Three-component synthesis and anticancer evaluation of polycyclic indenopyridines lead to the discovery of a novel indenoheterocycle with potent apoptosis inducing properties†‡

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A multicomponent reaction of indane-1,3-dione, an aldehyde and an amine-containing aromatic compound leading to the formation of indenopyridine-based heterocyclic medicinal scaffolds has been investigated. It was found that the yields significantly improve when oxygen gas is bubbled through the reaction mixture, facilitating the oxidation of the intermediate dihydropyridine-containing compounds to their aromatic counterparts. Investigation of the reaction scope revealed that formaldehyde, as well as various aliphatic, aromatic and heteroaromatic aldehydes, works well as the aldehyde component. In addition, substituted anilines and diverse aminoheterocycles can be utilized in this process as the amine-containing component. Preliminary biological evaluation of the synthesized library identified a pyrimidine-based polycycle, which rivals the anticancer drug etoposide in its toxicity and apoptosis inducing properties toward a human T-cell leukemia cell line.

Introduction

Heterocycles, fused with an indenone ring system, represent important biological and medicinal scaffolds. Thus, the indenopyridine skeleton is present in the 4-azafluorenone group of alkaloids, represented by its simplest member onychnine (Fig. 1).[1] Indenopyrazoles (**A**) and indenopyridazines (**B**) have been investigated as cyclin-dependent kinase[2] and selective monoamine oxidase B (MAO-B)[3] inhibitors respectively.

Further, indenopyridines (**C**) exhibit cytotoxic,[4a] phosphodiesterase inhibitory,[4b] adenosine A2a receptor antagonistic,[4c] antiinflammatory/antiallergic,[4d] coronary dilating[4e] and calcium modulating activities.[4f] These compounds have also been investigated for the treatment of hyperlipoproteinemia and arteriosclerosis[4g] as well as neurodegenerative diseases.[4h] Lastly, indenopyridone NSC 314622 is serving as a lead compound for the development of anticancer agents targeting topoisomerase I. Its polycyclic planar structure allows for DNA intercalation and inhibition of DNA religation by topoisomerase I in a manner similar to the polycyclic natural product camptothecin and its clinically useful derivative topotecan.[5]

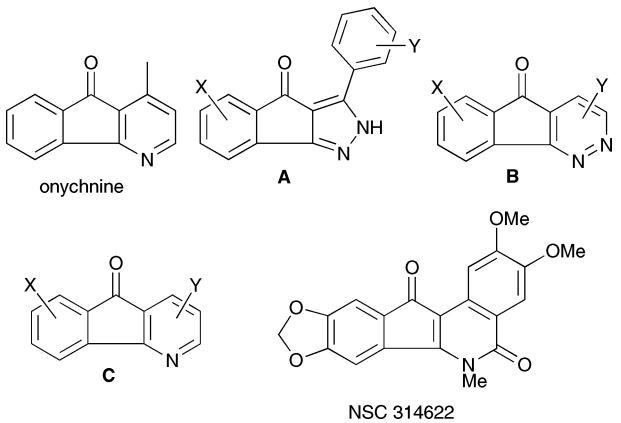

Fig. 1 Naturally occurring and medicinally important indenone-fused heterocycles.

As part of a program aimed at developing multicomponent synthetic routes to heterocyclic scaffolds with medicinal utility,[6] we have been exploring novel approaches to indenoheterocycles. More specifically, we investigated a three-component process involving cyclocondensation of indane-1,3-dione, anilines or aminoheterocycles and various aldehydes, leading to the formation of polycyclic indenopyridines (Fig. 2). Such compounds, in which X is a benzene ring, have been under intense scrutiny as DNA intercalators and topoisomerase inhibitors.[7] Therefore, it is expected that a simple one-step synthesis of these compounds will have an impact on this area of research. Furthermore, compounds with heterocyclic X rings have been only scarcely investigated.[8]

Herein, we describe the results of our study and preliminary biological evaluation of the polycyclic indenopyridines. Although

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† Electronic supplementary information (ESI) available: Full compound characterization data, copies of ^{1}H NMR spectra for all new compounds and X-ray data for compound **27**. See DOI: 10.1039/b713820b

‡ CCDC reference number 660957. For crystallographic data in CIF or other electronic format see DOI: 10.1039/b713820b

