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Synthesis and Evaluation of 4/5-Hydroxy-2,3-diaryl(substituted)-cyclopent-2-en-1-ones as *cis*-Restricted Analogues of Combretastatin A-4 as Novel Anticancer Agents

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A new series of 2,3-diaryl-4/5-hydroxy-cyclopent-2-en-1-one analogues replacing the *cis* double bond of combretastatin A-4 (CA-4) by 4/5-hydroxy cyclopentenone moieties was designed and synthesized. The analogues displayed potent cytotoxic activity ($IC_{50} < 1$ μg/mL) against a panel of human cancer cell lines and endothelial cells. The most potent analogues **11** and **42** belonging to the 5-hydroxy cyclopentenone class were further evaluated for their mechanism of action. Both of the analogues led to cell cycle arrest at G2/M phase and induced apoptosis in endothelial cells. Antitubulin property of **42** was superior to **11** and comparable to CA-4. The compound **42** had better aqueous solubility, metabolic stability, and pharmacokinetic profile than CA-4 and also demonstrated significant tumor regression in the human colon xenograft model. Our data suggests that *cis*-restricted analogues of CA-4 are a new class of molecules that have the potential to be developed as novel agents for the treatment of cancer.

Introduction

Microtubules are long, filamentous tube-shaped polymers of α and β tubulin that are essential in all eukaryotic cells. They are crucial in the development and maintenance of cell shape, in the transport of vesicles, mitochondria and other components throughout the cells, in cell signaling, and in cell division or mitosis.[1] Their importance in mitosis and cell division make microtubules an obvious and important target for anticancer drugs.[1] The dynamic process of assembly and disassembly of microtubules to tubulin is blocked by various agents that bind to distinct sites such as binding sites for colchicines, taxol, and vinca alkaloids, finally leading to arrest in mitosis and cell death by apoptosis or necrosis.[2] Several microtubule targeting drugs,-namely, Paclitaxel, Epothilones, and Dolastatin are in clinical use or in various stages of clinical development.[2]

Combretastatin A-4 (**1′**; Figure 1), a natural product isolated from the bark of the South African tree *Combretum caffrum* is highly cytotoxic against a variety of human cancer cells including multidrug resistant cancer cell lines.[3] The molecule binds at or near colchicine binding site and inhibits tubulin polymerization ultimately leading to cell death.[3] It displays selective toxicity toward tumor vasculature by inhibiting the blood supply to the tumor leading to cell death. Currently two phase II clinical trials are ongoing for **1** (combretastatin A-4 phosphate) for advanced anaplastic thyroid cancer, and one phase I study has recently begun in combination with bevacizumab for subjects with advanced solid tumors (www.clinicaltrials.gov). A phase Ib trial of **1** in combination with carboplatin or paclitaxel chemotherapy conducted in patients with advanced cancer revealed that the combination of **1** with either carboplatin or paclitaxel was well tolerated.[4]

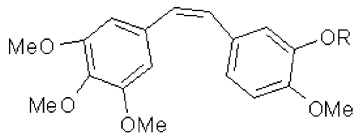

Figure 1. Structures of CA-4 phosphate (**1**) and CA-4 (**1′**). R = H combretastatin A-4 (CA-4, **1′**), R = PO_3Na_2 CA-4 disodium phosphate ester (CA-4P, **1**).

Preclinical studies have shown that **1** induces blood flow reductions and subsequent tumor cell death in a variety of preclinical models.[5]

The encouraging antivascular and anticancer activity of **1** has stimulated significant interest in a number of diverse ligands designed to mimic **1**. It has been established through structure−activity relationship (SAR) studies in several research laboratories[6−8a,b] that the *cis*-configuration of the double bond between the two aryl rings and the 3,4,5-trimethoxy systems on the A ring are fundamental requirements for its biological activity. However, the main problems associated with this compound have been the ready isomerization of the double bond to its inactive *trans*-form and low water solubility.[9,10] As an outcome of the exhaustive study on **1′**, it has been demonstrated[11,12] that **1** could provide the solution to overcome the problem of low water solubility. In our laboratories we have been actively engaged in designing several strategies to improve the potency and overcome double bond isomerization of **1**. One of the key approaches taken has been to rigidify the olefin by a heterocyclic compound,[13−17] five-membered and six-membered rings, as also previously shown by us.[18] In our opinion, the hydroxy-cyclopentenone moiety can be the surrogate of the *cis*-double bond separating the two phenyl rings to combat with the problems of double bond isomerization.

A family of 2,3-diaryl-4/5-hydroxy-cyclopent-2-en-1-one analogues replacing the *cis*-double bond by 4/5-hydroxy cyclopentenone moieties was designed and synthesized to study

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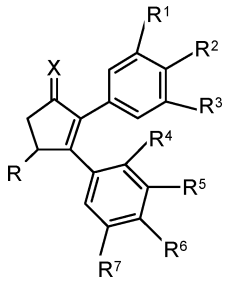
Table 1. 2,3-Diaryl-4-hydroxycyclopent-2-en-1-one Analogues of **1′**



Sr. No	cmpd	R	R^1	R^2	R^3	R^4	R^5	R^6	R^7	X
1	**14**	OH	OMe	OMe	OMe	H	H	OMe	H	O
2	**15**	OH	OMe	OMe	OMe	H	Me	OMe	Me	O
3	**16**	OH	OMe	OMe	OMe	OMe	H	H	OMe	O
4	**17**	OTBS	OMe	OMe	OMe	H	H	OMe	H	O
5	**18**	OTBS	OMe	OMe	OMe	H	H	H	H	O
6	**19**	OTBS	OMe	OMe	OMe	H	Me	OMe	Me	O
7	**20**	OTBS	OMe	OMe	OMe	OMe	H	H	OMe	O
8	**21**	OH	OMe	OMe	OMe	H	H	OMe	H	N−OH
9	**22**	OAc	OMe	OMe	OMe	H	H	OMe	H	N−OH
10	**23**	OTBS	OMe	OMe	OMe	OMe	H	H	OMe	N−OH
11	**24**	OTBS	H	H	H	H	H	H	H	O
12	**25**	OTBS	OMe	OMe	OMe	H	COOMe	OMe	H	O
13	**26**	OTBS	OMe	OMe	OMe	H	OMe	OMe	H	O
14	**27**	OAc	OMe	OMe	OMe	H	Me	OMe	Me	O
15	**28**	OAc	OMe	OMe	OMe	H	Me	OMe	Me	N−OH
16	**29**	OTBS	OMe	OMe	OMe	H	OMe	OMe	H	N−OH
17	**30**	OH	H	H	H	H	H	H	H	N−OH
18	**31**	OAc	H	H	H	H	H	H	H	N−OH
19	**32**	OTBS	OMe	OMe	OMe	H	OMOM	OMe	H	O
20	**33**	OH	OMe	OMe	OMe	H	OH	OMe	H	O
21	**34**	OTBS	OMe	OMe	OMe	H	OMOM	OMe	H	N−OH
22	**35**	OTBS	OMe	OMe	OMe	H	OH	OMe	H	O
23	**36**	OH	OMe	OMe	OMe	H	OH	OMe	H	N−OH
24	**37**	OAc	OMe	OMe	OMe	H	OH	OMe	H	N−OH
25	**38**	OH	OMe	OMe	OMe	H	H	O COOEt	H	O
26	**39**	OH	OMe	OMe	OMe	H	COONa	OMe	H	O
27	**40**	OAc	OMe	OMe	OMe	H	H	OMe	H	N−OH

their potential cytotoxicity. Introduction of hydroxy cyclopentenone moiety indeed proved to be advantageous with respect to the stability, being a *cis*-restricted structural modification. The hydroxy group at the 4- or 5-position in the cyclopentenone ring provided a handle to design several stable analogues. The synthetic methods developed allowed us to generate 28 analogues each of 4-hydroxy and 5-hydroxy cyclopentenone compounds. The analogues were screened for cytotoxicity using nine human cell lines. They were selected on the basis of in vitro cytotoxicity data and were then evaluated for their potential to inhibit tubulin polymerization, cause cell cycle arrest, and induce apoptosis. Additionally, the aqueous solubility, metabolic stability, pharmacokinetics, and in vivo efficacy in the tumor xenograft model were also evaluated for the selected analogues. In the present manuscript, it is shown that the replacement of the *cis*-double bond by the hydroxy-cyclopentenone moiety produced several stable cytotoxic compounds. The SAR and pharmacodynamic profiles of the analogues is also described here.

