Design, Synthesis, Characterization, and *In Vitro* Evaluation of Isatin-Pomalidomide Hybrids for Cytotoxicity against Multiple Myeloma Cell Lines

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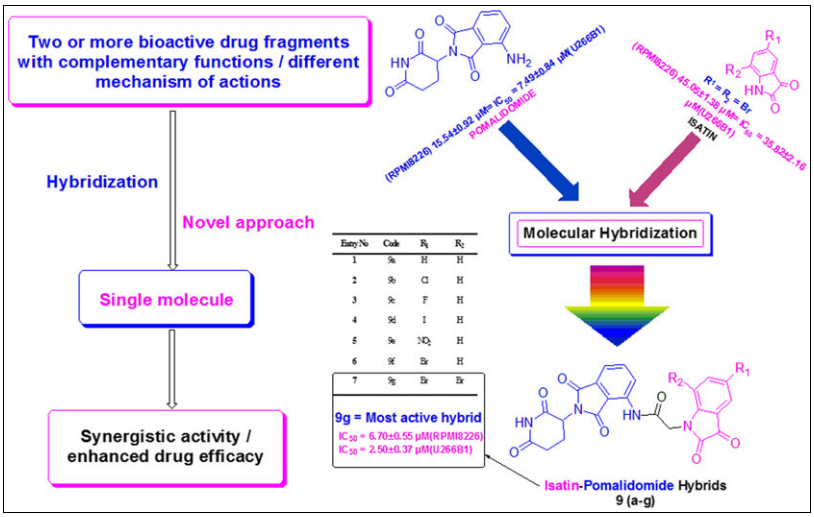


Inspired by the concept of molecular hybridization, a series of new isatin-pomalidomide hybrids (**9a**–**9g**) were designed, synthesized, characterized, and evaluated for *in vitro* cytotoxic activity against U266B1 and RPMI 8226 multiple myeloma cell lines. Sandmeyer methodology and N-halomethylketo alkylation reaction are the two important reactions involved in the synthesis of isatin-pomalidomide hybrids (**9a**–**9g**). All the synthesized compounds (**3a**–**3d**, **4**, **5**, **6**, and **9a**–**9g**) were characterized by using IR, mass, ^{1}H-NMR, and ^{13}C-NMR spectral techniques. The efficacy of all the synthesized compounds was tested against the aforementioned cell lines by employing MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) standard protocols while using pomalidomide as a standard. The test concentrations used in the MTT assay were 1, 10, 20, 30, and 40 μM, and the period of incubation was 24 h. All the synthesized compounds were found to have moderate to greater cytotoxic activity against the aforementioned cell lines. Among them, synthesized hybrids **9f** (IC_{50}, U266B1 = 5.15 ± 0.72 μM, RPMI8226 = 11.66 ± 0.79 μM) and **9g** (IC_{50}, U266B1 = 2.50 ± 0.37 μM, RPMI8226 = 6.70 ± 0.55 μM) displayed better cytotoxic activity against both the cell lines used in the present study.

INTRODUCTION

Design of new hybrid molecules by the covalent conjugation of two or more bioactive fragments with complementary or different mechanism of actions into a single new hybrid moiety is called molecular hybridization [1]. It is one of the simple and effective ways of drug discovery with high incidence of success rate, time saving, and economic as opposed to the conventional process of drug discovery. Numerous literature reports suggest that molecular hybrids are known to possess additive or synergistic biological activities unlike their parent moieties [2,3]. Some known examples of such hybrid molecules with enhanced biological activities are presented and shown in Table 1 and Figure 1.

The concept underlying this approach is minimizing the dose of each individual drug to facilitate limited drug exposure, reducing dose-dependent toxicity, and decreasing the emergence of multidrug resistance [9]. Estramustine and nitric oxide-amino salicylic acid hybrids are some of the best examples for the marketed and under clinical trial products of molecular hybridization.

The isatin (1*H*-indole-2,3-dione) is a molecule with immense and diverse biological profile, offering wide array of applications in the field of medicinal chemistry with ease of its synthesis. Some of the reported activities of isatin in the literature are antibacterial [10], antifungal

Table 1

Cytotoxic activities of the recently reported isatin-based hybrids.

S. no.	Name of the hybrids	Cytotoxic activity against cancer cell lines	Ref.
1.	Isatin-benzothiazole hybrids	MDA-MB231, MDA-MB468, and MCF7; also against two non-cancer cell lines, 184B5 and MCF10A.	[2]
2.	Isatin-thiazolidinone hybrids	MDA-MB239 and MDA-MB435 (breast cancer cell lines variations)	[4]
3.	Isatin-4-piperazinylquinoline hybrids	MDA-MB468 (a PTEN defective, p53 positive, EGFR positive breast adenocar-cinoma cell line) and MCF7 (p53+/−, invasive ductal breast carcinoma) and two non-cancer breast epithelial cell lines (184B5 and MCF10A)	[5]
4.	Curcumin-thalidomide hybrids	Human multiple myeloma MM1S, RPMI8226, U266 cells, and human lung cancer A549 cells.	[6]
5.	Isatin-quinazoline hybrids	Liver HepG2, breast MCF-7, and colon HT-29 cancer cell lines, more inhibition against HepG2 cell lines	[7]
6.	Isatin-pyridine hybrids	HepG2 hepatocellular carcinoma, A549 lung cancer, and MCF-7 breast cancer cell lines	[8]