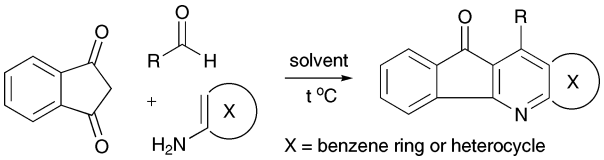


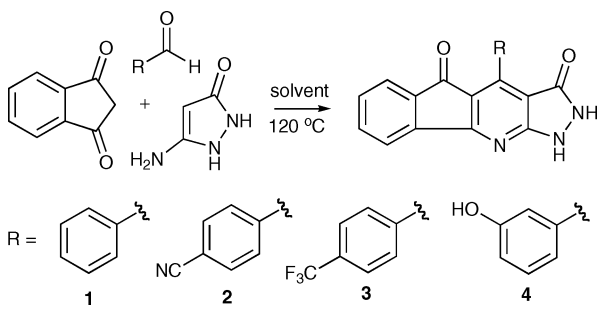
Fig. 2 Multicomponent synthesis of polycyclic indenopyridines.

related reactions utilizing various 1,3-dicarbonyl compounds have been reported by several groups,[6e,9] our investigation represents the first systematic study of the use of indane-1,3-dione in this process. Furthermore, this work has led to the discovery of a novel indenoheterocycle with potent cytotoxic and apoptosis inducing properties.

Results and discussion

As a starting point of this investigation we explored the reaction of 5-amino-1,2-dihydropyrazol-3-one with a series of four aldehydes. The reactions were expected to produce novel tetracyclic systems **1–4** (Table 1). After the formation of the desired products was not observed in refluxing ethanol, we explored refluxing in *n*-BuOH, which allowed for the reaction to be run at a higher temperature. The desired tetracycles **1** and **2**, but not **3** and **4**, were formed in low yields when the reflux was conducted under a nitrogen atmosphere (entry 1). However, when the reaction flask was opened to the atmosphere, all four tetracycles were formed in similar yields (entry 2). We hypothesized that atmospheric oxygen might have a role in this improvement and conducted the reaction with continuous bubbling of oxygen through the solution. This change of experimental conditions further resulted in a small but uniform increase of reaction yields for all four products (entry 3). Next, we screened a number of solvent systems using the same oxygenation procedure and identified ethylene glycol, DMF and a 2 : 1 mixture of acetic acid with ethylene glycol as solvents of choice that improved the reaction yields to *ca.* 60% (entries 5, 7, 8). In most cases the pure heterocycles **1–4** precipitated directly from the reaction mixtures with the exception of just a few cases, in which both pyridine and dihydropyridine products co-precipitated (entry 6).

Table 1 Optimization of reaction conditions



Entry	Solvent	Conditions or additives	% Isolated yield 1	2	3	4
1	*n*-BuOH	N_2 atm.	27	19	<5	<5
2	*n*-BuOH	Open flask	24	18	18	20
3	*n*-BuOH	O_2 bubbling	37	22	20	23
4	AcOH–*n*-BuOH (2 : 1)	O_2 bubbling	25	50	35	27
5	Glycol	O_2 bubbling	52	62	56	50
6	AcOH	O_2 bubbling	n/a[a]	31	27	n/a[a]
7	DMF	O_2 bubbling	66	67	55	45
8	AcOH–glycol (2 : 1)	O_2 bubbling	60	55	65	64
9	AcOH–glycol (2 : 1)	Open flask	41	27	33	40
10	AcOH–glycol (2 : 1)	N_2 atm.	37	32	35	47
11	AcOH–glycol (2 : 1)	Chloranil	71	60	72	n/a[b]

[a] Mixtures of the pyridine products **1** and **4** with the corresponding dihydropyridines were obtained. [b] The presence of the phenolic hydroxyl in **4** resulted in overoxidation of the product.

We recently reported a mechanistic study of a multicomponent reaction involving the cyclocondensation of aldehydes with thiols and two equivalents of malononitrile, resulting in 3,5-dicyanopyridines.[6a,6c] (Fig. 3). The yields of the target pyridines did not exceed 50% in all cases and we identified the oxidation of dihydropyridines to pyridines by the intermediate Knoevenagel adducts as the reason for this phenomenon.

