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Indomethacin hybrids as novel cytotoxic compounds

Farzaneh Yazdi[1], Maryam Abbasi[2], Hojjat Sadeghi-Aliabadi[1]*

[1] Department of Medicinal Chemistry, Faculty of Pharmacy, Isfahan University of Medical Sciences, Isfahan, Iran
[2] Department of Medicinal Chemistry, Faculty of Pharmacy, Hormozgan University of Medical Sciences, Bandar Abbas, Iran

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ABSTRACT

Cancer is still one of the most invasive health problems around the world although many researches have been done in this field. Different kinds of drugs are developed and used to improve cancer therapy. Some evidence has shown that nonsteroidal anti-inflammatory drugs (NSAIDs) have anticancer activity in addition to anti-inflammatory effects. To improve the safety profile of NSAIDs and enhance anticancer potency, different strategies such as hybridization are used in several studies. Indomethacin is a lead compound in NSAIDs classes' demonstrated inhibitory activity for some malignancies including breast and ovarian cancer. Two kinds of hybrids were proposed and synthesized using diamine linkers via amid bonds: indomethacin-indomethacin (hybrid A) and indomethacin-methotrexate (MTX; hybrid B). To confirm the structures of newly synthesized hybrids, melting points, IR, H NMR were applied. The cytotoxic effects of synthesized hybrids against Hela and MCF-7 cancer cells were evaluated by MTT assay. The results showed that both hybrids were more cytotoxic than indomethacin and MTX alone.

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Keywords
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Corresponding to:
Hojjat Sadeghi-Aliabadi,
Department of Medicinal Chemistry, Faculty of Pharmacy, Isfahan University of Medical Sciences, 81746-73461 Isfahan, Iran

Email:
sadeghi@pharm.mui.ac.ir

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INTRODUCTION

Cancer is still one of the most invasive health problems around the world although many studies have done and some breakthroughs in the cancer treatment were developed. Inflammation is a recognized sign of cancer that is contributed to the development of malignancies. Systemic inflammation and local immune response have important role in the development of tumors and survival of patients with cancer. Therefore, it provides a chance to target these inflammatory responses to improve cancer treatments [1,2].

Tumor response to use nonsteroidal anti-inflammatory drugs (NSAIDs) especially indomethacin is observed in different experimental, clinical, epidemiologic studies that show NSAIDs could be used as anticancer drugs [3]. These studies have been experimentally shown that NSAIDs have two mechanisms that help to repress malignant alteration and tumor growth such as inhibiting angiogenesis and stimulating apoptosis. Endothelial cells are affected by both selective and nonselective NSAIDs so the angiogenesis will be inhibited [4].

On the other hand, NSAIDs apply their activity by inhibition of prostaglandin synthesis catalyzed by cyclooxygenase (COX) enzyme. The COX enzyme has two isoforms, including: COX_1 with a cytoprotective role and COX_2 with a role in the inflammation and pain [5,6].

Some NSAIDs including indomethacin, aspirin and ibuprofen not only used as effective painkillers but they used for other diseases such as arthritis and cardiovascular diseases and recently, for the prevention of colon and breast cancers [4,7,8].

Relationship between NSAIDs consumption and

decreasing the rate of breast cancer is revealed in some studies back to 1980 [9].

The mechanism of suppressing breast cancer by indomethacin had been developed by other researchers. Results were represented that protein kinases, protein phosphatases or signaling peptides are indirect signaling ways that may influence choline phospholipid metabolism and as a result indomethacin may have inhibitory effects on the gene expression of breast cancer cells [10].

Animal experiments provided evidence that colorectal cancer has been controlled by some NSAIDs such as sulindac and selective COX-2 inhibitors [8]. In addition ibuprofen and indomethacin showed antitumor effect on K562 cells [11]. Although these two NSAIDs were effective agents against chronic myeloid leukemia but their antitumor properties would warrant further studies on other clinical applications of these drugs.