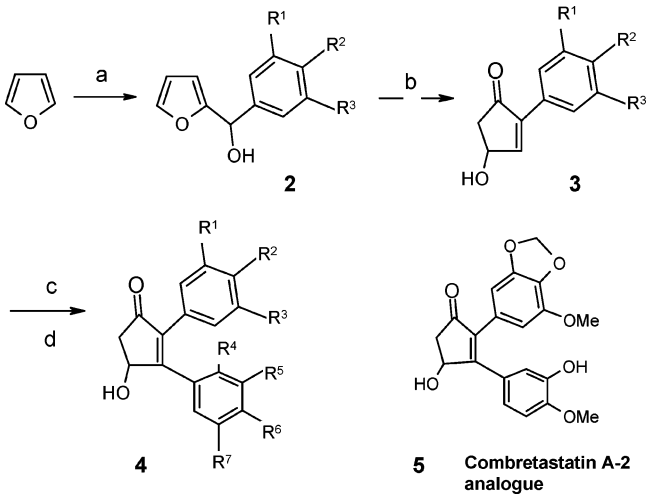
Chemistry

The 4-hydroxy-2,3-diaryl-cyclopentenone analogues **4** were synthesized starting from condensation of furyl lithium with corresponding substituted benzaldehydes. A simple and efficient one step method[18] was developed for the preparation of 2-aryl-4-hydroxy-cyclopentenone **3** from 2-furylmethanol **2** in excellent yield using zinc chloride as a weak Lewis acid at 110 °C. Protection of the 4-hydroxy group in **3** with *tert*-butyldimethylsilyl chloride gave the corresponding derivatives, which were subjected to the Heck reaction using palladium acetate and aryl halide in the presence of base to obtain the desired analogues **4**.

This methodology provided 28 analogues (27 compounds in Table 1 and compound **10** in Scheme 3) by employing various benzaldehydes, aryl halides, and further deprotection, followed by derivatization. This series included the synthesis of combretastatin A-2 analogue **5** (Scheme 1), for which 5-methoxy piperonaldehyde was treated with furyllithium, and the Heck reaction was performed using 4-iodo-1-methoxy-2-methoxymethyloxybenzene in our synthetic strategy described above. It was observed that oximation of the products thus obtained led to the more soluble substances.

The second group of compounds possessing a hydroxy group at the 5-position in the cyclopentenone ring was accomplished from the intermediate **6**. The intermediate **6** was treated (Scheme 2) with the desired aryl halide (bromide or iodide) in the presence of magnesium or *n*-butyllithium, which reacted to give a 1,2-addition product as the tertiary alcohol, in good to excellent yields. Additionally, reaction of the cyclopentenol **7** performed with pyridinium dichromate (PDC) in dichloromethane at 0 °C to room temperature afforded the corresponding 2,3-diaryl-5-*tert*-butyldimethylsilyloxycyclopentenones **8**. The desired 5-hydroxy analogues were obtained by deprotection of the corresponding 2,3-diaryl-5-*tert*-butyldimethylsilyloxycyclopentenones

Scheme 1[a]



[a] Reagents and conditions: (a) (i) *n*-BuLi, THF; (ii) substituted benzaldehyde; (b) $ZnCl_2$, dioxan/water, reflux, 24 h; (c) TBDMSCl, DMAP, DCM, Et_3N, 3 h; (d) $Pd(OAc)_2$ (12 mol %), K_2CO_3 (2 equiv), Bu_4NBr, CH_3CN.

8. The mechanism[19] of this rearrangement involves initial formation of the chromate ester A from the tertiary alcohol, followed by an allylic rearrangement of the chromate ester of the secondary alcohol. Typical fragmentation of the resultant chromate ester B delivered the ketone. In this series we have mainly synthesized the B-ring modified analogues of *cis*-restricted **1′** analogues in which the hydroxy group at 3-position of B-ring was replaced with substituents like H, Cl, F, O-isopropyl, O-allyl, NHAc, NHCOPh, NMe_2, NHCHO, and NMeCHO (Table 2).

To synthesize some water soluble analogues in this series of compounds and also to confirm the role of methoxy group at the 4-position in the B-ring, we considered replacing 4-methoxy in the B-ring of the **1′** analogue by employing 4-iodophenoxyacetic acid ethyl ester in the Heck reaction step, which furnished the intermediate **9** (Scheme 3). Alkaline hydrolysis of the ethyl ester **9** and sequential treatment with sodium hydroxide in distilled water gave the water-soluble sodium salt **10**.

A highly water soluble analogue of 5-hydroxy-2-(3,4,5-trimethoxyphenyl)-3-(4-methoxyphenyl)-cyclopent-2-ene-1-one **11** was accomplished as the disodium salt of its phosphate analogue **13**. The compound **11** was reacted with di-*tert*-butyl-*N*,*N*-diethylphosphoramidite and tetrazole in THF to get the corresponding phosphate analogue **12**. Further hydrolytic cleavage of *tert*-butyl groups with TFA in DCM followed by treatment with sodium hydroxide successfully formed the water-soluble analogue **13**. All compounds synthesized possessing 4/5-hydroxy functionality and their derivatives were characterized by spectroscopic methods, and purity was confirmed by analytical methods (high performance liquid chromatography (HPLC) > 95%).

Biological Activity Results and SAR. The synthesized cyclopentenone analogues were assessed for cytotoxicity in the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) cytotoxicity assay. 2,3-Diaryl-4-hydroxycyclopent-2-en-1-one analogues (Table 1) were synthesized and screened for cytotoxicity. In the designed analogues, ring A possessed 3,4,5-trimethoxy substitution, which was considered to be fundamental for the tubulin binding activity, whereas variations were made in the ring B. For every variation in the B-ring, we achieved 4−6 samples for SAR study by simple organic transformations like (1) removal of *tert*-butyldimethylsilyl (−OTBDMS) protecting group, (2) derivatization of this free hydroxy group by acylation, (3) oximation of the ketone group, and so on. Other derivatizations leading to analogues having a molecular weight of more than 500 were avoided to select better druglike molecules. The noteworthy observations based on the screening data (Table 3) of 28 samples are discussed below.

Some of the samples were purposely synthesized with absence of a methoxy group at 4-position in the B-ring, such samples, for example, the compounds **16**, **18**, and **20**, showed reduced cytotoxicity, which confirmed that the presence of a methoxy group at position 4 in the ring-B is mandatory for the biological activity.