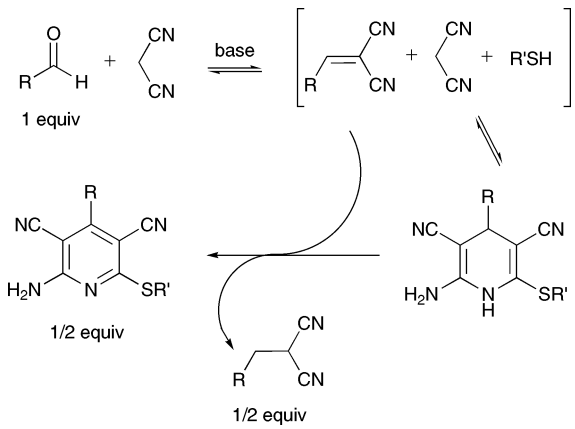


Fig. 3 Reductive consumption of a reaction intermediate resulting in a decrease in product yields by half.

A similar process would explain the effect of oxygen on the yields of tetracycles **1–4** and the occasional co-precipitation of these compounds with the corresponding dihydropyridine-containing tetracycles.[10] Indeed, when the reaction is conducted in AcOH–ethylene glycol (2 : 1) mixture, and oxygen is not bubbled through the reaction solution or the reaction is performed under a nitrogen atmosphere, the yields are cut almost in half (compare entries 9 and 10 with entry 8 in Table 1). In addition, conducting the reaction in the presence of a strong oxidizing agent, such as chloranil, raises the yields above 70% (entry 11). The mechanistic investigations aimed at understanding of this detrimental redox process are currently underway in our laboratories. We are now becoming increasingly convinced that this phenomenon is a common occurrence in many heterocycle-forming transformations which involve an ultimate oxidative aromatization step, and which *are often presumed to be effected with air oxygen or hydrogen release*.

To explore the scope of this process we reacted electron-rich and electron-deficient aromatic aldehydes as well as aliphatic and heterocyclic counterparts. While the yields varied, the reaction is general and highly practical, since the products precipitate directly from the reaction mixtures and require no further purification (Table 2).

Interestingly, the utilization of benzene-1,4-dicarbaldehyde leads to the formation of product **8**, containing nine rings, in

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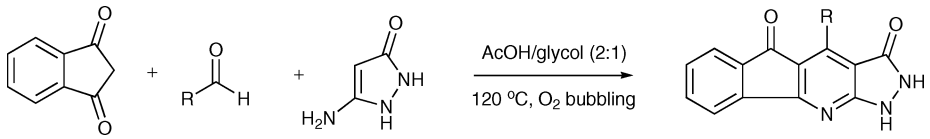
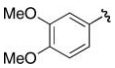
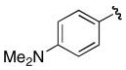
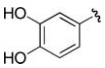
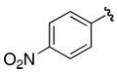
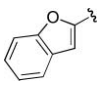
Table 2 Exploration of the reaction scope in relation to the aldehyde component



Indeno-pyridine	R	% Yield
5		63
6		67
7		70
8		80
9		52
10		42
11		68
12		61
13		55
14		53
15		46
16		55
17		73
18	Ethyl	33
19	Propyl	43

a five-component condensation process. The reaction also takes place smoothly when formaldehyde is used as the aldehyde component giving tetracycle **20** (Fig. 4). Surprisingly, the use of indole-3-carbaldehyde results in the loss of the indole moiety to yield the same ring compound **20**. Evidently, oxidation of the indole subunit with oxygen occurs before oxidation of the dihydropyridine portion of the molecule and results in C–C bond cleavage. Although we are unaware of reports describing such a transformation proceeding with the concomitant aromatization of a heterocyclic moiety, in general oxidative cleavage of a C-3 substituent on an indole ring is well-documented, especially in biological systems.[11]

The reaction was further extended to various other aromatic and heteroaromatic amine-containing starting components (Table 3). Thus, polycycles **21–23** are obtained from substituted anilines, **24–27** from diversely substituted 3- and 5-aminopyrazoles and pyrazolones, **28** from an aminotriazole, and **29** and **30** from 1-*N*-Me-6-aminouracil. The reactions generally proceed in good