Animal models of colon and breast cancers have shown a causal connection between COX_2 consumption and epithelial tumor genesis. COX_2 is an enzyme that over express at several epithelial cancers including breast cancer and controls prostaglandin synthesis [11-15].

To improve the safety profile of NSAIDs and enhance drugs potency and anticancer activity of them, different strategies including hybridization and targeted drug delivery like biovehicles are proposed. Drug hybridization strategies are valuable tools in the development of new drugs with either improved affinity for one bioreceptor or dual action on more than one. Hybridization methods have been applied, for example, to optimize therapy with available drugs and evaluate drug-resistance reversers (chemo sensitizers) as well as new chemotherapeutic targets [16].

Some hybrids are composed of two anti-tumor moieties with various mechanism of action. Up to now, many studies have been done according to hybrid drugs idea (e.g. conjugate of indomethacin and 5-fluorouracil) for the treatment of different diseases like cancer, malaria, inflammation and blood pressure [17-20]. Indomethacin-naphtalimid hybrid is another effective example of anticancer hybrids that are synthesized by Wu et al in 2010. Both indomethacin and naphthalimide are known as anticancer drugs and the biological assay of this hybrid showed higher cytotoxic activity than indomethacin against cancer cell lines such as HeLa, HL-60, HCT-8, and A375 [21].

The other purpose of hybridization could be synthesizing targeted drug. Conjugation of methotrexate (MTX) with an analog of luteinizing hormone (LH) is an example of targeted drug that is synthesized by Zhu et al in 2016. MTX and LH analog alone are effective chemotherapeutic drugs against some cancers; but the new synthesized conjugated compound inhibited the growth of prostate tumor more effectively [22].

The main aim of our investigation was synthesize and characterize a new hybrid drug consist of MTX and indomethacin. It could be more effective against cancer cell lines than indomethacin alone. Also in cancer treatment we prospect that hybridization of indomethacin with MTX could be a targeted drug with dual action because of this fact that tumor tissues are usually inflammated and indomethacin has also antitumor properties.

MATERIALS AND METHODS

All reagents were purchased from Sigma-Aldrich or Merck companies via local vendors. Chloroform was dried by distillation before using. H-NMR was obtained in chloroform using a Bruker AV-400 spectrometer (Germany); chemical shifts are given as ppm in δ scale (in CDCl3 and TMS as internal standard). IR spectra were recorded as KBr pellets by a Perkin-Elmer 2000 Fourier-transform IR (FTIR) instrument (Japan). Melting points were determined in capillaries using electro thermal 9200 melting point apparatus. Column chromatography was performed on 200–300 mesh silica gels.

Indomethacin and MTX free bases were kindly provided by Jalinous and Osveh pharmaceutical companies (Tehran, Iran), respectively.

Chemistry

Conjugation of indomethacin-1,12diaminododecan-indomethacin (hybrid A)

Indomethacin free base (1.43g, 4mmol) was dissolved in dry chloroform (50ml). To this solution, dicyclohexylcarbodiimid (4.1g, 20mmol) was added with stirring and the reaction mixture was stirred under nitrogen for 2 hours. 1, 12-diaminododecan (0.8g, 4mmol) was dissolved in chloroform (10ml) and added to the reaction mixture of activated indomethacin by dropping funnel. The reaction mixture was then stirred over night at refluxed temperature. Reaction progress was monitored by TLC until completion. Then the chloroform was distilled off under vacuum and the conjugated product was separated by column chromatography. The white crystalline product was obtained by 60% yield [23].

Conjugation of indomethacin-1,6diaminohexan

Indomethacin powder as free base (1.43g, 4mmol) was activated with DCC (4.1g, 20mmol) as mentioned above. This solution was then added drop wise to a solution of 1,6 diaminohexane (1.39g, 12mmol) dissolved in chloroform (25ml). The reaction mixture was stirred for 3 hours at room temperature. After completion (monitored by TLC), the reaction was quenched with water. To remove the water-soluble by products, the reaction mixture was washed 3 times by distilled water (3×100 ml). The organic layer was separated and dried under reduced pressure. The obtained residue was purified using column chromatography to give indometacin-1,6diaminohexan as a white product (mp: 110°C, 57% yield) [24].