In addition to having a methoxy functional group on B-ring, a carboxylic acid group was also attached to the B-ring to enhance the solubility of the analogues. These analogues were also amenable to salt formation. We synthesized a compound with −COOH at the 3-position of the B-ring, that is, the compound **39**. This option was not found beneficial as it lowered the cytotoxic activity. Similarly, the methoxy group at the 4-position in the ring B was replaced by carboxymethyloxy ($-OCH_2COOH$) group (Scheme 3) and its water-soluble sodium salt (compound **10**), which was less active.

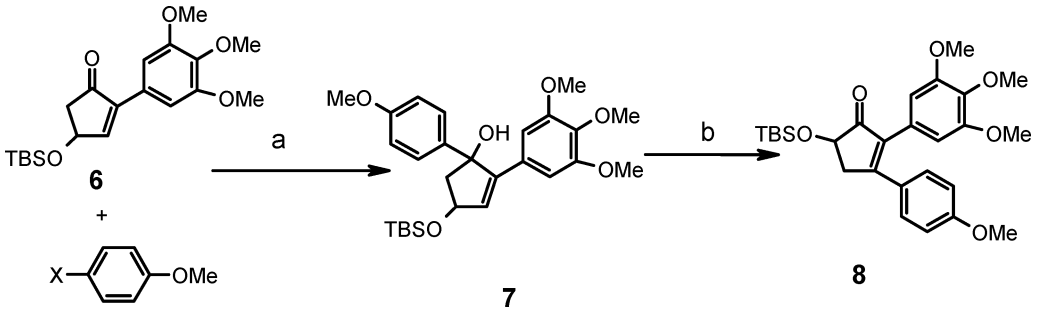
The **1′** mimic, that is, the compound **33** and its analogues **35**−**37**, displayed comparable or even better cancer cell growth inhibition against a number of cancer cell lines such as HT29 (colon), DU145 (prostate), KB (oral), L132 (lung), Hep2 (larynx), and PA1 (ovary). In most of the examples studied, oximation of the ketone in the cyclopentenone ring did not influence the IC_{50} values significantly, for example, **21**, **28**−**31**, and **34**, while compounds **22**, **23**, **36**, and **37** exhibited good cytotoxicity. The compound **40** was found to be the most active among the oximes studied, as shown in Table 3. The oxime analogues were preferred in some cases due to their higher solubility and served as prodrugs. The TBDMS protection of the hydroxy group did not influence the cytotoxicity.

Table 2 exhibits the 5-hydroxy cyclopentenone analogues screened for the cytotoxic activity, and the IC_{50} values are depicted in Table 4. Some of the compounds from this class, for example, **11**, **41**, **42**, **46**−**48**, and **66** showed strong cytotoxic activity. One of the additional features studied in this group of analogues was the halogenated B-ring compounds. The 3-chloro- and 3-fluoro-substituted B-ring compounds **53**−**57**, **59**, and **60** displayed marked tumor growth suppression ($IC_{50} < 1$ μg/mL) selectively against HT29, L132, and Hep2 cell lines.

On the contrary, the free hydroxy group as well as the methoxy group at the 3-position of the B-ring decreased the cytotoxic activity. Other alkyl ethers at the 3-position of ring B like allyl ether and isopropyl ether also abolished the activity, for example, compounds **44**, **45**, **58**, **63**, and **64**. Potent efficacy has been attributed to the replacement of the phenolic OH of **1′** with an amino group in the literature.[8b] We investigated the compounds with the NHAc, the NMeCHO, and the NHCHO group in place of the phenolic OH at 3-position in the B-ring. These groups were well tolerated, and the compound **66** was highly toxic to the tumor cells. This indicated that the nature of the substituent in the phenyl ring located at position 3 in 5-hydroxy-cyclopentenone analogues was crucial.

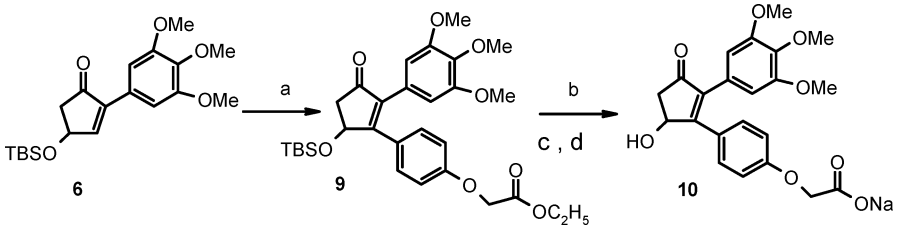
By interchanging the substitution pattern of rings A and B, we synthesized the compounds **49**−**51**. It was noteworthy that this pattern destroyed the efficacy of the compound to inhibit the growth of cancer cells, especially in the case of the compounds with an electron-withdrawing (nitro) group in place of the phenolic OH (in ring A in this example). The presence of a primary amine instead of a phenolic OH in the ring A (**51**)

Scheme 2[a]



[a] Reagents and conditions: (a) Mg, THF or *n*-BuLi, THF; (b) PDC, DCM, 0 °C−rt.

Scheme 3[a]



[a] Reagents and conditions: (a) $Pd(OAc)_2$ (12 mol %), K_2CO_3 (2 equiv), Bu_4NBr, CH_3CN, 36 h; (b) TBAF (1 M soln.), THF, 0 °C-rt, 1 h; (c) 10% NaOH, MeOH; H^+; (d) NaOH, dist. water.

was effective for inhibition (IC_{50} < 1 μg/mL) of at least two cell lines, namely, HT-29 and KB.

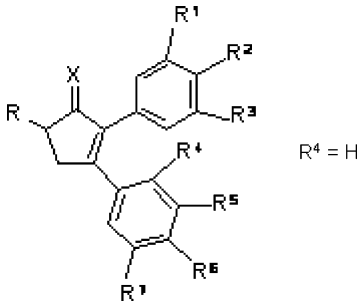
Finally, the 4-methoxy group in the B-ring was replaced by a 4-thiomethyl group, and it was observed that compound **48** still retained the cytotoxicity. However, this activity was lost to some extent when the TBDMS protection was removed (compound **52**). The role of the TBDMS group under biological systems has not been studied, however, it is noteworthy that, in some of the analogues possessing TBDMS instead of the free hydroxyl group in the cyclopentenone ring, enhanced cytotoxicity was observed, for example, compounds **23** vs **16**; **24** vs **30**; **47** vs **44**; and **45** and **48** vs **52**.

Compound **11** displayed a promising cytotoxicity profile in a number of cancer cell lines. In addition to **11**, the corresponding oxime **42** also exhibited strong cytotoxicity against a number of cancer cell lines such as HT29, KB, L132, MiaPaCa2, Hep 2, PA-1, and ECV 304. The overall biological activity studies of the compounds in this group indicated that the compound with the A-ring possessing 3,4,5-trimethoxy systems and the B-ring with a 4-methoxy substitution was a very potent cytotoxic compound. A water-soluble disodium phosphate derivative (**13**) of analogue retained the activity shown by the parent molecule (**11**).

Based on the in vitro cytotoxicity data, two cyclopentenone analogues **11** and **42** were selected. These analogues were assessed for their effects on cell cycle, ability to induce programmed cell death, and in vitro tubulin polymerization inhibition potential. The reported antivascular properties of **1′** prompted us to select endothelial cells (ECV304) as the model for investigating the mechanism of anticancer/antivascular action of cyclopentenone analogues.

Tubulin Polymerization. The cyclopentenone analogues **11** and **42** were assessed for their ability to inhibit tubulin polymerization. IC_{50} values for inhibition of tubulin polymerization for **1′**, **11** and **42** were found to be 0.98, 5.34 and 1.75 μM respectively (Table 5). The comparison of IC_{50} values of **1′** and **42** indicate that **42** was comparable to **1′** and may act as a cytotoxic agent through inhibition of tubulin polymerization. The antitubulin potential of **42** was evidently superior to **11**.