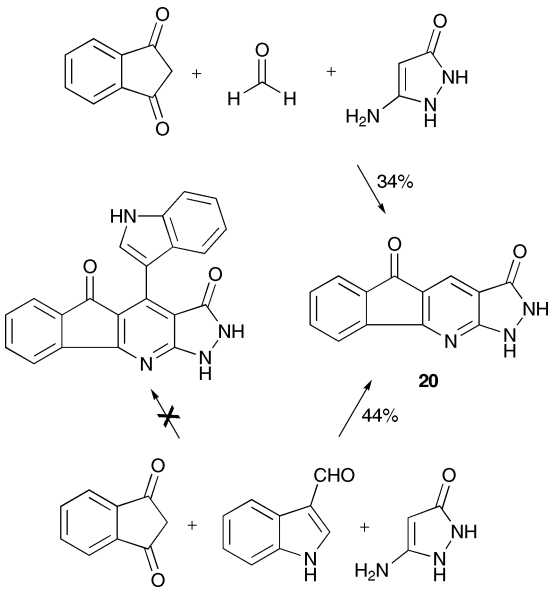


Fig. 4 Reactions of formaldehyde and indole-3-CHO resulting in the same product.

to moderate yields with both electron-rich (p-MeO-C_4H_4-CHO) and electron poor (p-NC-C_4H_4-CHO) aldehydes. Surprisingly, pyrazoles **31** and **32** as well as pyrimidinedione **33** are obtained in dihydropyridine form. In the absence of a mechanistic explanation for the resistance of these compounds toward oxidation, we attribute this unexpected outcome to their reduced solubility in the reaction solvent mixture, which leads to their precipitation before oxidation can occur.

The structures of the synthesized compounds were confirmed by ^{1}H and ^{13}C NMR analyses and high resolution MS. In addition, we obtained an X-ray crystal structure of compound **27** (Fig. 5), which shows the planar nature of the central tetracyclic core,

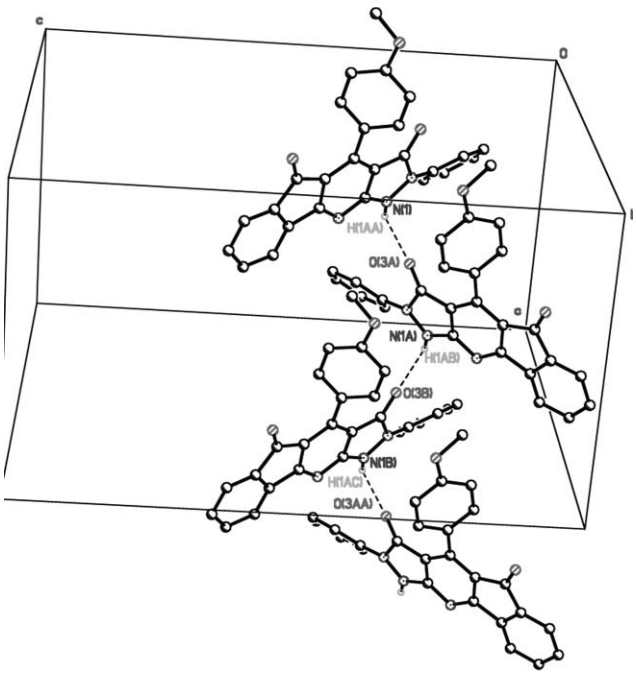


Fig. 5 X-Ray structure of **27**.

indicating the potential of the synthesized compounds to provide medicinal scaffolds in the search for novel DNA intercalators and topoisomerase inhibitors.[7]‡

We performed a preliminary biological evaluation of the synthesized library of compounds using flow cytometry. Using the Annexin-V/propidium iodide assay[12] the compounds were tested for their cell-killing and apoptosis inducing properties against the Jurkat cell line as a model for human T-cell leukemia. Cells were treated with DMSO solutions of the respective compounds at 25 μM final concentrations and the percentage of cells undergoing apoptosis was assessed after 36 h of treatment, while the remaining cell viability percentage was determined after 72 h of treatment (Table 4).