Conjugation of indomethacin-1,6diaminohexane-Methotrexate (hybrid B)

MTX (0.54g, 1.2mmol) was dissolved in dry chloroform (10ml) and dicyclohexylcarbodiimid (1.23g, 6mmol) was added and then the reaction mixture was stirred under

nitrogen for 6hours. After completion of the reaction (monitored by TLC), activated MTX was added drop wise to a solution of indomethacin-1,6 diaminohaxane conjugate in chloroform (10ml) over 10 min and the reaction mixture was stirred under nitrogen for 72 hours. Then chloroform was distilled off under vacuum and the residue was dissolved in dichloromethane (20ml) and extracted with hexane (10 ml) until the title compound (indomethacin-1,6diaminohexan-MTX) was precipitated as yellow crystals (mp: 150ºC, 50% yield) [25,26].

Cell lines

MCF-7 (breast cancer) and Hela (cervix cancer) cells were obtained from national cell bank of Iran, pastuer institute, Tehran, Iran. Cell were maintained in RPMI 1640 containing 100 µg/ml streptomycin, 100 units/ml penicillin supplemented with 10% heat-inactivated fetal bovine serum (FBS).

Biological evaluation of synthesized hybrids

Cytotoxic effects of novel synthesized compounds were tested against MCF-7 and Hela cells, using MTT assay as reported previously [26].

Briefly cells were plated in 96-well plate at a concentration of 5 x 10^4 cells/ml and incubated for 24 h. Different concentrations of the novel synthesized hybrid were added to each wells so that the final concentrations of compounds were 0.1, 1, 10, 50, 100 µM and incubated for further 48 h, in a humidified environment at 37℃ with 5% CO_2.

After incubation, each well was treated with 20 µL of MTT dye (5 mg/ml) and re-incubated for 3 h at the same condition. Then, the culture media replaced with 150 µl of DMSO to dissolve formazan crystals and the absorbance was recorded at 570 nm using an ELISA plate reader (BioTek, USA).20 µl of DMSO (1%) and doxorubicin (7.7µm) were added to the wells and used as negative and positive controls, respectively.

Each experiment was repeated three times and cell survival was calculated using following formula:

$$Cell\ survival\ (\%) = \frac{absorbance\ of\ treated\ well - absorbance\ of\ blank}{absorbance\ of\ control\ well - absorbance\ of\ blank} \times 100$$

IC_{50} values were determined by plotting the cell viability against compound concentrations, at 50% cell survival. All statistical analysis was performed with the Microsoft Office Excel 2010 and SPSS by one-way ANOVA method [26].

RESULTS

To confirm the structures of newly synthesized hybrids, melting points, IR, H-NMR were used (Fig. 1).