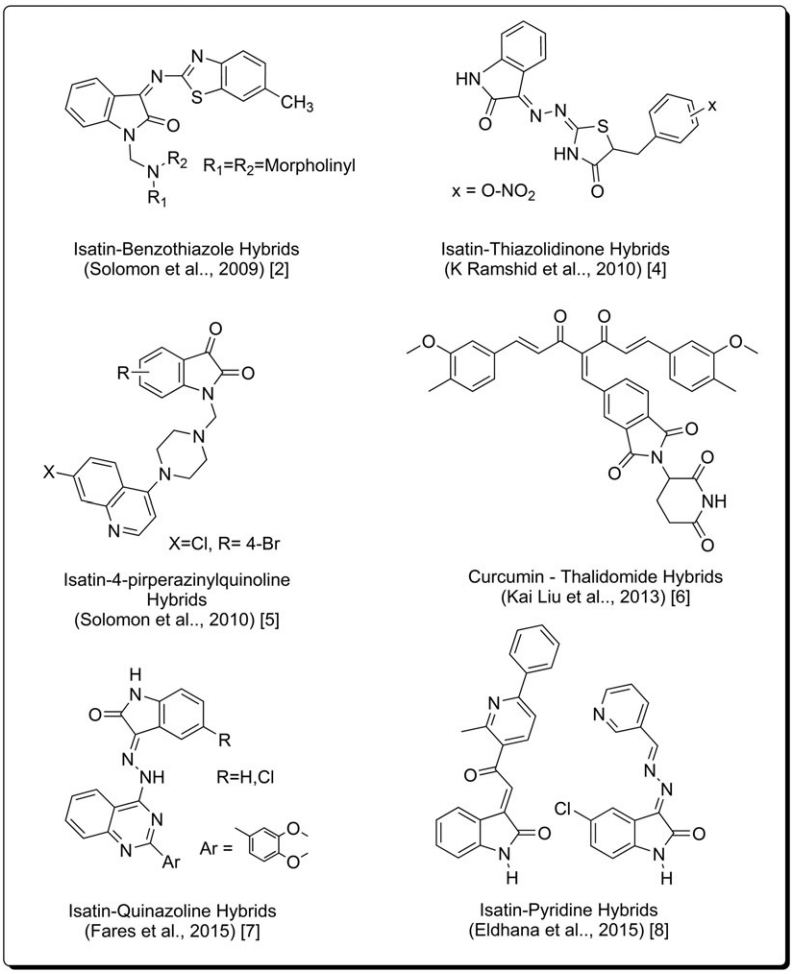


Figure 1. Few examples for the recently reported isatin-based hybrids.

[11], antiviral [12], anti-HIV [13,14], anti-mycobacterial [15], anticancer [16–20], anti-inflammatory [21], anticonvulsant [22], anti-TB [23–25], antimalarial, and anti-plasmodial [26–28] activities.

Gowda *et al*. [29] reported that isatin does possess anti-multiple myeloma activity by inhibiting one of the key enzymes of Bruton's tyrosine kinase involved in the pathogenesis of multiple myeloma. Thus, by combining

these two pharmacophore units (isatin-pomalidomide) with complimentary functions into single hybrid molecule, there is a possibility of enhancing the efficacy of envisaged hybrids.

Multiple myeloma is one of the hematological cancers manifested by the proliferation of malignant monoclonal B cells in the bone marrow. The other symptoms include anemia, renal insufficiency, hypercalcemia, and immunodeficiency [30]. The American Cancer Society's estimates for multiple myeloma in the USA are 30,770 diagnosed new cases (16,400 in men and 14,370 in women) and 12,770 deaths are expected to occur (6,830 in men and 5,940 in women) in the year 2018 [31]. Thalidomide, lenalidomide, pomalidomide, bortezomib, and its combination with dexamethasone are some of the important drugs used in the treatment of multiple myeloma [32]. Pomalidomide is a derivative of thalidomide being approved in the USA and Europe for the treatment of relapsed and refractory multiple myeloma in adults. Pomalidomide is available in the market under the brand name Pomalyst as capsule in the USA and as Imnovid in Europe [32].

Pomalidomide is chemically described as 4-amino-2-(2,6-dioxopiperodin-3-yl)-isoindoles-1,3-dione, and it belongs to the class of phthalimide derivatives such as thalidomide, lenalidomide, and pomalidomide whose structures are shown in Figure 2.

Pomalidomide is found to be effective than its precursors thalidomide and lenalidomide in the treatment of multiple myeloma at an oral dose of 2–15 mg/day with the inclusion of oxo and amino functional groups from thalidomide and lenalidomide, respectively [33]. Despite of much progress being made in the development of novel anti-myeloma drugs, none of them could counter effectively the emergence of more number of side effects, drug resistance, and eventual relapse in the treatment of multiple myeloma.

The present study attempts to address the aforementioned problem by reducing the dose of pomalidomide and replacing its part with the biologically safe moiety and without getting compromised on the anti-multiple myeloma activity against the multiple myeloma cell lines.

Research investigations on isatin nucleus underscored the importance of alkyl substitution with a carbon chain length of 2–3 at N-1 position of isatin nucleus as it is essential for the elicitation of maximum anticancer activity and claims that N-1 substitution on isatin favors anticancer/cytotoxic activity [34,35]. Further, substitution of electron-withdrawing groups at the 5th position of N-alkyl-substituted isatin is associated with increased biological activity [19,20].

Based on these observations, we designed the synthesis of isatin-pomalidomide hybrids (**9a**–**9g**) by using molecular hybridization technique [1]. According to this approach, a series of new isatin-pomalidomide hybrid molecules were engineered by the use of two different bioactive drug fragments, pomalidomide and isatin (1*H*-indole-2,3-dione), and the rationality behind the design of isatin-pomalidomide hybrids (**9a**–**9g**) is illustrated in Figure 3.

The focus of the present investigation is on the design, synthesis, and characterization of series of novel compounds obtained by hybridizing pomalidomide with bioactive natural product scaffold, isatin. It also involves the exploration of isatin-pomalidomide hybrids potential as effective anticancer agents against RPMI 8226 and U266B1 multiple myeloma cell lines. Thus, we here in report the unprecedented *in vitro* cytotoxic activities of pomalidomide and its hybrids on multiple myeloma cell lines.

RESULTS AND DISCUSSION

In the present study, isatin derivatives (**3a**, **3b**, **3c**, **3d**, **4**, **5**, and **6**) were synthesized *via* Sandmeyer methodology initially, and later, these isatin derivatives were conjugated with pomalidomide covalently through N-halo methyl keto alkylation reaction as shown in Schemes 1 and 2. The physical data of all the synthesized compounds are presented in the Experimental Methods section. A total of 14 compounds have been synthesized, out of which seven derivatives are unconjugated isatins (**3a**, **3b**, **3c**, **3d**, **4**, **5**, and **6**) and the other seven derivatives are pomalidomide conjugated isatin hybrids (**9a**–**9g**).