All 5-aminopyrazolone-derived indenoheterocycles **1–19** and **27** exhibit small levels of cytotoxicity (85–98% viability relative to the control cells treated with DMSO) and apoptosis induction (1–6% relative to the control cells treated with DMSO), whereas aniline (**21–23**), most pyrazole (**24–26**, **32**) and triazole-derived (**28**) counterparts are completely inactive. 3-Thiophenopyrazole **31** displayed somewhat enhanced cytotoxicity (*ca.* 80% cell viability). The most promising activity, however, is found with pyrimidinedione-containing dihydropyridine **33**, which manifests good levels of both toxicity and apoptosis induction. Since the biological evaluation of the occasionally formed dihydropyridines and pyridine–dihydropyridine mixtures associated with compounds **1–30**[10] did not reveal any enhanced anticancer properties of the dihydropyridine-based scaffolds (data not shown), we conclude that the pyrimidinedione moiety is the primary contributor to the anticancer activity displayed by indenoheterocycle **33**.

To evaluate the potency associated with compound **33**, we carried out dose-dependent experiments using a clinical anticancer agent etoposide, known to exert its toxic effect through topoisomerase II-dependent DNA cleavage,[13] as a control. Jurkat cells were treated with **33** and etoposide at a range of final concentrations, and percentages of apoptotic and viable cells were determined after 24 and 48 h of treatment (Fig. 6 and 7). Both **33** and etoposide manifest good dose-dependence for both cell-killing and apoptosis induction activities, with our indenopyrimidine compound being more effective at lower concentrations (cytotoxic $IC_{50} = 3$ μM).

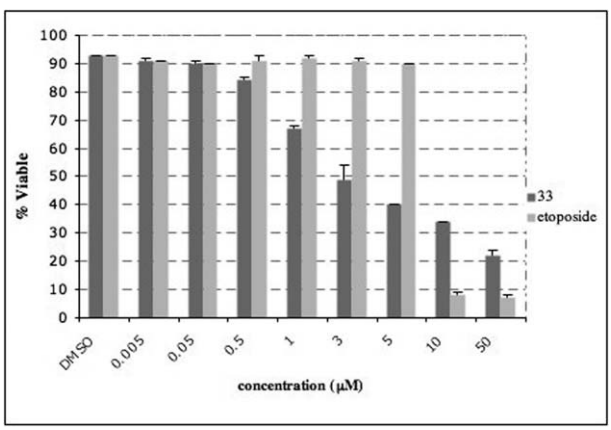


Fig. 6 Dose-dependent comparative evaluation of toxicities to Jurkat cells exhibited by **33** and etoposide. Error bars represent variations in two independent experiments, each performed in triplicate.

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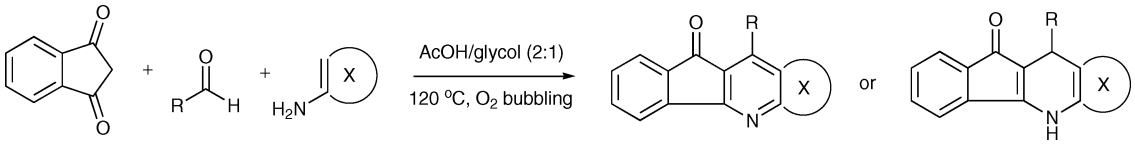
Table 3 Exploration of the reaction scope in relation to the amine-containing component



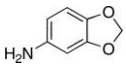
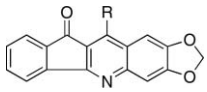
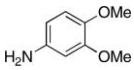
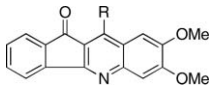
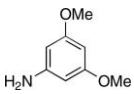
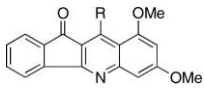
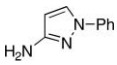
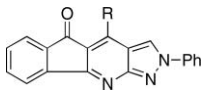
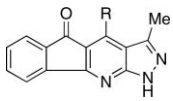
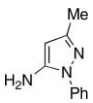
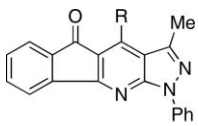
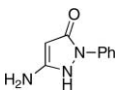
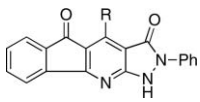
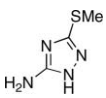
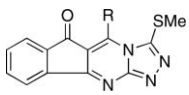
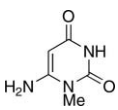
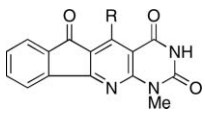
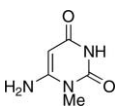
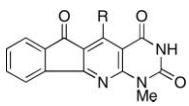
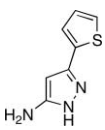
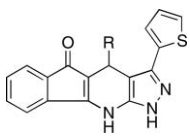
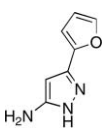
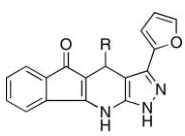
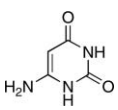
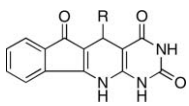
Product	R		Product structure	% Yield
21	*p*-MeO-C_6H_4-			34
22	*p*-MeO-C_6H_4-			35
23	*p*-MeO-C_6H_4-			30
24	*p*-MeO-C_6H_4-			41
25	*p*-MeO-C_6H_4-			30
26	*p*-MeO-C_6H_4-			72
27	*p*-MeO-C_6H_4-			63
28	*p*-NC-C_6H_4-			48
29	*p*-NC-C_6H_4-			51
30	*p*-MeO-C_6H_4-			56
31	*p*-NC-C_6H_4-			65
32	*p*-NC-C_6H_4-			61
33	*p*-MeO-C_6H_4-			72

