Letter

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Investigating the Anticancer Activity of Isatin/Dihydropyrazole Hybrids

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KEYWORDS: Anticancer agents, isatin-dihydropyrazole hybrids, apoptosis inducers.

ABSTRACT: A series of isatin-dihydropyrazole hybrids have been synthesized in order to assess their potential as anticancer agents. In particular, twelve compounds were evaluated for their anti-proliferative activity toward A549, IGR39, U87, MDA-MB-231, MCF-7, BT474, BxPC-3, SKOV-3, H1299 cell lines, and human foreskin fibroblasts. Four compounds exhibited an interesting anti-proliferative activity and were further examined to determine their EC_{50} values toward a panel of selected tumor cell lines. Best compounds were then investigated for their induced mechanism of cell death. Preliminary structure-activity relationship indicates that the presence of a substituent such as chlorine atom or a methyl moiety in the position 5 of the isatin nucleus is beneficial for the antitumor activity. **EMAC4001** resulted the most promising compound within the studied series with EC_{50} values ranging from 0.01 μM to 0.38 μM.

Several anticancer chemotherapeutic agents act by causing cell death either by directly inhibiting the synthesis of DNA or by interfering with its function. Unfortunately, they are generally not specific for tumor cells and therefore associated with a high toxicity. Not surprisingly, nowadays, the focus of the scientific community is oriented toward the development of new target-directed, more specific cytotoxic agents. Such agents must be capable to inhibit or modulate identified molecular targets that are involved in the control of cancer cells, such as signal transduction, apoptosis, transcription regulation, matrix invasion, and angiogenesis. Our research group is currently involved in several projects regarding the design and synthesis of anticancer agents directed toward several targets such as human carbonic anhydrases (hCA),[1-5] DNA G-quadruplex, and hCA/COX2 dual inhibitors.[6-9] However, it is commonly recognized that cancer is a complex multifactorial disease and, therefore, cannot be treated with a single drug therapy. Accordingly, new agents, combining diverse pharmacophores in a single hybrid molecule, might represent a goal for the treatment of cancer and indeed a big effort has been put into the identification of anticancer multi-target hybrids agents.[10-18] In this respect, isatin is commonly recognized as a privileged scaffold in drug design.[2, 19-24] Moreover, it is a highly represented structural motif in kinase inhibitors anticancer drugs (Figure 1).[25-28] The structure activity relationships of the isatin based multi-kinase inhibitor Nintedanib, as well as its drug development to phase III clinical trial has been recently reported.[28] More in detail the relevant role of the isatin nitrogen and of the carbonyl group in the position 1 and 2, as H-bond donor and acceptor network, was outlined. However, with respect to the structurally similar, multi-kinase inhibitor Sunitinib, the isatin nucleus was, in this case, decorated by a methoxycarbonyl group in the position 6, instead of a fluorine atom in the position 5. On this basis, we have synthesized a new series of dihydropyrazole isatin dihydrothiazole hybrids **EMAC4000**, **4001**, **4003**, **4005**, **4007**, **4008**, **4011**, **4012**, **4014**, **4015**, **4018**, and **4019** to evaluate their activity toward diverse cancer cell lines. Analogous compounds have been previously reported and the most relevant structural features that are essential and/or beneficial for the activity have been outlined.[23]