Table 2. 2,3-Diaryl-5-hydroxycyclopent-2-en-1-one Analogues of **1′**



Sr. No	cmpd	R	X	R^1	R^2	R^3	R^5	R^6	R^7
1	**11**	OH	O	OMe	OMe	OMe	H	OMe	H
2	**41**	OAc	O	OMe	OMe	OMe	H	OMe	H
3	**42**	OH	N−OH	OMe	OMe	OMe	H	OMe	H
4	**43**	OAc	N−OH	OMe	OMe	OMe	H	OMe	H
5	**44**	OH	O	OMe	OMe	OMe	OiPr	OMe	H
6	**45**	OH	N−OH	OMe	OMe	OMe	OiPr	OMe	H
7	**46**	OTBS	O	OMe	OMe	OMe	H	OMe	H
8	**47**	OTBS	O	OMe	OMe	OMe	OiPr	OMe	H
9	**48**	OTBS	O	OMe	OMe	OMe	H	SMe	H
10	**49**	OTBS	O	NO_2	OMe	H	OMe	OMe	OMe
11	**50**	OH	O	NO_2	OMe	H	OMe	OMe	OMe
12	**51**	OH	O	NH_2	OMe	H	OMe	OMe	OMe
13	**52**	OH	N−OH	OMe	OMe	OMe	H	SMe	H
14	**53**	OH	N−OH	OMe	OMe	OMe	Cl	OMe	H
15	**54**	OH	O	OMe	OMe	OMe	F	OMe	H
16	**55**	OAc	O	OMe	OMe	OMe	F	OMe	H
17	**56**	OH	N−OH	OMe	OMe	OMe	F	OMe	H
18	**57**	OH	O	OMe	OMe	OMe	Cl	OMe	H
19	**58**	OAc	O	OMe	OMe	OMe	OiPr	OMe	H
20	**59**	OAc	O	OMe	OMe	OMe	Cl	OMe	H
21	**60**	OAc	N−OH	OMe	OMe	OMe	Cl	OMe	H
22	**61**	OAc	O	OMe	OMe	OMe	OH	OMe	H
23	**62**	OH	N−OH	OMe	OMe	OMe	OH	OMe	H
24	**63**	OH	O	OMe	OMe	OMe	O-allyl	OMe	H
25	**64**	OH	N−OH	OMe	OMe	OMe	O-allyl	OMe	H
26	**65**	OH	O	OMe	OMe	OMe	NHCOPh	OMe	H
27	**66**	OAc	O	OMe	OMe	OMe	NHCHO	OMe	H

Hence, based on the superior inhibition of tubulin polymerization, **42** was selected for further preclinical studies.

Table 3. Cytotoxicity Data of Cyclopentenone Analogues from 2,3-Diaryl-4-hydroxycyclopent-2-en-1-one Series[a]

		IC_{50} (μg/mL)								
Sr. No.	cmpd of formula No.	HT29	DU145	KB	L132	MiaPaCa-2	Hep2	PA1	ECV 304	293
	1′	5	57	<1	<1	<1	5	<1	<1	5.7
1	**10**	ND	NA	NA	ND	NA	NA	ND	ND	NA
2	**14**	15	18.7	ND	10	NA	NA	3.38	NA	10
3	**15**	NA	NA	NA	NA	NA	NA	NA	NA	NA
4	**16**	NA	NA	NA	NA	NA	NA	NA	NA	NA
5	**17**	NA	NA	NA	NA	NA	NA	NA	NA	NA
6	**18**	NA	NA	NA	NA	NA	NA	NA	NA	NA
7	**19**	10	NA	40	NA	ND	26	32	15	29
8	**20**	5	100	10	100	NA	19	17	6.5	9.5
9	**21**	15	34.25	ND	11	NA	NA	2.35	NA	NA
10	**22**	1.5	20	5	20	1.0	3	20	4	32
11	**23**	50	NA	NA	NA	1	7	8.5	6	7
12	**24**	1	1.5	ND	40	1	4	1.61	4	5
13	**25**	NA	NA	NA	NA	NA	NA	NA	NA	NA
14	**26**	NA	NA	NA	NA	NA	NA	NA	NA	NA
15	**27**	NA	NA	NA	NA	NA	NA	NA	NA	NA
16	**28**	NA	NA	NA	NA	NA	NA	NA	NA	NA
17	**29**	20	60	15	60	15	32	ND	36	70
18	**30**	NA	NA	NA	NA	NA	NA	NA	NA	NA
19	**31**	NA	NA	9	NA	4.5	NA	NA	NA	92.3
20	**32**	43	34	33	32	50	12	13.5	40	NA
21	**33**	<1	8	<1	<1	NA	<1	<1	NA	NA
22	**34**	<1	30	27	28	6	13.7	8	16.5	18
23	**35**	38	75	6.5	<1	55	14.5	14	30	42
24	**36**	9	23	10	8	NA	<1	3.7	NA	NA
25	**37**	<1	23	<1.0	<1.0	<1.0	NA	<1	NA	6.06
26	**38**	NA	NA	NA	NA	NA	NA	NA	NA	NA
27	**39**	NA	NA	NA	NA	NA	NA	NA	NA	NA
28	**40**	<1	21	<1.0	<1.0	<1.0	<1.0	<1.0	60	22

[a] NA = not active, that is, the IC_{50} is greater than the maximal concentration tested (100 μg/mL). ND = not done.

Table 4. Cytotoxicity Data of Cyclopentenone Analogues from 2,3-Diaryl-5-hydroxycyclopent-2-en-1-one Series[a]

		IC_{50} (μg/mL)								
Sr. No.	cmpd of formula No.	HT29	DU145	KB	L132	MiaPaCa-2	Hep2	PA1	ECV 304	293
	1′	5	57	<1	<1	<1	5	<1	<1	5.7
1	**11**	<1	NA	<1	<1	<1	<1	<1	<1	6.3
2	**13**	ND	7.5	72	ND	<1	<1	ND	46	ND
3	**41**	<1	NA	72	NA	<1	18	<1	18	ND
4	**42**	<1	NA	<1	<1	<1	<1	<1	<1	4.2
5	**43**	1	NA	69	ND	<1	100	<1	NA	25.6
6	**44**	NA	NA	NA	NA	NA	NA	NA	NA	NA
7	**45**	69	81	95	75	98	40	61	96	36.77
8	**46**	5	28	7	9	<1	16	7	16	NA
9	**47**	9	6.3	7.5	7	NA	7.1	7.1	NA	NA
10	**48**	<1	NA	10	<1	<1	NA	<1	NA	NA
11	**49**	NA	NA	NA	NA	NA	NA	NA	NA	NA
12	**50**	NA	NA	NA	NA	NA	NA	NA	NA	NA
13	**51**	<1	41	<1	54	NA	16	20	NA	NA
14	**52**	36	2.5	15	NA	40	<1	25.3	57	27
15	**53**	<1	16	9.8	<1	33	<1	NA	18.5	50
16	**54**	<1	27	4.5	<1	64	<1	NA	19.5	NA
17	**55**	<1	16	33	<1	39.5	<1	NA	41	59
18	**56**	<1	10	<1	<1	94	<1	NA	43	NA
19	**57**	<1	40	2.5	<1	NA	<1	NA	20	NA
20	**58**	NA	NA	NA	NA	NA	NA	NA	NA	NA
21	**59**	<1	64	64	<1	24	<1	NA	3.5	60
22	**60**	<1	17	50	<1	10	<1	37.5	8	32
23	**61**	<1	NA	35	6	18.5	NA	6	NA	92
24	**62**	69	10	90	81	NA	20	26	NA	NA
25	**63**	NA	NA	NA	NA	NA	NA	NA	NA	NA
26	**64**	NA	NA	NA	NA	NA	NA	NA	NA	NA
27	**65**	NA	NA	NA	NA	NA	NA	NA	NA	NA
28	**66**	<1	NA	8	2	2	3.27	5.0	ND	4.16

[a] NA = not active, that is, the IC_{50} is greater than the maximal concentration tested (100 μg/mL). ND = not done.