Table 4 Preliminary anticancer evaluation with flow cytometric Annexin-V/propidium iodide assay using the Jurkat cell line[a]

Compound at 25 μM	% Cell viability[b]	% Apoptosis[c]
1	92 ± 1	2 ± 1
2	91 ± 2	4 ± 1
3	93 ± 0	2 ± 1
4	92 ± 1	6 ± 2
5	92 ± 0	4 ± 1
6	97 ± 2	2 ± 1
7	92 ± 3	2 ± 0
8	94 ± 1	5 ± 0
9	95 ± 0	2 ± 1
10	97 ± 3	3 ± 2
11	96 ± 1	2 ± 0
12	92 ± 2	4 ± 0
13	89 ± 3	4 ± 2
14	87 ± 0	4 ± 2
15	85 ± 2	3 ± 0
16	90 ± 3	5 ± 2
17	ND	ND
18	98 ± 0	1 ± 0
19	89 ± 1	3 ± 1
20	ND	ND
21	97 ± 3	1 ± 1
22	99 ± 1	1 ± 1
23	99 ± 1	1 ± 1
24	98 ± 2	1 ± 1
25	98 ± 1	1 ± 1
26	98 ± 1	1 ± 1
27	96 ± 4	1 ± 1
28	98 ± 1	2 ± 1
29	ND	ND
30	ND	ND
31	79 ± 6	8 ± 2
32	98 ± 2	1 ± 1
33	42 ± 1	34 ± 1

[a] Both cell viability and apoptosis data are obtained in two independent experiments, each performed in triplicate. ND = not determined. [b] Relative to 100% DMSO control after 72 h of treatment ± SD. [c] Relative to 0% DMSO control after 36 h of treatment ± SD.

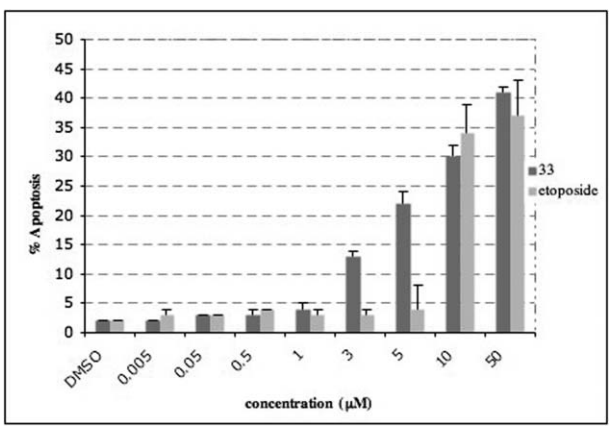


Fig. 7 Dose-dependent comparative evaluation of apoptosis induction in Jurkat cells by **33** and etoposide. Error bars represent variations in two independent experiments, each performed in triplicate.

Further, light microscopy clearly shows changes in cellular morphology, such as shriveling, as well as extensive formation of cellular debris when Jurkat cells are treated with compound **33** (C) or etoposide (D), but not with DMSO (A) or inactive compound **25** (B) (Fig. 8).