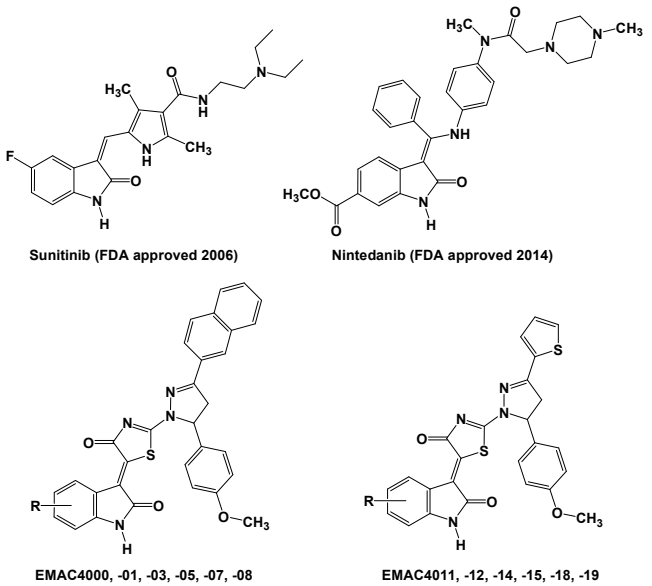
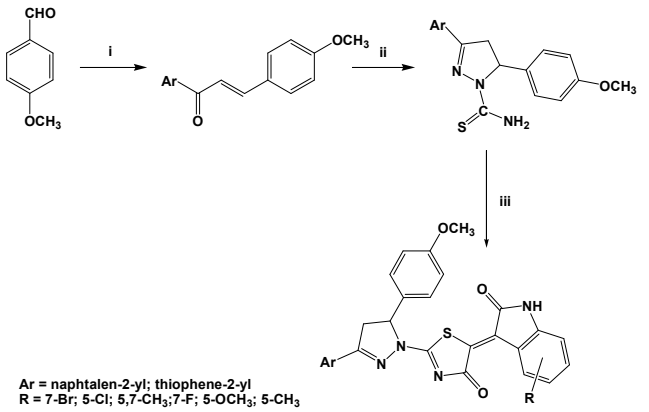


Figure 1. Structurally related multi-kinase inhibitor Sunitinib (VEGFR, PDGFR, KIT, RET), Nintedanib (VEGFR, EGFR, PDGFR), and **EMAC4000, 4001, 4003, 4005, 4007, 4008, 4011, 4012, 4014, 4015, 4018,** and **4019** derivatives.

Prompted by these observations, we aimed to further investigate the effect of both the introduction of diverse substituents on the isatin nucleus and of the replacement of the naphtalen-2-yl group with a thiazole-2-yl ring on the biological activity. **EMAC** compounds were synthesized slightly modifying previously reported methods (Scheme 1).[2, 29]

Scheme 1. Synthetic Pathway to Compounds EMAC4000, 4001, 4003, 4005, 4007, 4008, 4011, 4012, 4014, 4015, 4018, and 4019[a]



[a]Reagents and conditions: (i) 2-acetylnaphtalene or 2-acetylthiophene, ethanol, NaOH 10% water solution, 0°C; (ii), thiosemicarbazide, ethanol, KOH 5%, reflux; (iii) ethyl bromoacetate, R-isatin, dry sodium acetate, acetic acid, reflux.

Briefly, an ethanol solution of 2-acetylnaphthalene (for the synthesis of **EMAC4000**, **-01**, **-03**, **-05**, **-07**, **-08**) or 2-acetylthiophene (for the synthesis of **EMAC4011**, **-12**, **-14**, **-15**, **-18**, **-19**) was reacted at 0°C with an equimolar amount of 4-methoxybenzaldehyde in the presence of 1,2 equivalent of sodium hydroxide 10% water solution. The obtained solids were crystallized from ethanol. The obtained diarylpropenones were reacted with thiosemicarbazide in refluxing ethanol by adding a freshly prepared KOH 5% ethanol solution. The formation of the dihydrothiazole ring and the condensation of the substituted isatin was accomplished in a single three component step. The 3,5-diaryldihydropyrazole, ethyl bromoacetate and the appropriate isatin derivative were refluxed in acetic acid in the presence anhydrous sodium acetate to give the desired products **EMAC4000**, **-01**, **-03**, **-05**, **-07**, **-08, -11**, **-12**, **-14**, **-15**, **-18**, **-19**).

All compounds were characterized by means of analytical and spectroscopic methods (SI, Tables S1, S2, and Figures S2-S36) and then evaluated for their ability to inhibit tumor cell growth. Firstly, the activity of the new derivatives was evaluated for the anti-proliferative activity in the MTT assay at a fixed concentration of 10 μM) toward a panel of nine human cancer cell lines, namely A549 (lung carcinoma), IGR39 (melanoma), U87 (glioblastoma), MDA-MB-231 (triple-negative breast cancer), MCF-7 (breast adenocarcinoma), and BT474 (invasive ductal carcinoma), H1299 (non-small cell lung carcinoma), SKOV-3 (ovarian cancer) , BxPC-3 (pancreatic adenocarcinoma) cell lines, and human foreskin fibroblasts. When tested toward cancer cell lines some of the compounds exhibited anti-proliferative activity. In particular, compounds bearing a 2-naphtyl substituent in the position 3 of the dihydropyrazole ring were generally more active than their corresponding 2-thiophenyl analogues. Although with some differences, compounds **EMAC4001**, **EMAC4007**, and **EMAC4008** resulted as the most active toward the entire cell panel.