Arrest of Endothelial Cells in G2/M and Inhibition of Mitosis. The ability of cyclopentenone analogues **11** and **42** to modulate endothelial cell cycle progression was evaluated using asynchronous proliferating endothelial cultures (ECV304) exposed to the drug, which were then stained with propidium iodide (PI) and analyzed by flow cytometry. Cells were treated

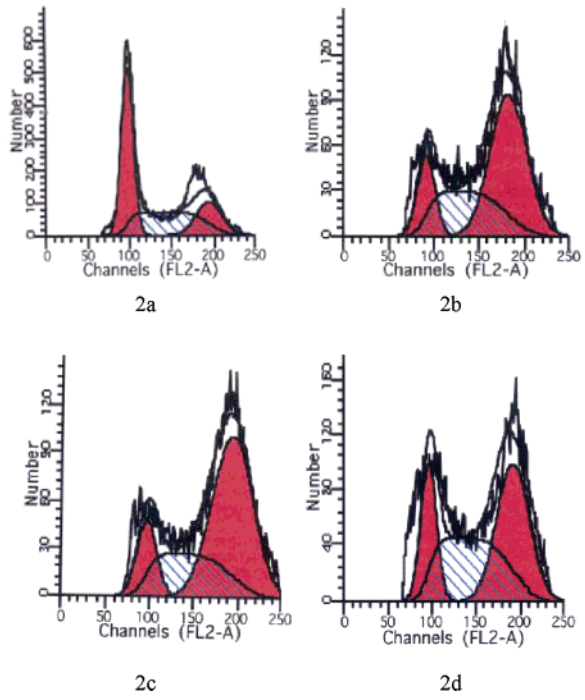


Figure 2. Effect on the cell cycle as determined by flow cytometry. Untreated cells were taken as control (2a). Cells were treated with analogues **11** (2b), **42** (2c), and **1′** (2d) at the concentration of 1 μM.

Table 5. In Vitro Tubulin Polymerization Inhibition Potential of **1′**, **11**, and **42**

cmpd	IC_{50} (μM)
1′	0.98
11	5.34
42	1.75

Table 6. Physical Properties of **1′**and **42**

cmpd	solubility (μM)	metabolic stability (% compound remaining)
1′	122.8	84.8
42	359.6	96.4

with **1′**, **11**, and **42** at the concentration of 1 μM for 24 h. There was an accumulation of 41, 54, and 59% of cells in the G2/M phase of the cell cycle observed with **1′**, **11**, and **42** as compared to that of the untreated control (Figure 2) in which only 18% accumulation was observed. Hence, both the derivatives **11** and **42** at a concentration of 1 μM led to enhanced mitotic arrest in G2/M phase in endothelial cell cultures.

DNA Fragmentation Analysis. Proliferating endothelial cells were treated with **1′**, **11**, and **42** at concentrations ranging from 10 nM to 1 μM for 24 h. After the treatment, many rounded, loosely adherent cells accumulated in the cultures. Evaluation of apoptosis by DNA fragmentation was carried out in both strongly and loosely adherent cells. No DNA fragmentation was observed in strongly adherent cells at any of the concentrations studied. However, the treatment with **1′**, **11**, and **42** led to

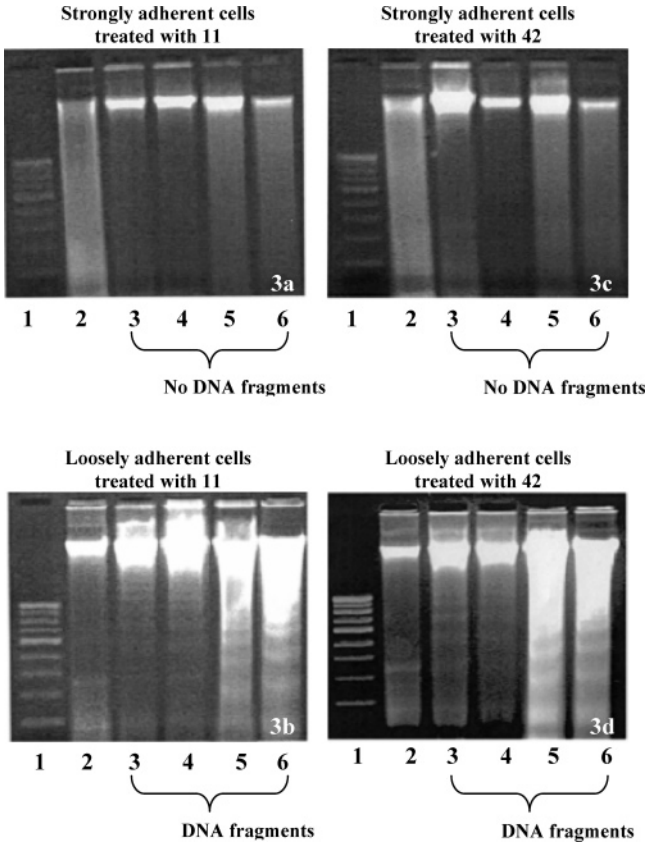


Figure 3. Evaluation of apoptosis by DNA fragmentation in ECV-304 cells. (3a and 3b) Depiction of the DNA fragmentation pattern observed with treatment of cells with **11** and (3c and 3d) depiction of the DNA fragmentation pattern observed with treatment of cells with **42** in strongly adherent and loosely adherent cells, respectively, at concentrations of 10, 100, and 1000 nM. **1′** was included as a reference standard at the concentration of 100 nM. Lane 1, 100 bp DNA marker; Lane 2, control cells; Lanes 3−5, cells treated with 10, 100, and 1000 nM of analogues; and Lane 6, 100 nM **1′**. Lanes 3, 4, 5, and 6 of 3b and 3d depict discrete DNA fragments, which are characteristic morphological marker of apoptosis.

evident DNA fragmentation in the loosely adherent cells at all the concentrations studied. (Figure 3). Similar apoptotic effects of **1** (100 nM) were previously reported[28] in human umbilical vein endothelial cells.

DAPI Staining for Visualization of Apoptosis. CA-4 (**1′**) and analogues **11** and **42** were found to cause apoptosis as seen by staining micronuclei with DAPI (4′-6-diamidino-2-phenylindole) in loosely adherent ECV304 cells, in a dose-dependent manner at concentrations ranging from 10 nM to 1 μM (Figure 4).

Multiple damaged, fragmented, and unevenly displayed micronuclei were observed in a significant number of cells. Hence, the cyclopentenone analogues **11** and **42** may possess similar apoptotic effects in endothelial cells that were comparable to that observed for **1′**.