Molecular hybridization is one of the simple and easiest methods of the drug design to bring the best features from their parent moieties to its corresponding progeny. The main aim of the present study is to examine the role of covalent conjugation and combination on the cytotoxic

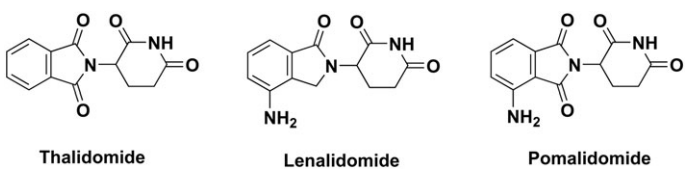


Figure 2. Chemical structures of thalidomide derivatives.

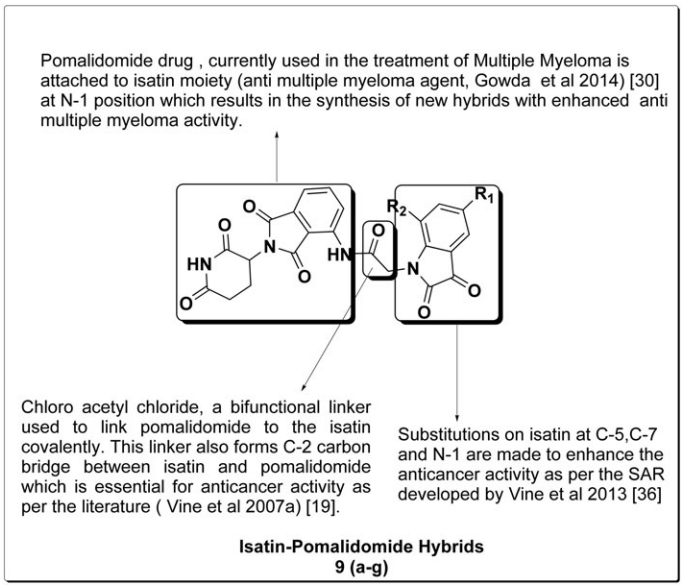
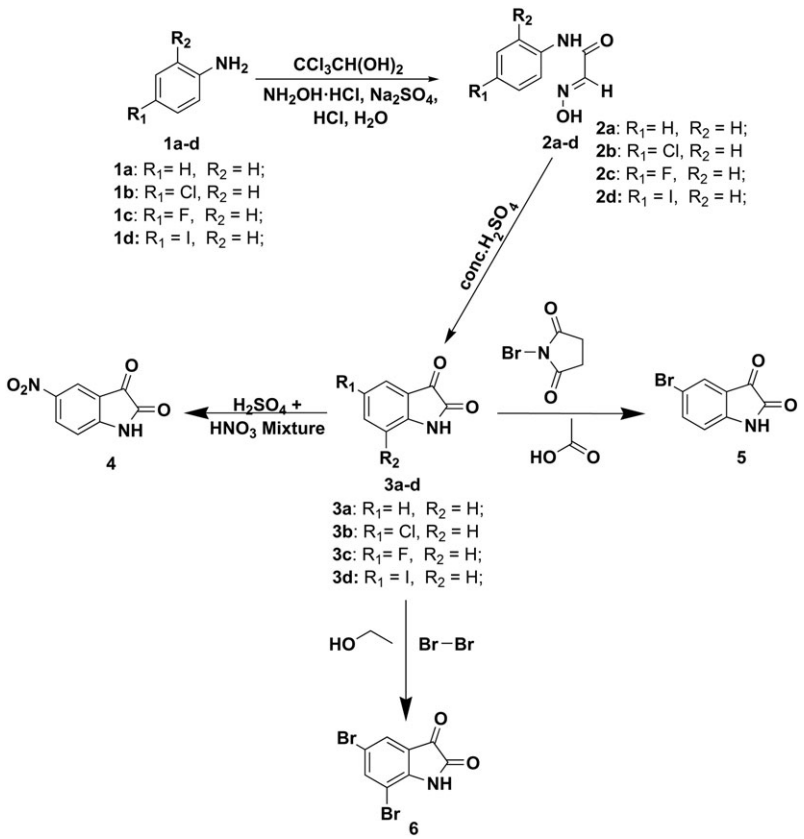


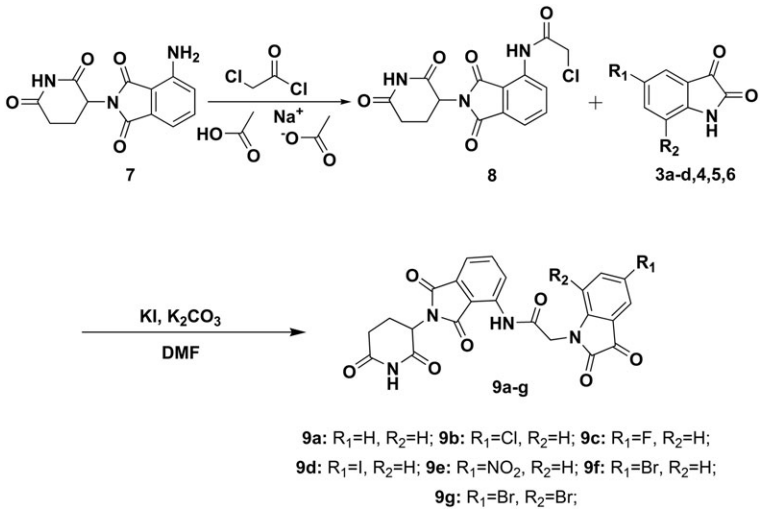
Figure 3. Rationale behind the design of isatin-pomalidomide hybrids (**9a**–**9g**).

Scheme 1. Synthesis of isatin and isatin derivatives (**3a**–**3d**, **4**, **5**, and **6**) from p-aniline (**1a**–**1d**) and chloral hydrate.



activities of the isatin derivatives (**3a**, **3b**, **3c**, **3d**, **4**, **5**, and **6**) as well as their corresponding hybrids with pomalidomide (**9a**–**9g**) against U266B1 and RPMI 8226 multiple myeloma cell lines. In order to highlight the role of covalent conjugation and combination, their cytotoxic activities have been evaluated separately as unconjugated moieties (**3a**, **3b**, **3c**, **3d**, **4**, **5**, and **6**) and conjugated moieties (**9a**–**9g**) against U266B1 and RPMI 8226

Scheme 2. The synthetic pathway of isatin-pomalidomide hybrids (**9a**–**9g**).



multiple myeloma cell lines, which are presented in Table 2. In addition to this, the hybrid (**9g**) with the best IC_{50} value was selected, and its effect on the cytotoxicity was evaluated as a mixture (1:1 molar ratio) so as to understand the efficacy of the covalent conjugation over the physical combination.