Interestingly, **EMAC4012** and **EMAC4019** were the most active within the 2-thiophenyl series when tested on IGR39, U87 and IGR39 respectively, with anti-proliferative activity comparable with 2-naphtyl analogues. Nevertheless, it should be noted that these derivatives are the analogues of the two most potent compounds of the 2-naphtyl series **EMAC4001** and **EMAC4008**, indicating that the presence of the 5-chloro, or of its isoster 5-CH_3 substituent is optimal for the anti-proliferative activity within this class of compounds. Prompted by these encouraging results we measured the EC_{50} values of the most active compounds of the 2-naphtyl series **EMAC4001**, **EMAC4007**, **EMAC4008**, and of the best performing derivative within the 2-thiophenyl series **EMAC4012** on a panel of selected cancer cell lines (Table 2).

All compounds exhibited EC_{50} values in the low micromolar/high nanomolar range. **EMAC4001** resulted as the most potent within all the tested compounds with EC_{50} values ranging from 0.01 μM against H1299 to 0.38 μM against U87 cells (Table 2). The substitution of the 2-naphtyl moiety with the 2-thiophenyl group in the position 3 of the dihydropyrazole ring in **EMAC4012**, led to an evident decrease of the potency and to EC_{50} values of 2.97 μM and 5.76 μM toward IGR39 and U87 cell lines respectively. Interestingly, when tested on A549, IGR39, and U87, **EMAC4008** exhibited the highest activity with respect to compounds **EMAC4001**, **EMAC4007**, **and EMAC4012**, with EC_{50} values of 0.18 μM, 0.14 μM, and 0.23 μM, respectively. On the basis of these results it can be observed that the 5-Cl-isatin is generally the most efficient, but, at least in some cases, its isosteric replacement with the 5-CH_3-isatin is well tolerated or even more advantageous. Furthermore, to better characterize the biological behavior of these derivatives we investigated the mechanism of cellular death when the cells are treated with half of the EC_{50}

concentration of compounds **EMAC4001** and **EMAC4008**. Results are presented in Figure 1, and the percentage of apoptotic and necrotic cells is reported in Figure 2.

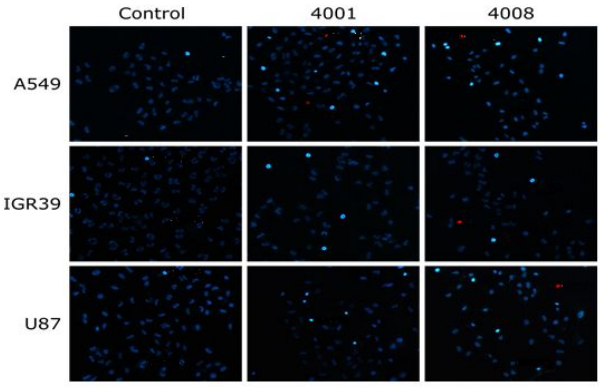


Figure 1. Visualization of apoptotic (bright blue) and necrotic (red) cells after treatment with ½ EC_{50} of **EMAC4001** and **EMAC4008**.

Results were more than encouraging. In all the three considered cell lines the percentage of apoptotic cells ranges between 13.5 and 27%. Conversely, when the number of necrotic cells is considered, either **EMAC4001** or **EMAC4008** induced the necrosis of less than 1% of the cell population. Results show that tested compounds induce cell death mostly through apoptosis. Overall these results indicate that a specific mechanism, such as the inhibition of a signaling pathway, might be the target of **EMAC** derivatives.

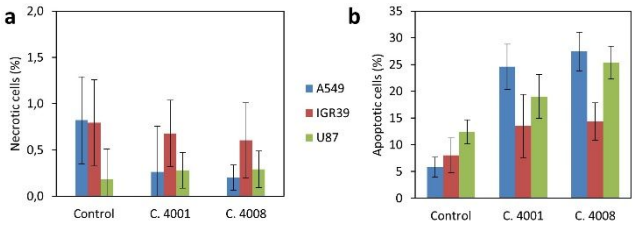
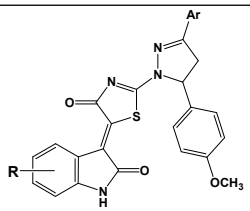


Figure 2. a) Percentage of necrotic cells after treatment with ½ EC_{50} of **EMAC4001** and **EMAC4008**; b) Percentage of apoptotic cells after treatment with ½ EC_{50} of **EMAC4001** and **EMAC4008**.