Physical Properties. Based on better inhibition of tubulin, **42** was selected for studying physical properties such as

Table 7. Pharmacokinetic Parameters of **1′** and **42**

cmpd	half-life, $t_{1/2}$ (h)	maximum plasma concn., C_{max} (μg/mL)	area under curve, AUC (h·μg/mL)	volume of distribution, V_d (mL)	clearance, Cl (mL/h)	mean residence time, MRT (h)
1′	0.19 ± 0.08	3.83 ± 1.0	0.92 ± 0.2	80.82 ± 47.8	291.36 ± 65.5	0.15 ± 0.04
42	0.47 ± 0.1	7.05 ± 1.2	3.84 ± 0.3	46.56 ± 14.05	68.94 ± 3.2	0.53 ± 0.09

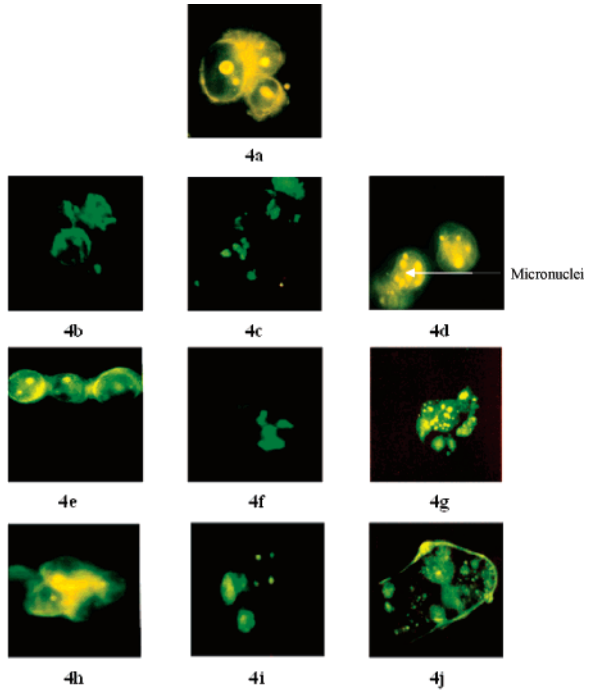


Figure 4. Visualization of micronuclei in ECV 304 cells by DAPI staining. Untreated cells were taken as control (4a). Cells were treated with **1′** (4b, 4c, 4d), **11** (4e, 4f, 4g), and **42** (4h, 4i, 4j) at the concentrations of 10 nM, 100 nM, and 1 μM, respectively.

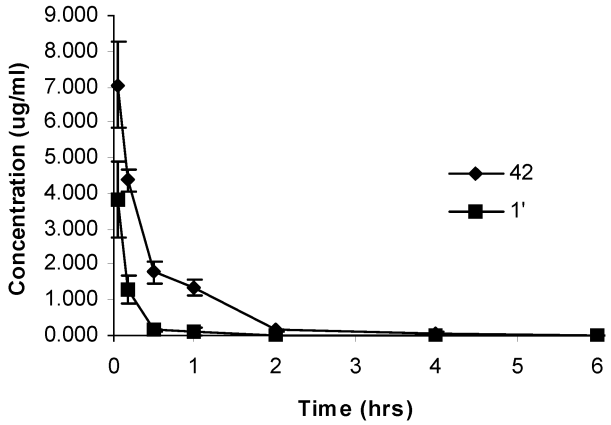


Figure 5. Plasma concentration profile of **1′** and **42** upon intravenous administration in Swiss albino mice (each point represents mean ± SD, $n = 3$).

solubility and metabolic stability. The data is summarized in Table 6. The aqueous solubility of **42** and **1′** was 359.6 μM and 122.8 μM, respectively, in phosphate buffer at pH 7.4, as estimated using the DMSO precipitation method. In terms of stability, **42** appeared to have better metabolic stability (96.4% of parent compound remaining) as compared to **1′** (84.8% of parent compound remaining) after incubation for 60 min at 37 °C with human liver microsomes. These results show that cyclopentenone analogue **42** has better aqueous solubility and metabolic stability as compared to **1′**.

Pharmacokinetics. The plasma concentration curve of compounds **1′** and **42** in mice is shown in Figure 5. Pharmacokinetic parameters are given in Table 7. The compounds were rapidly eliminated in mice following i.v. administration with a short terminal half-life of 0.19 h (**1′**) and 0.47 h (**42**). The C_{max} coincides with the first sample collection point at 3 min and is low for both **42** (7.05 μg/mL) and **1′** (3.83 μg/mL). The AUC, volume of distribution, clearance, and mean residence time

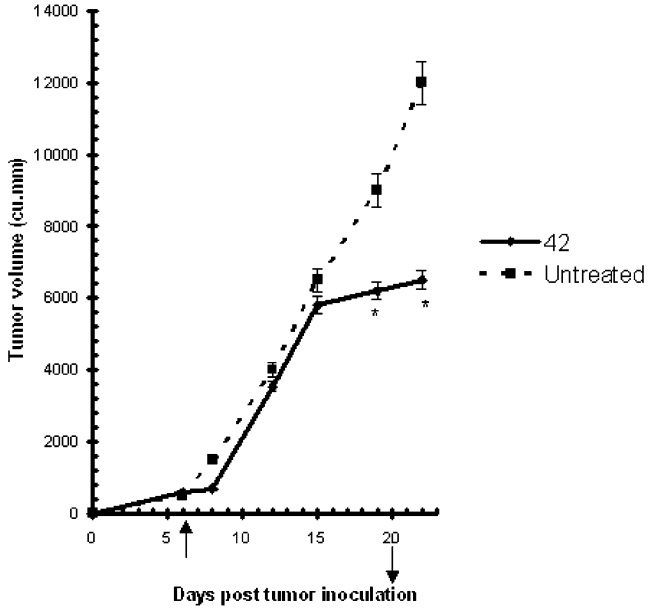


Figure 6. In vivo antitumor activity of **42** in human colon (PTC) xenograft model. Treatment was initiated on day 6 (tumor volume, approx. 500 mm^3). Values represent mean ± SD, $n = 3$. (↑) Represents initiation of treatment, (↓) represents withdrawal of treatment, and (*) represents $p < 0.05$.

values for **42** were 3.84 h·μg/mL, 46.56 mL, 68.94 mL/h, and 0.53 h, respectively, and for **1′** were 0.92 h·μg/mL, 80.82 mL, 291.36 mL/h, and 0.15 h, respectively. The pharmacokinetic parameters in mice show that **42** stays in plasma for a longer time, as shown by a marginally higher half-life and mean residence time and provides a higher drug exposure as compared to **1′**, as shown by a higher C_{max} and AUC and a lower clearance from plasma.

In Vivo Activity in Xenograft Model. Previous studies have shown that **1** and **1′** were inactive in colon SW1222,[20] colon 26,[21] and HT-29[22] tumor xenografts in nude mice, suggesting that these compounds may not be endowed with anticancer activity specific to colon cancers. The promising anticancer activities of our analogues in colon cancer cell lines prompted us to explore their potential in vivo. Based on the in vitro cytotoxicity ($IC_{50} = 271$ ng/mL) of **42** in primary tumor colon cells (PTC[a]) obtained from patients with advanced metastatic colon cancer, we tested the in vivo antitumor activity of **42** in PTC xenografted in nude mice.

Different treatment regimes have been reported for **1** and **1′**, varying from single dose,[21] daily doses,[23] once in two days,[24] and weekly.[25] It has also been suggested that continuous dosing with **1** gives better response compared to single dose, probably by preventing revascularization and repopulation of the tumor that is initially necrosed by drug treatment.[23] Based on the above studies, we have determined the antitumor activity of **42** at the maximum tolerated dose of 20 mg/kg by daily administration for 14 days. Figure 6 shows the tumor kinetics up to day 22 in the treated and untreated animals. Daily treatment with **42** by an intravenous route induced tumor regression in the xenografts leading to significantly decreased tumor volume ($P < 0.05$) on days 19 and 22 and *T*/*C*% of 45.8% on day 22 post tumor inoculation as compared to untreated controls.