Table 2

The half maximal effective concentration (IC_{50} in μM) of the isatin and its derivatives (**3a**–**3d**, **4**, **5**, and **6**) and isatin-pomalidomide hybrids (**9a**–**9g**) against U266B1 and RPMI 8226 multiple myeloma cell lines.

Entry no.	Code	IC_{50} μM[a,b]	
		U266B1	RPMI8226
1	**3a**	35.82 ± 2.16***	45.05 ± 1.38**
2	**3b**	31.21 ± 1.23***	38.13 ± 1.22**
3	**3c**	25.84 ± 1.72***	35.41 ± 1.03**
4	**3d**	24.44 ± 1.76***	31.56 ± 1.52**
5	**4**	22.42 ± 1.24***	33.61 ± 0.94**
6	**5**	21.35 ± 2.44***	30.71 ± 1.10**
7	**6**	15.37 ± 2.01***	24.09 ± 0.99**
8	**9a**	12.96 ± 1.25***	21.33 ± 1.49**
9	**9b**	10.89 ± 1.02*	18.57 ± 1.48*
10	**9c**	9.73 ± 1.17*	16.36 ± 0.76*
11	**9d**	9.39 ± 0.39*	15.68 ± 0.57*
12	**9e**	8.23 ± 0.78*	15.32 ± 0.90*
13	**9f**	5.15 ± 0.72*	11.66 ± 0.79**
14	**9g**	2.50 ± 0.37***	6.70 ± 0.55***
15	PM + DBIS (1:1)	6.12 ± 1.05**	11.57 ± 0.42**
16	Pomalidomide (PM, Std)	7.49 ± 0.84	15.54 ± 0.92

IC_{50} was calculated from dose–response curves. Statistical analysis was performed by applying two-way analysis of variance, nonparametric, followed by Dunnet's multiple comparisons test.
[a]Sigmoidal dose–response curves (variable slope) were generated using GraphPad Prism V. 6.00 (GraphPad Software Inc.).
[b]Values are the mean of triplicates of at least three independent experiments.
*Not significant.
**P-value < 0.05.
***P-value < 0.001 when compared with pomalidomide.

The IC_{50} values of unconjugated isatin derivatives (**3a**, **3b**, **3c**, **3d**, **4**, **5**, and **6**) and isatin-pomalidomide hybrids (**9a**–**9g**) were found to be in the range of 35.82 ± 2.16 μM to 2.50 ± 0.37 μM (U266B1) and 45.05 ± 1.38 μM to 6.70 ± 0.55 μM (RPMI 8226). This shows that the synthesized derivatives are sensitive towards U266B1 cells than RPMI 8226 cells. The cytotoxic assay of covalently conjugated isatin-pomalidomide hybrids (**9a**–**9g**) as well as unconjugated isatin derivatives (**3a**–**3d**, **4**, **5**, and **6**) indicated that isatin nucleus conjugation and combination with pomalidomide have enhanced the cytotoxic activity as observed from their IC_{50} values in general. However, the extent of cell growth inhibition varies from one conjugate to the other conjugate when compared with pomalidomide. As per the statistical analysis, it is understood that conjugation of unsubstituted isatin (**3a**) and 5-substituted isatins (**3b**, **3c**, **3d**, **4**, and **5**) with pomalidomide have less effect on the cytotoxicity against U266B1 and RPMI 8226 cell lines as evident from its corresponding IC_{50} values of the cytotoxic assay.

The IC_{50} data of the present study (from Figs. 4 and 5) revealed the similarity in the cytotoxic activities of the 5-substituted isatin-pomalidomide hybrids (**9b**, **9c**, **9d**, **9e**, and **9f**) and pomalidomide. But, there is significant difference ($^{**}P$-value < 0.001, when compared with pomalidomide) in the IC_{50} value of the **9g** hybrid/conjugate on both the cell lines U266B1 (2.50 ± 0.37 μM) and RPMI 8226 (6.70 ± 0.55 μM) when compared with pomalidomide IC_{50} value on both the cell lines U266B1 (7.49 ± 0.84 μM) and RPMI 8226 (15.54 ± 0.92 μM), suggesting that 5,7 substitutions on the isatin derivatives and its conjugation with

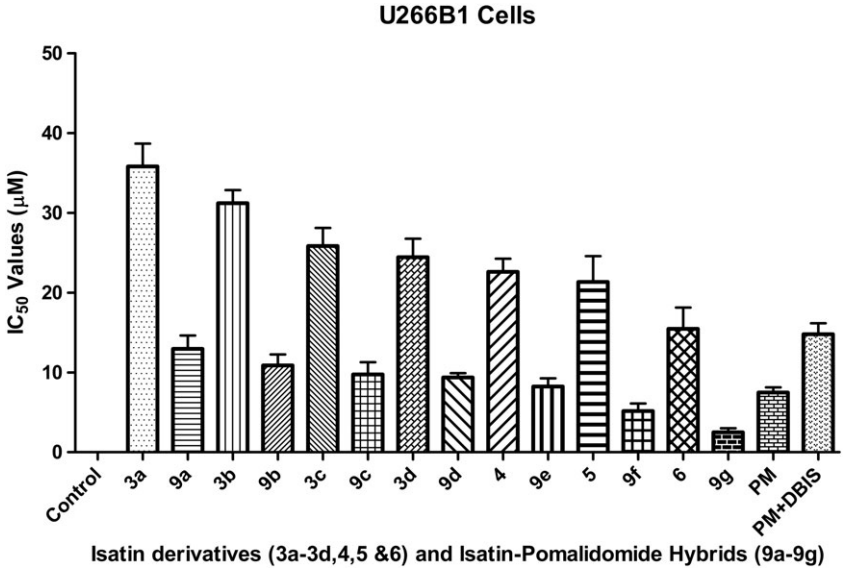


Figure 4. A comparative view of the IC_{50} values of isatin derivatives (**3a**–**3d**, **4**, **5**, and **6**) and its pomalidomide hybrids (**9a**–**9g**) against U266B1 multiple myeloma cell line.

