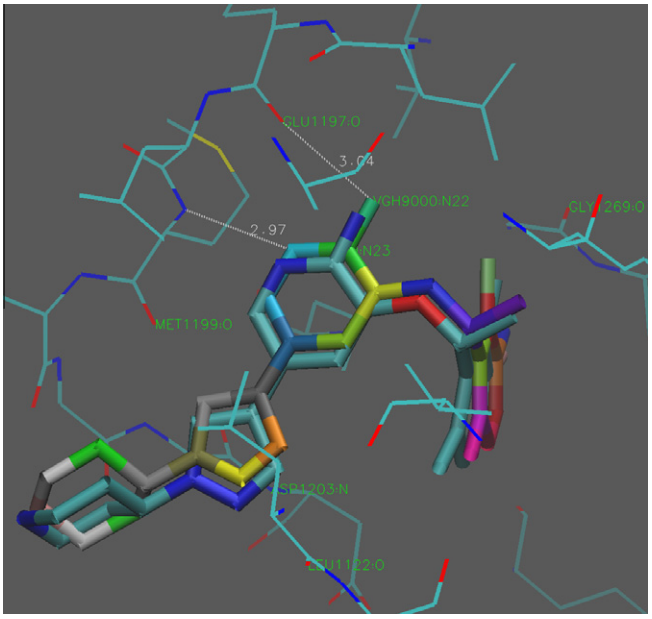


Figure 2. Molecular docking of bound and docked crizotinib in ALK, where the docked ligand was discriminated by showing two hydrogen bond interactions.

N−Me, 3′-CN and 5-Cl, 3′-CN groups, respectively, exhibited maximum cytotoxicity. A slight loss of activity was observed in compounds **4a**, **4k–m** and appeared to be the second most active series with IC_{50} values falling between 52.8 and 53.1 μM. A moderate inhibitory potency was observed in compounds **4n–q** and **4s** (IC_{50} = 54.6–55.9). A much reduced activity was exhibited by compounds **4f** and **4r** with IC_{50} values of 59.4 and 62.8 μM, respectively. However, compounds **4b**, **4d**, **4g–j** emerged as the least active series, exhibiting IC_{50} values of >200 μM.

To investigate the potential binding mode of inhibitors, all the compounds were subjected to molecular docking using the AUTODOCK 1.5.4 docking program. Because of the critical roles of aberrant signaling in cancer, anaplastic lymphoma kinase (ALK) receptor is an attractive oncology target for therapeutic intervention. To this end, the X-ray crystal structure of ALK in complex with crizotinib was downloaded from the protein data bank (PDBID: 2XP2) and was used for the docking study.[20] Ligand 2D structures were drawn using ChemDraw Ultra 7.0 (ChemOffice 2002). Chem3D Ultra 7.0 was used to convert 2D structure into 3D and the energy minimized using semi-empirical AM1 method. Minimize energy to minimum RMS gradient of 0.100 was set in each iteration. All structures were saved as .pdb file format for input to AutoDockTools (ADT) version 1.5.4.[21a] All the ligand structures were then saved in PDBQT file format, for input into AUTODOCK version 1.5.4.[21b]

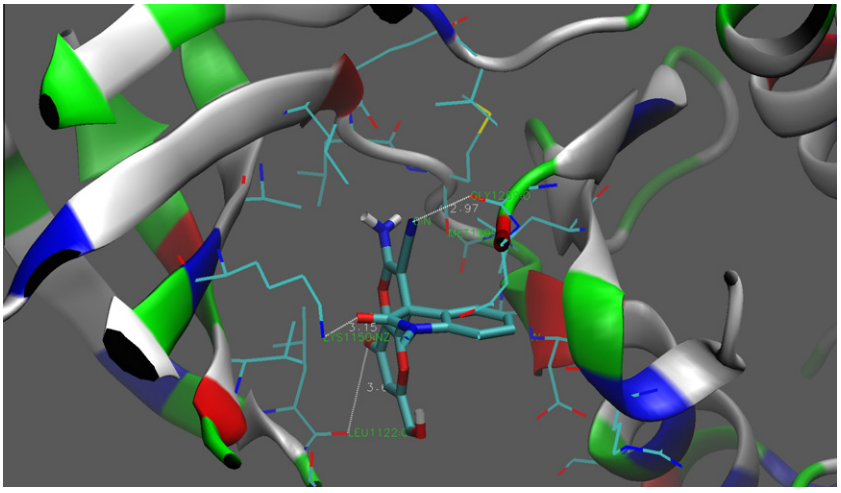


Figure 3. Docking of the most active compound **4e** (FEB = −5.99 kcal/mol) in ALK (dotted lines showing hydrogen bonding interactions).