Conclusion

Microtubules are the key components of cytoskeleton and are acted upon by a large number of chemically diverse

[a] Abbreviation: PTC, primary tumor colon cells.

compounds, many of which are derived from natural products that inhibit the cell proliferation in mitosis.[2] Hence, microtubules are an attractive target for the treatment of cancer.[1] Combretastatin A-4 (**1′**), a natural product isolated from *Combretum caffrum* has potent anticancer and antivascular properties. It destabilizes microtubules and disrupts rapidly the vascular network of tumors leading to secondary tumor cell death. The two main problems associated with **1′** are the ready isomerization of the *cis* double bond to its inactive *trans* form and low water solubility.[9,10] To combat some of these problems, we have reported here the development of successful protocols to synthesize 2,3-diaryl (substituted)-4 or 5-hydroxy-cyclopent-2-en-1-ones, which provided *cis*-restricted combretastatin analogues. A common intermediate **3** was synthesized starting from bench chemicals in three steps. Heck reaction using appropriate aryl halide on this intermediate accomplished the synthesis of 4-hydroxy analogues, whereas 1,2-addition of aryl lithium or the corresponding Grignard reagent followed by double rearrangement in single step employing PDC resulted into the 5-hydroxy analogues. The protocols efficiently produced 56 new chemical entities, which were assessed for cytotoxicity and other biological parameters such as antitubulin and apoptotic properties. Rigidifying **1′** with the hydroxycyclopentenone system did not alter the reported SAR; on the contrary, it was in full agreement and exhibited enhanced broad-spectrum cytotoxicity. In vitro cytotoxicity data prompted us to select two promising analogues **11** and **42** belonging to 2,3-diaryl-5-hydroxy-cyclopent-2-en-1-one series that were highly cytotoxic ($IC_{50} < 1$ μg/mL) in a panel of human cancer cell lines comprising of HT29 (colon), KB (oral), L132 (lung), MiaPaCa-2 (pancreas), Hep 2 (larynx), PA1 (ovary), and endothelial cell line ECV304.

Both **11** and **42** led to cell cycle arrest in proliferating endothelial cells. At the concentration of 1 μM, the analogues displayed better activity than the parent compound **1′**. The analogues also induced apoptosis in ECV304 cells as shown by DNA fragmentation and DAPI staining of micronuclei of proliferating ECV304 cells, thereby indicating that the analogues may have similar apoptotic activity as that of **1′**.

When assessed for their ability to inhibit tubulin polymerization, there was a marked difference between **11** and **42** for inhibition of tubulin polymerization. The compound **42** was evidently a more potent antitubulin than **11**, as evident from its low IC_{50} value of 1.75 μM. Also the antitubulin activity of **42** was found to be comparable with **1′**, which may indicate a similarity in the mechanism of action with combretastatin A-4. Hence, **42** was selected for further evaluation of solubility, stability, pharmacokinetics, and in vivo efficacy studies.

Previous studies had indicated that **1′** and **1** do not cause significant tumor regression of human colon tumor xenografts in mice,[20−22] while **42** displayed good cytotoxicity in colon cancer cells. This prompted us to evaluate the anticancer potential of selected analogue **42** in colon carcinoma. PTC of human colon adenocarcinoma implanted tumor xenografts in nude mice were employed. The compound **42** caused significant ($p < 0.05$) tumor regression in colon tumors and delayed tumor growth as compared to untreated controls. The antitumor activity further confirmed the potency seen in the in vitro cytotoxicity assay. It may be concluded that *cis*-restricted analogues of **1′**, particularly **42**, possess promising anticancer activity combined with good aqueous solubility, stability, and acceptable pharmacokinetic profile. This compound is now undergoing further lead optimization in our laboratories for development as an anticancer agent.

Experimental Section

Biological Assays. MTT, PI, DAPI, guanosine 5′-triphosphate (GTP), and PIPES were obtained from Sigma, U.S.A. DMEM (Dulbecco's modified eagles medium) and fetal bovine serum (FBS) were procured from Gibco BRL, U.S.A. DMSO was from Merck, India, and antibiotic solution (containing penicillin and streptomycin) was obtained from Hyclone, U.S.A. Purified bovine brain tubulin (>99% pure) was procured from Cytoskeleton and NP-40 was purchased from Calbiochem. Combretastatin A-4 was synthesized at the National Chemical Laboratory, Pune. Human cancer cell lines HT29 (colon), DU145 (prostate), KB (oral), L132 (lung), MiaPaCa-2 (pancreas), Hep2 (larynx), PA-1 (human ovary), and 293 (kidney) were procured from NCCS, Pune, India. The ECV304 (endothelial) cell line was generously gifted by Dr. Takahashi (Tokyo University, Tokyo, Japan). Cell lines were grown in DMEM, containing L-glutamine and 25 mM HEPES, and supplemented with 10% FBS, penicillin (100 units/mL), streptomycin (100 μg/mL), and amphotericin B (0.25 mg/mL) at 37 °C, 5% CO_2, and 100% humidity.

Cytotoxicity Assay. Various concentrations of cyclopentenone analogues were tested for in vitro cytotoxic activity on human cancer cell lines representing colon (HT29), prostate (DU145), oral (KB), lung (L132), pancreas (MiaPaCa-2), larynx (Hep2), ovary (PA1), kidney (293), and normal endothelial cells (ECV304). Cytotoxicity was measured by MTT assay, which is based on the principle of uptake of MTT by the metabolically active cells, where it is metabolized by active mitochondria into a blue-colored formazan product that is read spectrophotometrically.[26] Briefly, tumor cells were seeded (5000−10 000 cells/well) in 96-well culture plates and incubated at 37 °C in a CO_2 incubator with various concentrations of cyclopentenone analogues ranging from 1 to 100 μg/mL, with relevant controls in triplicate wells. After 72 h, the assay was terminated by the addition of 25 μL of MTT solution (5 mg/mL) in each well. Percentage cytotoxicity was calculated as given below. The IC_{50} values were determined by nonlinear regression using Prism software v 4.01.

$$\text{percentage cytotoxicity} = 100 \times [1 - (X/R_1)]$$

where X = absorbance of treated sample at 540 nm and R_1 = absorbance of control sample at 540 nm.

In Vitro Tubulin Polymerization Assay. The tubulin assembly reaction was carried out spectrophotometrically at 37 °C in 1 mL of buffer containing 80 mM PIPES (pH 6.9), 0.5 mM EGTA, 1.0 mM GTP, 2 mM $MgCl_2$, and 20% glycerol by the procedure modified from the method of Sampath et al.[27] Highly purified bovine brain tubulin was used at the concentration of 1 mg/mL in the presence or absence of **1′** and the cyclopentenone analogues **11** and **42** were used at different concentrations. The compounds were dissolved in 100% DMSO, and the final concentration of DMSO was kept less than 0.4%. Tubulin polymerization was followed by measuring the increase in absorbance of the solution at 340 nm every 60 s. The amount of tubulin polymerized is directly proportional to the AUC, which was calculated using the prism software v 4.01. AUC for the control was set to 100% polymerization, and the concentration of the compound that inhibited tubulin polymerization by 50% (IC_{50}) was determined (Table 5).