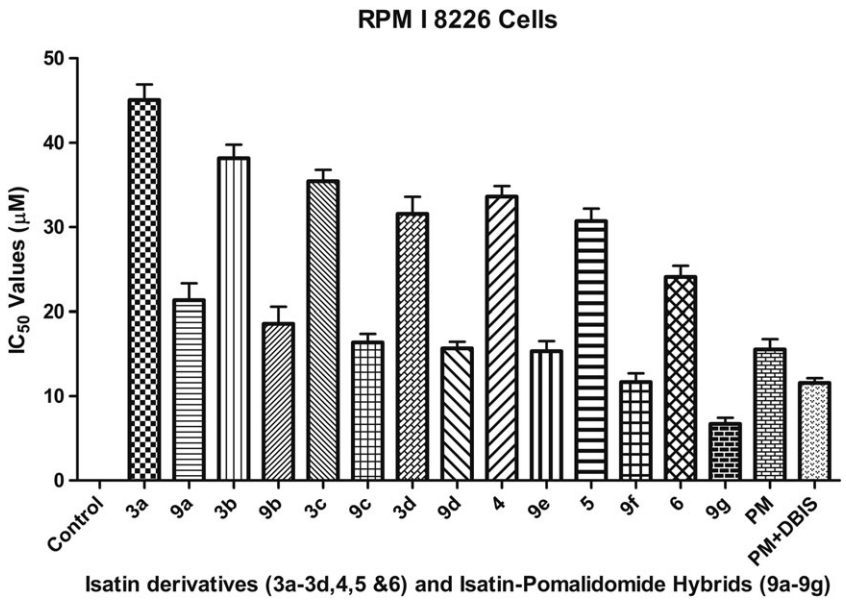


Figure 5. A comparative view of the IC_{50} values of isatin derivatives (**3a**–**3d**, **4**, **5**, and **6**) and its pomalidomide hybrids (**9a**–**9g**) against RPMI8226 multiple myeloma cell line.

pomalidomide have increased the cytotoxic activity on both the cell lines by significantly decreasing their corresponding IC_{50} values.

In addition to this, combined effects of pomalidomide (PM) and 5,7-di-bromoisatin (**6**) physical mixture (1:1) were also tested against the cell line used in the current study. The IC_{50} value of the physical mixture (PM and **6**) was compared with the IC_{50} value of its corresponding covalent conjugate (**9g**). The results obtained from their IC_{50} values demonstrated that covalent conjugation (**9g**) has superior cytotoxicity [2.50 ± 0.37 μM (U266B1); 6.70 ± 0.55 μM (RPMI8226)] than its physical mixture (PM + DBIS) [6.12 ± 1.05 μM (U266B1); 11.57 ± 0.42 μM (RPMI8226)].

As shown in Figures 4 and 5, the cytotoxic activity of unconjugated isatin derivatives has been increased after covalent conjugation with pomalidomide (**9a**–**9g**), which is evident from their IC_{50} values, being decreased to half of its value after covalent conjugation than its values prior to conjugation. This confirms that the conjugation

of two pharmacophores with complementary functions yields better activity than its original activity prior to covalent conjugation.

The obtained results showed excellent consistency with previously reported studies by Panga *et al*. [36], Modi *et al*. [34], and Vine *et al*. [19], where substitutions at 5th and 7th positions of isatin nucleus and conjugation at N-1 position conferred better cytotoxicity. Similarly, in the present study, the potent compounds **9g** and **9f** also have all the stated characteristics as mentioned in the previous studies.

CONCLUSION

In the present study, molecular hybridization technique was used to synthesize the isatin-pomalidomide hybrids (**9a**–**9g**) with enhanced anti-multiple myeloma activity than its parent compounds isatin derivatives (**3a**–**3d**, **4**, **5**, and **6**) and pomalidomide (PM). All the synthesized derivatives (14) that consists of unconjugated isatin derivatives and conjugated isatin derivatives with pomalidomide have been evaluated for their cytotoxic activity against U266B1 and RPMI 8226 multiple myeloma cell lines by using MTT assay. It is observed that among all the covalent hybrids of isatin and pomalidomide, pomalidomide conjugation with 5,7-di-bromoisatin derivative was found to be effective in modifying the cytotoxic activity of the pomalidomide as evident from its IC_{50} values. In addition to this, efforts were laid to prove the superiority of covalent conjugation over the physical combination by testing the most active compounds against the aforementioned cell lines used in the present study. The IC_{50} value obtained from this study showed that the covalent conjugation of 5,7-di-bromoisatin with pomalidomide has low IC_{50} value than that of its physical mixture [5,7-di-bromoisatin and pomalidomide (PM + DBIS)], concluding that covalent conjugated compound (**9g**) was most effective against the aforementioned two cell lines used in the present study. Thus, it can be deduced from the aforementioned study that the conjugation of isatin and pomalidomide was proved to be beneficial. However, advanced studies such as the stability of the hybrids in the systemic circulation and their mode of action must be explored in the near future to witness the real applications of molecular hybridization in drug discovery and delivery.

EXPERIMENTAL METHODS

General procedure for the preparation of compounds (3a–3d, 4, 5, and 6). Preparation of isatin and its derivatives (**3a**–**3d**, **4**, **5**, and **6**) was performed as per the already reported procedures [19,20,37–39].

Preparation of 2-chloro-*N*-(1,3-dioxo-2-(2,6-dioxopiperidin-3-yl)isoindolin-7-yl)-acetamide (8). Pomalidomide (**7**) (2.73 g, 0.01 moles) was dissolved in a mixture of 25 mL of glacial acetic acid and 25 mL of saturated solution of sodium acetate. To this, chloroacetyl chloride (1.5 mL, 0.013 moles) was added drop wise to avoid the vigorous reaction. After half an hour, the product was filtered with suction, washed several times with cold water, and then infrared dried. The product was crystallized from methanol [34].