Although further studies are needed to further clarify and identify the exact mechanism of action of such derivative, our data indicates that the hybridization of 5-chloroisatin with 3,5-diaryldihydropyrazoles by the interposition of a dihydrothiazole spacer is a promising approach to the identification of anticancer agents. With this information in our hands, we are encouraged to further investigate these scaffolds in order to optimize their activity and pharmacokinetic properties.

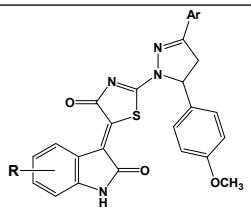
Table 1. Anti-proliferative activity of compounds **EMAC4000**, **-01**, **-03**, **-05**, **-07**, **-08**, **-11**, **-12**, **-14**, and **-15**, **-18**, **-19** at 10 μM concentration.



EMAC Compound	Ar	R	% growth									
			A549	IGR39	U87	MDA-MB-231	MCF-7	BT474	BxPC-3	SKOV-3	H1299	Fibroblasts
4000		7-Br	94.18	98.64	96.71	68.15	101.82	88.24	55.31	89.86	81.02	47.28
4001		5-Cl	3.84	1.30	1.59	6.87	3.81	24.22	5.62	6.69	9.76	14.40
4003		5,7-CH_3	43.8	63.40	64.20	72.98	120.38	62.18	44.87	26.55	87.83	55.56
4005		7-F	86.55	109.13	86.19	87.29	118.14	112.38	73.07	67.65	60.49	89.44
4007		5-OCH_3	3.93	1.20	1.13	9.67	3.64	49.12	5.13	41.43	9.85	13.31

4008	 5-CH_3	6.21	0.46	14.20	10.77	4.81	40.25	5.02	66.61	19.34	11.84
4011	 7-Br	86.11	100.94	57.01	73.90	125.55	94.35	53.68	87.98	89.15	46.28
4012	 5-Cl	22.38	0.90	4.35	80.40	112.02	86.78	59.64	56.58	116.69	47.54
4014	 5,7-CH_3	103.73	116.76	126.25	110.92	114.44	103.19	72.26	79.92	119.87	80.32
4015	 5-F	63.59	28.15	14.40	81.47	112.42	102.48	77.24	94.06	68.35	20.03
4018	 5-OCH_3	62.52	98.60	44.85	99.30	119.79	98.43	67.58	49.54	85.88	64.36
4019	 5-CH_3	22.74	1.37	12.08	76.91	112.02	94.97	62.44	38.35	83.97	48.31

Table 2. EC_{50} values of **EMAC4001**, **EMAC4007**, **EMAC4008**, and **EMAC4012** toward a panel of selected tumor cells.



EMAC Compounds	Ar	R	EC_{50} values (μM)								
			A549	IGR39	U87	MCF-7	BT474	BxPC-3	SKOV-3	H1299	Fibro blasts
4001		5-Cl	0.34	0.33	0.38	0.07	0.09	0.06	0.06	0.01	//
4007		5-OCH_3	0.73	0.50	0.67	0.27	0.24	0.10	//	0.15	0.27
4008		5-CH_3	0.18	0.14	0.23	0.31	//	0.10	//	//	0.15
4012		5-Cl	//	2.97	5.76	//	//	//	//	//	//
Sunitinib			//	//	//	0.96	0.90	2.5	1.36	1.54	0.30

ASSOCIATED CONTENT

Supporting Information

(Experimental procedures and compounds characterization are reported (PDF). The Supporting Information is available free of charge on the ACS Publications website at DOI:

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Author Contributions

‡These authors contributed equally. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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ABBREVIATIONS

hCA, human carbonic anhydrase; COX2, cyclooxygenase2; VEGFR, vascular endothelial growth factor; PDGFR, platelet-derived growth factor receptors; KIT, Mast/stem cell growth factor receptor; RET, proto-oncogene tyrosine-protein kinase receptor; MTT, 3-(4,5-Dimethylthiazol-2-Yl)-2,5-diphenyltetrazolium bromide.

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