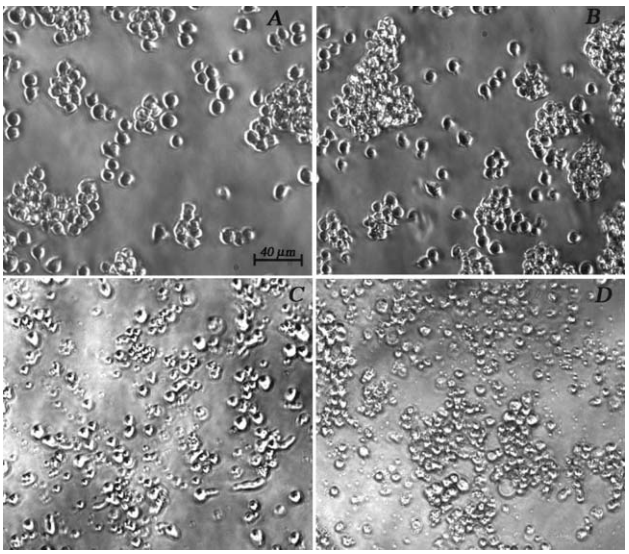


Fig. 8 Light microscopy pictures of Jurkat cells taken after 48 hours of treatment with DMSO control (A) or compounds **25** (B), **33** (C) or etoposide (D) at the final concentrations of 25 μM.

Conclusions

Optimization of the reaction conditions, and specifically the discovery of the beneficial effect of oxygenation of the reaction mixtures, has led to an efficient multicomponent process to prepare various indenoheterocycles. The reaction scope is broad, permitting the use of formaldehyde, aliphatic, aromatic and heteroaromatic aldehydes as well as diverse aromatic and heteroaromatic amine-containing compounds. Due to the well-recognized utility of such polycycles as DNA intercalators and topoisomerase inhibitors, many libraries of compounds can be prepared for biological evaluation using these indenoheterocycles as structural scaffolds for further diversification. Such efforts, as well as exploration of additional scaffolds that may be accessible through this multicomponent reaction, are underway.

Most pleasingly, preliminary anticancer evaluation of the synthesized library led to the discovery of the pyrimidine-based indenoheterocycle **33**, whose cytotoxic and apoptosis inducing potencies compare favorably with the clinical anticancer agent etoposide. A library of analogues based on this heterocyclic scaffold is currently being prepared and evaluated in the search for nanomolar potencies. A major impediment to further medicinal evaluation of these compounds is their poor water solubility. Therefore, further work in this area will encompass the preparation of compounds containing solubilizing residues, such as positively charged ammonium moieties. Because of the expectation that these compounds target DNA, such charged polar residues would also have a beneficial effect on their affinity toward DNA through the attractive interaction with the negatively charged phosphate backbone.

Experimental

General methods

All aldehydes, anilines, aminoheterocycles, indane-1,3-dione, chloranil, acetic acid and ethylene glycol were purchased from

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commercial sources and used without purification. The reactions were performed under nitrogen, open to the atmosphere or by bubbling oxygen gas through reaction mixtures. ^{1}H and ^{13}C NMR spectra were recorded on a 300 MHz spectrometer.

General procedure for the synthesis of compounds 1–33

A selected aldehyde (2.1 mmol, or 1.05 mmol for the synthesis of **8**), aromatic or heterocyclic amine (2.1 mmol) and 1,3-indanedione (0.3 g, 2 mmol) are suspended in a mixture of acetic acid and ethylene glycol (20 mL, 2 : 1). The reaction mixture is heated for 4 h at 120 °C and then allowed to cool to room temperature. The formed precipitate is isolated by filtration and washed with ethanol and diethyl ether. In most cases the products are >95% pure as judged by NMR analysis. Compounds **17**, **24**, **31** and **33** were additionally purified by recrystallization from DMF–H_2O.

Selected characterization data

4-Phenyl-1,2-dihydro-5*H*-indeno[1,2-*b*]pyrazolo[4,3-*e*]pyridin-3,5-dione (1). 60%; ^{1}H NMR (DMSO-d_6) δ 7.86 (d, 1H, $J = 7.4$ Hz, Ind-*H*), 7.71 (t, 1H, $J = 7.1$ Hz, Ind-*H*), 7.58–7.44 (m, 7H); ^{13}C NMR δ 189.3, 165.5, 157.1, 154.1, 146.8, 141.9, 137.4, 135.3, 132.0, 131.0, 130.6, 129.5, 127.5, 123.4, 121.2, 117.7, 102.5; HRMS *m/z* (ESI) calcd for $C_{19}H_{11}N_3NaO_2$ $(M + Na)^+$ 336.0749, found 336.0744.