Procedure for the preparation of pomalidomide (7) and 5,7-di-bromoisatin (6) physical mixture (PM + DBIS) in micromolar concentration. The test solutions (in μM) of pomalidomide (**7**) and 5,7-di-bromoisatin (**6**) physical mixture (PM + DBIS) were prepared in the ratio of 1:1 by using the following formula.

$$M1 \times V1 = M2 \times V2,$$

where M1 = molarity of 5,7-di-bromoisatin (**6**) [weight = mass/density; density = 2.2 g/cm^3]; V1 = volume of 5,7-di-bromoisatin (**6**); M2 = molarity of pomalidomide (**7**); and V2 = volume of pomalidomide (**7**) [weight = mass/density; density = 1.6 g/cm^3].

The aforementioned equation is valid when the densities of the compounds are equal. Hence, weights of the compounds were divided by the densities of pomalidomide (**7**) and 5,7-di-bromoisatin (**6**).

General procedure for the preparation of isatin-pomalidomide hybrids (9a–9g). Compounds **3a**–**3d**, **4**, **5**, and **6** (1 g) were taken up in 30 mL anhydrous DMF and ice cooled with stirring [34]. Solid K_2CO_3 (1 g, 0.0072 moles) was added in one portion, and the dark-colored suspension was raised to room temperature and stirred for a further 1 h. The appropriate amount of N-acylated pomalidomide (**8**) (1 g, 0.0028 moles) and KI (0.5 g, 0.006 moles) was added, and the reaction mixture was stirred at 80°C for 2–24 h, until the reaction was over, which was confirmed using TLC. The reaction mixture was poured into six times its volume of water, and then diluted hydrochloric acid was added until the pH value is between three and four stirred for a further 10 min. The crude final product was filtered with suction, washed several times with cold water, and then infrared dried. The product was crystallized from methanol. The yield, melting point, and spectral data of each compound are given as follows.

1H-indole-2, 3-dione (3a). Yield: 60%; M.P.: 193–195°C; IR (KBr, t cm^{-1}): 3195 (NH of isatin NH), 1730 (C═O of isatin), 1696 (─NH─CO); ^{1}H-NMR (300 MHz, δ ppm, DMSO-d_6): 6.95–8.03 (m, 4H, Ar─H), 10.20 (s, 1H, ─NH); CHN *Anal*. Calcd for $C_8H_5NO_2$: C (65.31), H (3.43), N (9.52). Found: C (65.11), H (3.20), N (9.71); MS *m/z* (+ve):148.1 (M^+).

5-Chloro-1H-indole-2,3-dione (3b). Yield: 68%; M.P.: 255–258°C; IR (KBr, t cm^{-1}): 3346 (NH of isatin), 1710 (C═O of isatin), 1683 (─NH─CO); ^{1}H-NMR (300 MHz, δ ppm, DMSO-d_6): 7.50–7.80 (m, 3H, Ar─H), 8.94 (s,

1H, —NH); CHN *Anal*. Calcd for $C_8H_4ClNO_2$: C (52.92), H (2.22), N (7.71). Found: C (52.88), H (1.96), N (7.82); MS *m/z* (+ve): 181.9 (M^+), 183.9 (M + 2).

5-Fluoro-1H-indole-2,3-dione (3c). Yield: 56%; M.P.: 224–227°C; IR (KBr, t cm^{-1}): 3338 (NH of isatin), 1740 (C═O of isatin), 1672 (—NH—CO), 1227 (C–F); ^{1}H-NMR (300 MHz, δ ppm, DMSO-d_6): 6.95–7.70 (m, 3H, Ar—H), 11.00 (s, 1H, —NH); CHN *Anal*. Calcd for $C_8H_4FNO_2$: C (58.19), H (2.44), N (8.48). Found: C (58.10), H (2.38), N (8.69); MS *m/z* (+ve): 166.1 (M^+).

5-Iodo-1H-indole-2,3-dione (3d). Yield: 40%; M.P.: 276–280°C; IR (KBr, t cm^{-1}): 3216 (NH of —NH—CO), 1744 (C═O of isatin), 1658 (—NH—CO); ^{1}H-NMR (300 MHz, δ ppm, DMSO-d_6): 7.60–8.70 (m, 3H, Ar—H), 8.86 (s, 1H, —NH); CHN *Anal*. Calcd for $C_8H_4INO_2$: C (35.19), H (1.48), N (5.13). Found: C (35.12), H (1.41), N (5.20); MS *m/z* (+ve): 273.9 (M^+).

5-Nitro-1H-indole-2,3-dione (4). Yield: 50%; M.P.: 251°C; IR (KBr, t cm^{-1}): 3238 (NH of —NH—CO), 1730 (C═O of isatin), 1663 (—NH—CO), 1500–1480 and 1350–1318 (—NO_2); ^{1}H-NMR (300 MHz, d ppm, DMSO-d_6): 7.98–8.72 (m, 3H, Ar—H), 10.01 (s, 1H, —NH); CHN *Anal*. Calcd for $C_8H_4N_2O_4$: C (50.10), H (2.10), N (14.58). Found: C (49.99), H (1.86), N (14.92); MS *m/z* (+ve): 193.02 (M^+).

5-Bromo-1H-indole-2,3-dione (5). Yield: 42%; M.P.: 247–250°C; IR (KBr, t cm^{-1}): 3260 (NH of —NH—CO), 1728 (C═O of isatin), 1695 (—NH—CO), 678 (C—Br); ^{1}H-NMR (300 MHz, δ ppm, DMSO-d_6): 6.97–7.80 (m, 3H, Ar—H), 10.00 (s, 1H, —NH); CHN *Anal*. Calcd for $C_8H_4BrNO_2$: C (42.51), H (1.78), N (6.20). Found: C (42.30), H (1.70), N (6.50); MS *m/z* (+ve): 225.9 (M^+), 227.9 (M + 2).