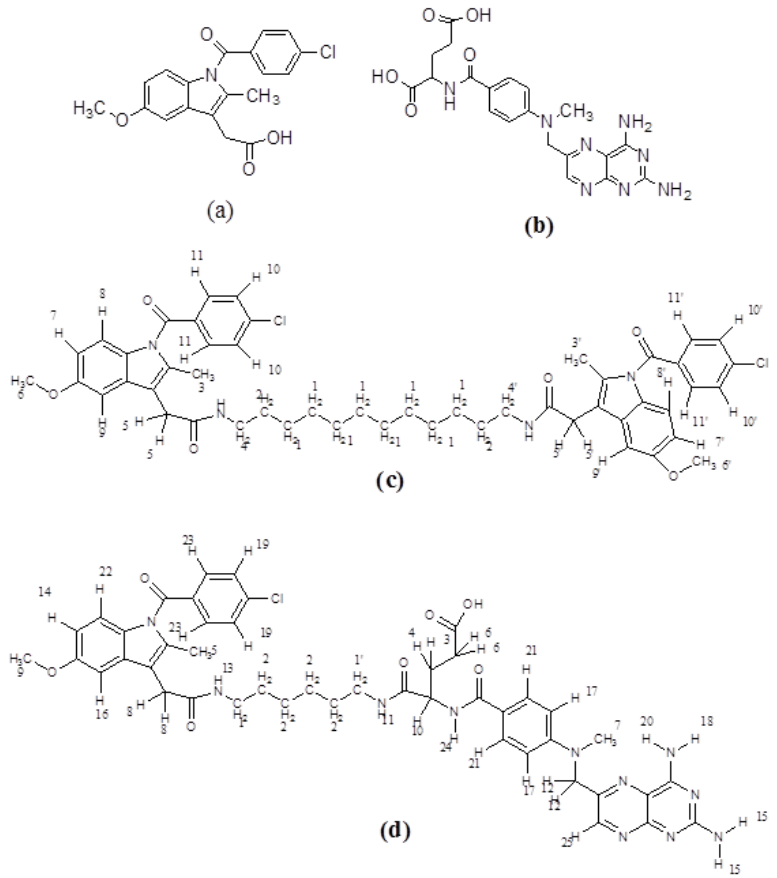

Figure 1. Structures of the tested compounds; Indomethacin (a); Methotrexate (b); Hybrid A (c); Hybrid B(d)

Chemical results

Hybrid A

Melting point:165°C;IR (KBr, *v*): 3309(N-H), 2927.4(C-H), 1679(C=O), 1638.23(C=O) cm-1; H NMR (CDCl3, 400 MHz), δH: (ppm): 1.09(16H,brS, 8×CH-1), 1.31(4H,q,2×CH-2), 2.3(6H,S, 2×CH-3,3'), 3.1(4H,q, 2×CH-4,4'), 3.56(4H,S, 2×CH-5,5'), 3.7(6H,S, 2×CH-6,6'),5.5(2H,t,2×NH), 6.6(2H,d, 2×CH-7,7'),6.7(2H,d, 2×CH-8,8'),6.8(2H,S, 2×CH-9,9'),7.4(4H,d,4× CH-10,10'),7.6(4H, d, 4× CH-11,11')

Hybrid B

Melting point:150°C;IR (KBr, ν): 3319.86(N-H), 2930.31(C-H), 1649.8(C=O), 1613.16 (C=O) cm-1; H NMR (CDCl3, 400 MHz), δH: (ppm): 1.2(4H, S, 2×CH-1,1'), 1.3(8H,S, 4× CH-2),1.9(1H, d, CH-3), 2.05(1H,d, CH-4), 2.3(5H, S, 2×CH-5,6), 3.1(3H,S,CH-7), 3.56(3H, S, CH-8), 3.7(4H, S, CH-9,10), 4.48(1H, t, NH-11), 4.68(2H, S, CH-12), 5.79(1H, t, NH-13), 6.61(1H, d,CH-14), 6.68(2H, d, NH-15), 6.79(1H,S, CH-16), 6.834(2H, d, CH-17), 7.26 (1H, d, CH-18), 7.4(2H, d, CH-19), 7.46(1H, d, CH-20), 7.58(3H, d, CH-21,22),7.65(2H, d, CH-23), 7.9(1H,S, CH-24), 8.5(1H, S, CH-25).

Biological results

The cytotoxic effects of synthesized hybrids were examined by MTT assay. Indomethacin was used as control. In both cell lines, the cell viability reduced gradually in a concentration dependent manner. Results of inhibitory activity were represented by IC_{50}. As shown in Table 1, hybrid B was much more cytotoxic than indomethacin alone against both cell lines.

Effect of Indomethacin against HeLa cells

Logarithmic concentrations of 0.1,1,10 and 100 µm of Indomethacin against Hela cells reduced cell viability to 81, 79, 70 and 50%, respectively (Fig. 2).