Analysis of Cell Cycle by Flow Cytometry. Cell cycle of human endothelial cancer cells (ECV304) treated with drugs was analyzed by flow cytometry.[28] Cells were plated at a density of 1.5×10^6 cells in 90 mm tissue culture dishes in DMEM medium supplemented with 10% FCS and incubated overnight. Subsequently, the medium was replaced with fresh medium and cells were treated with **1′** and cyclopentenone analogues **11** and **42** at the concentration of 1 μM for 24 h. Cells were trypsinized, washed in phosphate buffered saline (PBS), and fixed with ice cold 70% ethanol. Fixed samples were extensively washed in wash buffer (3.5 mM trisodium citrate, 0.1% NP-40, 0.5 mM tris chloride, and 1.5 mM spermine tetrahydrochloride pH 7.6). The pellet was resuspended in 250 μL of solution A (30 μg/mL trypsin prepared in wash buffer), mixed

by tapping, and incubated at RT for 10−15 min. After 15 min, 200 μL of solution B (500 μg/mL trypsin inhibitor and 100 μg/mL RNase prepared in wash buffer) was added to the cell suspension and further incubated for 15 min. Subsequently, 200 μL of ice-cold solution C (420 μg/mL PI, 3.33 mM spermine tetrahydrochloride) was added to the suspension and incubated in the dark at 4−10 °C for 1 h. Stained cells were acquired on a FACS calibur flow cytometer (Becton Dickinson). The low-level gate was set at 10% of the value of the G1 peak and the percentages of cells within the G1 and G2/M phases of the cell cycle were determined by analysis with Modfit software (Becton Dickinson; Figure 2).

Analysis of Apoptosis by DNA Fragmentation. DNA fragmentation was taken as an index of apoptosis.[29] Human endothelial cancer cells (ECV304) were plated at a density of 2×10^6 cells in 90 mm tissue culture dishes in DMEM medium supplemented with 10% FCS. The cells were incubated for 5−6 h to allow complete reattachment to the dishes. Subsequently, the medium was changed to fresh medium supplemented with 10% FCS and cells were treated with analogues **11** and **42** at concentrations ranging from 10 nM to 1 μM for 24 h. The compound **1′** (100 nM) was used as a positive control for the experiment. After the treatment, both the strongly adherent and the loosely adherent cells were harvested, pelleted at 2500 rpm, and lysed in buffer containing 1 mM Tris-Cl, 20 mM EDTA (pH 8), and 1% NP-40. The fragmented DNA was separated from intact chromatin using 5 M NaCl and isopropanol. The mixture was centrifuged at 12 000 rpm for 20 min to obtain the DNA pellet. The pellet was washed with 70% ethanol and analyzed by gel electrophoresis using 1.75% agarose gel (Figure 3).

Visualization of Apoptotic Bodies by DAPI Staining. Apoptotic bodies were visualized by staining the nuclei with DAPI.[28] ECV 304 cells were plated at a density of 1×10^6 cells per well in a six-well plate in DMEM medium and incubated in a CO_2 incubator at 37 °C for 6 h. Cells were treated in the presence or absence of **1′** and cyclopentenone analogues **11** and **42** at concentrations ranging from 10 nM to 1 μM for 24 h. The compound **1′** and analogues were dissolved in 100% DMSO, and the final concentration of DMSO was kept less than 0.1%. The loosely adherent cells were harvested, washed with PBS, and fixed in 70% ice-cold ethanol for 2 h. DAPI stock of 5 mg/mL was prepared in water and stored at −20 °C. The fixed cells were stained with 500 μL of 10 μg/mL DAPI prepared in methanol and incubated at 37 °C for 20 min. Subsequently, the cells were washed and resuspended in 50 μL of PBS. A 10 μL amount of suspension was taken on a slide and observed under the NIKON fluorescence microscope (emission wavelength of 344 nm and excitation wavelength of 450 nm) for visualization of multiple damaged, fragmented, and unevenly displayed micronuclei (Figure 4).

Physical Properties. The physical properties were determined by following standard methods and measured using a previously standardized HPLC method (as described in Pharmacokinetics in the Experimental Section). Briefly, solubility of the analogues was determined using the DMSO precipitation method.[30] Test compounds were dissolved in DMSO and kept with shaking for 1.5 h in phosphate buffer (pH 7.4). The soluble portion was filtered and measured by HPLC. The metabolic stability of the analogues was determined as described previously[31] by calculating the percentage of test substance remaining unmetabolized using HPLC analysis, following incubation for 60 min in pooled human liver microsomes.

Animal Studies. All animal experiments were carried out as per the guidelines of the Institutional Animal Ethics Committee (IAEC), Dabur Research Foundation.

Tumor Xenograft Assay. Human tumor xenograft assay was adapted from previously published methods.[32,33] Balb/C athymic nude mice, age 6−8 weeks, weighing around 20 g were obtained from National Centre for Laboratory Animals Sciences (NCLAS, NIN, Hyderabad, India) and maintained in sterile isolators at the small animal facility, Dabur Research Foundation. Briefly, PTC[34] (colon) tumor xenografts were established in Balb/c athymic mice by subcutaneous inoculation of a single cell suspension of PTC cells (15×10^6 cells/100 μL). The tumor bearing animals were divided into two groups of three animals each (treated and control). Treatment with compound **42** was initiated when the average tumor volumes, as measured using a vernier caliper, were around 500 mm^3. The test compound was formulated using a cosolvent formulation, diluted in 5% dextrose, and administered intravenously to the treatment group of tumor-bearing animals at a dose of 20 mg/kg once daily for a period of 14 days. Control animals received an equivalent volume of vehicle alone for the same period. The antitumor activity of the compound was monitored by measuring tumor volumes every fourth day using the formula $W \times W \times L \times 0.4$ (W = smaller diameter, L = larger diameter). Tumor regression was calculated using the formula $(1 - \text{tumor volume}_{(\text{treated})}/\text{tumor volume}_{(\text{control})}) \times 100$. Additionally, tumor volumes of different groups were compared with the multiple comparison procedure using LSMEANS, with ADJUST = Dunnett in PROC GLM of SAS 9.1.3. A value of $p < 0.05$ was considered significant.

Pharmacokinetics. Compounds were formulated using a cosolvent formulation, diluted in 5% dextrose, and administered intravenously in Swiss albino mice, age 6−8 weeks, weighing around 25 g, and maintained at the small animal facility, Dabur Research Foundation, India. After the dose administration, blood samples (200 μL) were collected by orbital bleeding under mild ether anesthesia at time points of 3, 10, 30 min, 1, 2, 4, 6, 8, and 24 h in EDTA-containing tubes. Not more than five bleeds were collected from a single mouse. The plasma was separated, extracted using organic solvents, and centrifuged, and the supernatant was evaporated to dryness. It was reconstituted with 200 μL in diluent (45:55 acetonitrile/water) and analyzed using HPLC (L2010, Shimadzu, Japan) to determine plasma concentrations. The mobile phase consisted of 0.005 M phosphate buffer (55%) and acetonitrile (45%). The HPLC column used was C_{18}, 250 × 4.6 mm, 5 μm, YMC-Pack ODS-A. The flow rate was adjusted to 1 mL/min. The retention time for **1′** and **42** was 15.4 and 7.5 min, respectively. The concentration of the test compound was determined by calculating the ratio of its peak to the internal standard peak and then comparing the ratio to a simultaneously performed standard curve. The limit of quantitation for both **1′** and **42** was 0.15 μg/mL (75 μL injection). The pharmacokinetic parameters were determined with WinNonlin v 5.0.1 software by performing noncompartmental analysis.

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Supporting Information Available: Synthetic procedures, characterization (mp, IR, and ^1H and ^{13}C NMR data) and elemental analyses of all new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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