5,7-Di-bromo-1H-indole-2,3-dione (6). Yield: 36.2%; M. P.: 252–255°C; IR (KBr, t cm^{-1}): 3386 (NH of —NH—CO), 1725 (C═O of isatin), 1698 (—NH—CO); ^{1}H-NMR (300 MHz, δ ppm, DMSO-d_6): 7.80 (s, 1H, Ar—H), 7.90 (s, 1H, Ar—H), 9.81 (s, 1H, —NH); CHN *Anal*. Calcd for $C_8H_3Br_2NO_2$: C (31.51), H (0.99), N (4.59). Found: C (31.42), H (0.90), N (4.73); MS *m/z* (+ve): 304.8 (M^+), 302.8 (M − 2), 306.8 (M + 2).

N-(1,3-dioxo-2-(2,6-dioxopiperidin-3-yl)isoindolin-7-yl)-2-(2,3-dioxoindolin-1-yl)-acetamide (9a). Yield: 52%; M.P.: 220–222°C; IR (KBr, t cm^{-1}): 3250 (NH of —NH—CO), 1695 (C═O of isatin), 1666 (—NH—CO); ^{1}H-NMR (300 MHz, δ ppm, DMSO-d_6): 2.00–2.30 (m, 4H, 2 × CH_2), 3.83 (s, 2H, H_2C—CO), 4.49–4.53 (t, 1H, —CH), 7.20–8.15 (m, 7H, Ar—H), 9.92 (s, 1H, —NH), 10.0 (bs, 1H, —CONHCO—); ^{13}C-NMR (300 MHz, δ ppm, DMSO-d_6): 21.0, 29.3, 53.2, 54.1, 117.4, 122.0, 123.0, 124.0, 124.4, 125.0, 130.0, 132.4, 132.3, 134.8, 140.4, 148.1, 160.4, 168.0, 168.2, 169.0, 172.4, 184.0; CHN *Anal*. Calcd for $C_{23}H_{16}N_4O_7$: C (60.00), H (3.50), N (12.17). Found: C (59.60), H (3.48), N (12.10); MS *m/z* (+ve): 461.04 (M^+), 462.1 (M + 1).

2-(5-Chloro-2,3-dioxo-2,3-dihydro-indol-1-yl)-N-[2-(2,6-dioxo-piperidin-3-yl)-1,3-dioxo-2,3-dihydro-1H-isoindol-4-yl]-acetamide (9b). Yield: 50%; M.P.: 205–207°C; IR (KBr, t cm^{-1}): 3300 (NH of —NH—CO), 1720 (C═O of isatin), 1680 (—NH—CO), 560 (—C—Cl); ^{1}H-NMR (300 MHz, d ppm, DMSO-d_6): 2.10–2.35 (m, 4H, 2 × CH_2), 3.92 (s, 2H, H_2C—CO), 4.00–4.20 (t, 1H, —CH), 7.50–8.13 (m, 6H, Ar—H), 9.80 (s, 1H, —NH), 10.2 (bs, 1H, —CONHCO—); ^{13}C-NMR (300 MHz, d ppm, DMSO-d_6): 21.2, 29.8, 54.0, 54.8, 119.0, 123.0, 123.4, 124.2, 125.0, 130.0, 130.5, 132.1, 132.6, 135.0, 140.3, 146.0, 160.4, 168.0, 168.4, 169.2, 173.0, 183.8; CHN *Anal*. Calcd for $C_{23}H_{15}ClN_4O_7$: C (55.83), H (3.06), N (11.32). Found: C (55.76), H (3.02), N (11.25); MS *m/z* (+ve): 461.2 (M^+), 462.2 (M + 2).

N-[2-(2,6-dioxo-piperidin-3-yl)-1,3-dioxo-2,3-dihydro-1H-isoindol-4-yl]-2-(5-fluoro-2,3-dioxo-2,3-dihydro-indol-1-yl)-acetamide (9c). Yield: 52%; M.P.: 281–283°C; IR (KBr, t cm^{-1}): 3300 (NH of —NHCO), 1715 (C═O of isatin), 1668 (—NH—CO), 1260 (—C—F); ^{1}H-NMR (300 MHz, δ ppm, DMSO-d_6): 2.05–2.32 (m, 4H, 2 × CH_2), 3.90 (s, 2H, H_2C—CO), 4.38–4.42 (t, 1H, —CH), 7.46–8.08 (m, 6H, Ar—H), 9.40 (s, 1H, —NH), 9.98 (bs, 1H, —CO—NH—CO—); ^{13}C-NMR (300 MHz, δ ppm, DMSO-d_6): 20.9, 29.0, 53.3, 54.0, 115.0, 119.3, 121.5, 123.0, 124.0, 125.1, 131.9, 132.6, 140.4, 144.0, 159.0, 160.3, 168.0, 168.4, 169.5, 172.4, 183.2; CHN *Anal*. Calcd for $C_{23}H_{15}FN_4O_7$: C (57.75), H (3.16), N (11.71). Found: C (53.03), H (3.11), N (6.32); MS *m/z* (+ve): 479.2 (M^+), 479.1 (M + 1).

N-[2-(2,6-dioxo-piperidin-3-yl)-1,3-dioxo-2,3-dihydro-1H-isoindol-4-yl]-2-(5-iodo-2,3-dioxo-2,3-dihydro-indol-1-yl)-acetamide (9d). Yield: 36%; M.P.: 248–250°C; IR (KBr, t cm^{-1}): 3400 (OH of —NH—CO), 1730 (C═O of isatin), 1660 (—NH—CO), 580 (—C—I); ^{1}H-NMR (300 MHz, δ ppm, DMSO-d_6): 1.98–2.29 (m, 4H, 2 × CH_2), 3.86 (s, 2H, H_2C—CO), 3.98–4.01 (t, 1H, —CH_2), 7.58–8.23 (m, 6H, Ar—H), 9.90 (s, 1H, —NH), 10.50 (bs, 1H, —CO—NH—CO—); ^{13}C-NMR (300 MHz, δ ppm, DMSO-d_6): 21.0, 29.6, 52.8, 54.2, 89.6, 119.0, 123.2, 124.0, 124.4, 125.2, 132.2, 133.0, 138.0, 140.5, 144.0, 147.0, 160.4, 168.0, 168.4, 169.0, 172.3, 184.3; CHN *Anal*. Calcd for $C_{23}H_{15}IN_4O_7$: C (47.12), H (2.58), N (9.50). Found: C (47.18), H (2.40), N (9.23); MS *m/z* (+ve): 587.0 (M^+), 588.0 (M + 1).