Effect of Indomethacin against MCF-7 cells

MCF-7 cells were treated with indomethacin at concentrations of 0.1, 1, 10 and 100 µm and cell survival

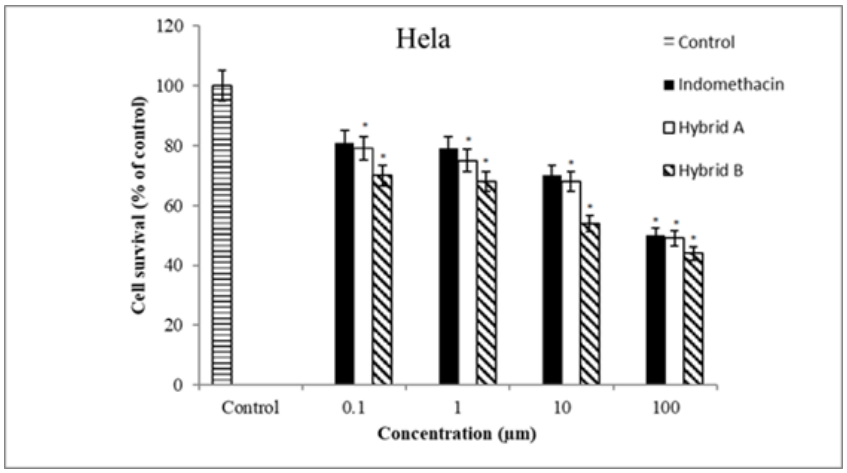

Figure 2. Percent cell survival of HeLa cells exposed to different concentrations (0.1, 1, 10,100µm) of indomethacin, hybrid A and hybrid B. The cytotoxicity was determined by MTT assay. Data are presented as percent of inhibition compared to negative control. Significant results were shown with an asterisk (*) on histograms (Anova, $p<0.05$). The error bars represent Mean ± SD.

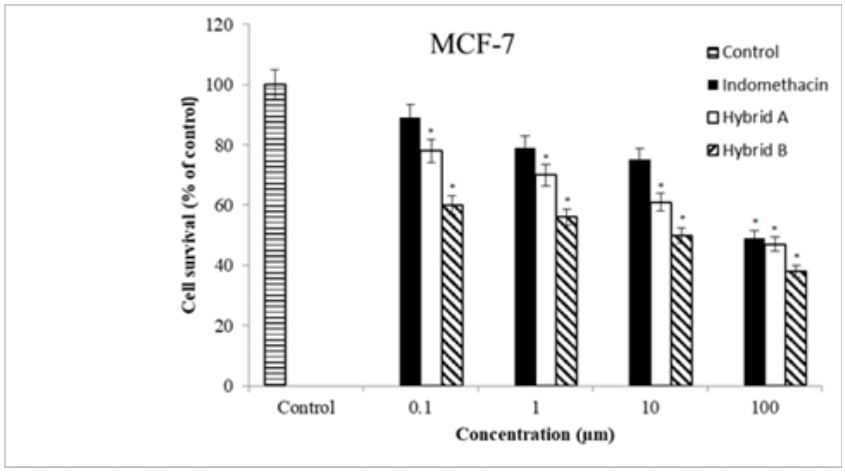

Figure 3. Percent cell survival of MCF-7 cells exposed to different concentrations (0.1, 1, 10,100µm) of indomethacin, hybrid A, hybrid B. The cytotoxicity was determined by MTT assay. Data are presented as percent of inhibition compared to negative control.* shows significant effects using one way ANOVA ($p<0.05$). The error bars represent Mean ± SD.

was obtained 89%, 79%, 75% and 49%, respectively. The cell viability results were shown in a concentration dependent manner (Fig. 3).

Effect of hybrid A against MCF-7 cells

Cell survival reduced in a concentration dependent manner to 78, 70, 61 and 47% after 48h exposure to hybrid A at concentrations of 0.1, 1, 10 and 100 µm, respectively. As seen in Figure 3, hybrid A at concentrations of 10 and 100 µm were decreased cell viability significantly ($p<0.05$).