4-(3,5-Dioxo-1,2-dihydro-5*H*-indeno[1,2-*b*]pyrazolo[4,3-*e*]pyridin-4-yl)benzonitrile (2). 55%; ^{1}H NMR (DMSO-d_6) δ 7.94 (d, 2H, $J = 8.0$ Hz, Ar-*H*), 7.84 (d, 1H, $J = 7.1$ Hz, Ind-*H*), 7.74 (d, 2H, $J = 8.0$ Hz, Ar-*H*), 7.69 (t, 1H, $J = 7.3$ Hz, Ind-*H*), 7.59–7.46 (m, 2H, Ind-*H*); ^{13}C NMR δ 189.3, 165.4, 156.9, 154.4, 144.2, 142.1, 137.3, 137.2, 135.6, 132.4, 131.5, 123.7, 121.4, 119.3, 117.9, 112.1, 102.2; HRMS *m/z* (ESI) calcd for $C_{20}H_{10}N_4NaO_2$ $(M + Na)^+$ 361.0701, found 361.0699.

5-(4-Methoxyphenyl)-5,11-dihydro-1*H*-indeno[2′,1′:5,6]pyrido-[2,3-*d*]pyrimidine-2,4,6(3*H*)-trione (33). 72%; ^{1}H NMR (DMSO-d_6) δ 10.83 (s, 1H, N*H*), 7.46–7.14 (m, 8H), 6.77 (d, 2H, $J = 7.7$ Hz, Ar-*H*), 4.62 (s, 1H, C-*H*), 3.67 (s, 3H, OCH_3); ^{13}C NMR δ 191.4, 163.3, 158.2, 153.7, 150.3, 144.9, 138.0, 136.4, 133.1, 132.6, 130.8, 129.1, 121.3, 119.4, 117.1, 113.9, 110.4, 91.8, 55.5; HRMS *m/z* (ESI) calcd for $C_{21}H_{15}N_3O_4$ $(M + H)^+$ 374.1141, found 374.1151.

Cell culture

A human T-cell leukemia cell line (Jurkat cells, Clone E6–1) was purchased from the American Type Culture Collection (ATCC #TBI-152, USA) and was cultured in RPMI-1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS), 100 mg L^{-1} penicillin G, 100 mg L^{-1} streptomycin, 1.0 mM sodium pyruvate (all from Gibco, Invitrogen: Life Technologies, USA), 1.5 g L^{-1} sodium bicarbonate, and 4.5 g L^{-1} glucose (Sigma) at 37 °C in a humidified atmosphere with 10% CO_2. Cells were diluted at a ratio of 1 : 5 every 2–3 days.

Flow cytometric Annexin-V/propidium iodide assay

Flow cytometry was used to quantitatively measure apoptosis and cell viability. After being cultured with medium alone or medium containing 0.1% (v/v) DMSO, or one of the test compounds at the indicated final concentration for a required time period, 2×10^5 Jurkat cells were centrifuged at 2200 rpm (400 G) for 1 min. The supernatant was discarded and the cells were resuspended in 100 μL per sample of Annexin-V-FITC/propidium iodide solution in Heinz-Hepes buffer (HHB: 30 mM HEPES, 110 mM NaCl, 10 mM KCl, 1 mM $MgCl_2$ and 10 mM glucose) (HHB, 3 μL $CaCl_2$ (1.5 M) per mL HHB, 2 μL (10 mg mL^{-1}) propidium iodide (Sigma) per mL HHB and 20 μL Annexin-V-FITC (Southern Biotech, Birmingham, AL) per mL HHB). The samples in the labeling solution were transferred into Falcon tubes and incubated in a water bath at 37 °C for 20 min. Values of relative fluorescence intensity were measured with FACScan Flow Cytometry (Becton-Dickinson) and analyzed by Cell Quest software. The results were then calculated and expressed as percentages of apoptotic or viable cells using Microsoft Excel 6.0 software.

Acknowledgements

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