N-[2-(2,6-dioxo-piperidin-3-yl)-1,3-dioxo-2,3-dihydro-1H-isoindol-4-yl]-2-(5-nitro-2,3-dioxo-2,3-dihydro-indol-1-yl)-acetamide (9e). Yield: 40%; M.P.: 236–238°C; IR (KBr, t cm^{-1}): 3360 (NH of —NH—CO), 1720 (C═O of isatin), 1640 (—NH—CO), 1348 (—C—NO_2); ^{1}H-NMR (300 MHz, δ ppm, DMSO-d_6): 2.11–2.39 (m, 4H, 2 × CH_2), 3.94 (s, 2H, H_2C—CO), 4.35–4.38 (t, 1H, —CH), 7.70–8.75 (m, 6H, Ar—H), 9.80 (s, 1H, —NH—CO), 10.03 (bs, 1H, —CO—NH—CO—); ^{13}C-NMR (300 MHz, δ ppm, DMSO-d_6): 21.2, 29.5, 53.0,

54.5, 119.0, 122.8, 123.0, 124.4, 124.9, 125.3, 132.2, 132.6, 140.7, 144.8, 154.3, 160.0, 168.3, 168.8, 169.3, 173.0, 184.6; CHN *Anal*. Calcd for $C_{23}H_{15}N_5O_9$: C (54.66), H (2.99), N (13.86). Found: C (54.40), H (3.01), N (13.78); MS *m/z* (+ve): 506.1 (M^+), 507.1 (M + 1).

2-(5-Bromo-2,3-dioxo-2,3-dihydro-indol-1-yl)-N-[2-(2,6-dioxo-piperidin-3-yl)-1,3-dioxo-2,3-dihydro-1H-isoindol-4-yl]-acetamide (9f). Yield: 50%; M.P.: 210–212°C; IR (KBr, t cm^{-1}): 3300 (—NH—CO), 1710 (C═O of isatin), 1683 (C═O of —NH—CO), 650 (C—Br); ^{1}H-NMR (300 MHz, δ ppm, DMSO-d_6): 1.80–2.32 (m, 4H, 2 × CH_2), 3.84 (s, 2H, H_2C—CO), 4.61–4.64 (t, 1H, —CH_2), 7.20–8.19 (m, 6H, Ar—H), 8.90 (s, 1H, —NH—CO), 10.20 (s, 1H, —CO—NH—CO—); ^{13}C-NMR (300 MHz, δ ppm, DMSO-d_6): 22, 31, 54, 55, 120, 123.1, 123.3, 123.8, 131.9, 132.0, 141.4, 141.8, 144.8, 160.0, 168.1, 169.1, 169.9, 171.8, 184.8; CHN *Anal*. Calcd for $C_{23}H_{15}BrN_4O_7$: C (51.22), H (2.80), N (10.39). Found: C (50.98), H (2.73), N (10.40); MS *m/z* (+ve): 539.0 (M + 1), 541.0 (M + 2).

2-(5,7-Di-bromo-2,3-dioxo-2,3-dihydro-indol-1-yl)-N-[2-(2,6-dioxo-piperidin-3-yl)-1,3-dioxo-2,3-dihydro-1H-isoindol-4-yl]-acetamide (9g). Yield: 38%; M.P.: 201–202°C; IR (KBr, t cm^{-1}): 3460 (NH of —NH—CO), 1717 (C═O of isatin), 1666 (—NH—CO), 648 (—C—Br); ^{1}H-NMR (300 MHz, δ ppm, DMSO-d_6): 2.11–2.39 (m, 4H, 2 × CH_2), 3.94 (s, 2H, H_2C—CO), 4.35–4.38 (t, 1H, —CH), 7.70–8.75 (m, 6H, Ar—H), 9.80 (s, 1H, —NH—CO), 10.03 (bs, 1H, —CO—NH—CO—); ^{13}C-NMR (300 MHz, δ ppm, DMSO-d_6): 19.2, 21.8, 51.5, 51.8, 121.5, 122.0, 122.6, 124.0, 124.4, 134.8, 141.2, 141.9, 146.7, 148.0, 160.0, 161.8, 168.2, 169.4, 171.9, 183.4; CHN *Anal*. Calcd for $C_{23}H_{14}Br_2N_4O_7$: C (44.69), H (2.28), N (9.06). Found: C (44.60), H (2.12), N (9.20); MS *m/z* (+ve): 617.9 (M + 1), 615.9 (M − 2), 619.9 (M + 2).

CYTOTOXIC ASSAY

Cell lines.

- Multiple myeloma cell lines (NCCS, Pune, India)
  - U266B1
  - RPMI 8226

Cell number for subculture: 1 million cells for flask (30-mL capacity).

Cell loading into plate: 1000–2000 cells per well (96-well plate).

Test solutions employed: 1, 10, 20, 30, and 40 μM.

Cell culture conditions. U266B1 and RPMI 8226 cell lines were obtained from the National Center for Cell Science, Pune, India. Each cell line was maintained in RPMI-1640 culture medium (Sigma Aldrich, Bangalore, India) supplemented with L-glutamine and 10% heat-inactivated fetal bovine serum (Himedia, Mumbai, India) in 25-cm^2 tissue culture flasks. All cells were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

MTT assay for cytotoxicity evaluation of the test compounds. To detect growth inhibition by the series of compounds, cells were seeded onto 96-well plates at a density of 5 × 10^3 per well before compound treatment. Cytotoxicity of the series of compounds in a variety of cell lines was determined using the MTT3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay after incubation of cells with these compounds at 1-, 10-, 20-, and 40-μM concentrations for 48 h. IC_{50} (the concentration of 50% inhibition of cell growth) was determined from the dose–response curves [40].

Statistical analysis. Graphs were plotted by taking Log concentration on *X*-axis and % inhibition on *Y*-axis. The IC_{50} values were obtained by nonlinear regression using GraphPad Prism 6.0. IC_{50} measurements for each compound were performed three times. All the values were expressed as mean ± SD. Two-way analysis of variance was performed to validate the data obtained from cytotoxic assay. All the values were found to be statistically significant at $P < 0.05$.

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