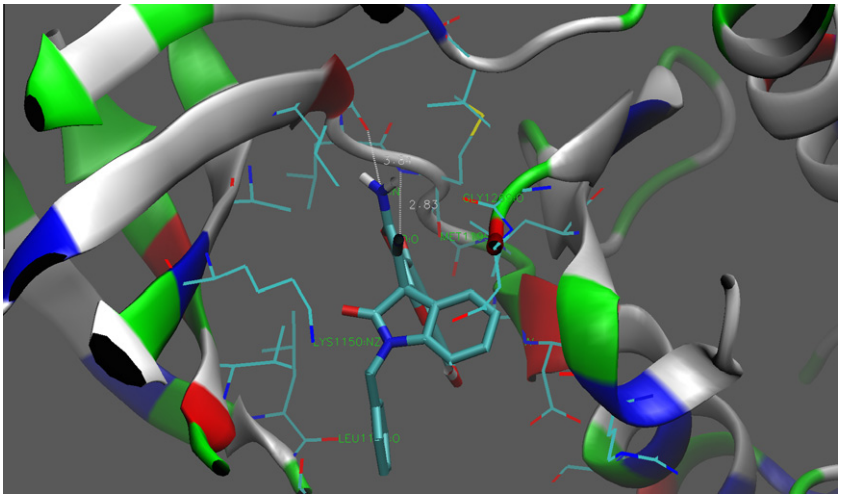


Figure 4. Docking of the least active compound **4h** (FEB = −4.18 kcal/mol) in ALK (dotted lines showing hydrogen bonding interactions).

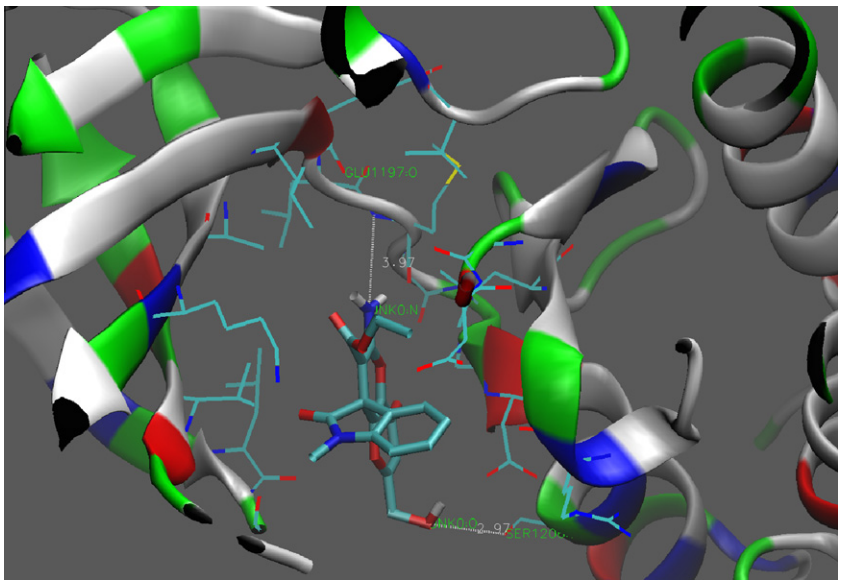


Figure 5. Docking of the intermediary active compound **4n** (FEB = −4.96 kcal/mol) in ALK (dotted lines showing hydrogen bonding interactions).

For the molecular docking study, protein structure was obtained from the Protein Data Bank; the ALK structure PDB ID was 2XP2. The co crystallized ligand (crizotinib) in the ALK structure was removed. For the protein structure, all hydrogen atoms were added, lower occupancy residue structures were deleted, and any incomplete side chains were replaced using the ADT version 1.5.4. Further ADT was used to remove crystal water, added Gagteiger charges to each atom, and merged the non-polar hydrogen atoms to the protein structure. The distance between donor and acceptor atoms that form a hydrogen bond was defined as 1.9 Å with a tolerance of 0.5 Å, and the acceptor–hydrogen-donor angle was not less than 120°. The structures were then saved in PDBQT file format, for input into AUTODOCK version 1.5.4. A grid box with dimension of 40 × 40 × 40 $Å^3$ and was centred on 29.608, 47.370, 9.753 was created around the binding site of crizotinib on ALK protein using AutoDockTools. The centre of the box was set at crizotinib and grid energy calculations were carried out. For the AUTODOCK docking calculation, default parameters were used and 50 docked conformations were generated for each compound. In order to verify reproducibility of the docking calculations, the bound ligand (crizotinib) was extracted from the complexes and submitted for one-ligand run calculation. This reproduced top scoring conformations of 10 falling within root-mean-square deviation (rmsd) values of 0.696–1.064 Å from bound X-ray conformation for ALK, suggesting this method is valid enough to be used for docking studies of other compounds. The outputs were exported to VMD and Pymol for visual inspection of the binding modes and interactions of the compounds with amino acid residues in the active sites (Fig. 2).

Docking of different ligands to protein was performed using AUTODOCK, same protocols used in as that of validation study. All docking were taken into 2.5 million energy evaluations were performed for each of the test molecules. Docked ligand conformations were analyzed in terms of energy, hydrogen bonding, and hydrophobic interaction between ligand and receptor protein ALK. Detailed analyses of the ligand–receptor interactions were carried out, and final coordinates of the ligand and receptor were saved as pdb files. For display of the receptor with the ligand binding site, VMD software was used. From the docking scores, the free energy of binding (FEB) of all compounds were calculated (Table 3). The results of which revealed that compound **4e** as the most active with a calculated binding energy of −5.99 kcal/mol. The least binding energy was exhibited by compound **4h** with a binding energy of −4.18 kcal/mol. The intermediary active compound **4n** showed −4.96 kcal/mol of binding energy. The binding interactions of these compounds were shown, respectively in Figures 3–5.

In conclusion, we have demonstrated the catalytic efficiency of $Cu(OTf)_2$ for the three component reaction between isatin, malononitrile and kojic acid. This method utilizes catalytic quantity of $Cu(OTf)_2$ in comparision with harsh and stoichiometric amine bases. Evaluation of in vitro anticancer properties towards A549 cancer cell lines revealed that thirteen compounds exhibited good to moderate cytotoxicity, out of which, two compounds **4e** and **4c** exhibited good anticancer potency with an IC_{50} value of 51.1 and 51.4 μM, respectively. The level of anticancer potential was studied by automated docking of ligands to the binding sites of ALK. The results revealed that compound **4e** showed minimum binding energy (−5.99 kcal/mol), which indicates its strong affinity towards ALK protein. Further investigation concerning an enantioselective version of this reaction and the mechanism of apoptosis induced by these compounds in A549 cancer cell lines is currently ongoing and will be reported in due course.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2013.02.086.

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15. *Representative procedure for the synthesis of* ***4k***: To a stirred solution of 5-chloroisatin (181 mg, 1.0 mmoL), ethylcyanoacetate (113 mg, 1.0 mmoL) and $Cu(OTf)_2$ (18.1 mg, 5 mol %) in 1,2-dichloroethane was added kojic acid (142 mg, 1.0 mmoL) at room temperature (25 °C). After refluxing for 25 min, the reaction mixture was cooled to room temperature and treated with 25 mL of water and extracted with dichloromethane (3×25 mL). The combined organic layers were dried over anhydrous sodium sulfate and concentrated under reduced pressure and purified by column chromatography over silica gel (100–200 mesh) to afford pure product of **4k** as off-white solid; IR (KBr): 3306, 1732, 1643, 1476, 1299, 1220, 1037, 869 cm^{-1}.^{1}H NMR (400 MHz, DMSO-d_6): δ_H 0.77 (t, J = 7.0 Hz, 3H), 3.73–3.84 (m, 2H), 4.00 (tdd, J = 15.9 and 5.7 Hz, 2H), 5.62 (t, J = 5.7 Hz, 1H), 6.35 (s, 1H), 6.86 (d, J = 8.2 Hz, 1H), 7.26 (d, J = 8.2 Hz, 1H), 7.29 (s, 1H), 8.16 (br s, 2H,), 10.75 (br s, 1H).^{13}C NMR (100 MHz, DMSO-d_6) δ_C 13.2, 51.4, 59.1, 59.2, 73.2, 110.8, 111.5, 123.9, 125.8, 128.7, 135.6, 136.6, 141.6, 147.1, 160.0, 166.9, 168.3, 169.5, 177.1. MS (ESI): m/z = 417 $[^{35}M_{Cl}+H]^+$, 419 $[^{37}M_{Cl}+H]^+$. Anal. Calcd for $C_{19}H_{15}ClN_2O_7$: C, 54.49; H, 3.61; N, 6.69. Found C, 54.43; H, 3.60; N, 6.72.
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17. Crystallographic data of the structure of compound **4k** have been deposited with the Cambridge Crystallographic Data Centre as supplementary publication no. CCDC-919587. Copies of the data can be obtained, free of charge on application to CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (fax: +44 01223 336033 or email: deposit@ccdc.cam.ac.uk).
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