Effect of hybrid A against Hela cells

The effect of hybrid A against Hela cells (Fig. 2) was concentration dependent and at tested concentrations (0.1, 1, 10,100 µm) cell viability was reduced significantly to 79, 75, 68 and 49%, respectively ($p<0.05$).

Effect of hybrid B against MCF-7 cells

A reduction in cell survival was seen in MCF-7 cell lines after treated with Hybrid B. Cell viability reduced to 60, 55, 50 and 38% at tested concentrations (0.1, 1, 10, 100 µm) respectively. This reduction in cell survival was statistically significant at concentrations of 10 and 100 µm ($p<0.05$)

Effect of hybid B against Hela cells

Logarithmic concentrations of hybrid B, (0.1, 1, 10 and 100 µm) were tested against HeLa cell line for 48 h. The results showed, as concentrations increased the percent of cell survival reduced to 70, 68, 54 and 44%, respectively which was statistically significant at concentrations in comparison with negative control ($p<0.05$) (Fig. 2).

DISCUSSION

Cancer and inflammation are closely linked to each other, so that many anti-cancer agents are also used to treat inflammatory diseases, such as rheumatoid arthritis and vice versa. Moreover, chronic inflammation increases the risk for various cancers, indicating that eliminating inflammation may represent a valid strategy for cancer prevention and therapy [6, 8, 27-29].

Indomethacin as a lead of NSAIDs compounds has been studied as anticancer agent and the link between using indomethacin and increasing tumor inhibitory activity have been shown in several studies in the cancer fields [11, 20, 21]. To increase indomethacin anticancer effect, new hybrids of indomethacin have been synthesized. Biological evaluation of these synthesized hybrids showed better cytotoxic effects against breast and ovarian cancer cells. For example, combination of naphthalimide as a known anticancer drug when hybrided with indomethacin showed the synergic effects against some cancer cell lines such as Hela [21]. In the same regards Singh et al in 2009 synthesized new anticancer hybrid drug conjugated of indomethacin and 5-fluorouracil. In vitro evaluation of this hybrid showed higher antiproliferative activity against some cell lines including breast, ovarian, colon and renal cancers [20]. According to the results obtained from indomethacin hybrids, we first proposed to synthesize a new hybrid (hybrid A) which is consist of two molecules of indomethacin conjugated via 1,12 diaminododecan as linker.

MTX is one of the effective chemotherapeutic drugs that is used clinically in different cancer therapy including breast, cervix, lung and prostate malignancies [22]. MTX has also potential to be used in clinic for the treatment of some chronic inflammatory diseases such as rheumatoid arthritis, dermatoses, ocular inflammation etc [30-32]. The anti-inflammatory effect of MTX is possibly a combination of different mechanisms including inhibition of pyrimidine and purine synthesis, reduction of antigen-dependent T-cell proliferation, suppression of transmethylation, reactions with accumulation of polyamines and promotion of adenosine release with adenosine-mediated suppression of inflammation. The latter mechanism of MTX has been supported by the in vitro, in vivo and clinical data. Generally, these mechanisms make MTX a good choice for hybridization drugs to treatment of inflammatory diseases and cancers [33-34]. Elmorsi and co-workers showed that NSAIDs such as indomethacin combined with MTX could increase MTX absorption and subsequently increase its cytotoxic effects [35]. Since most of cancers are contributed with inflammation [1, 14], in this study, we proposed to synthesize a new hybrid (hybrid B), that is a conjugation of indomethacin and MTX.

As shown in Table 1, hybrid A had better inhibitory activity than indomethacin against MCF-7 and HeLa cell lines which may relate to the increased molar ratio of indomethacin in the new hybrid. Consistent results from others showed that novel hybrid compounds including inorganic salts such as indomethacin-ZrO2-caprolacton [36], biotin-Pt-indomethacin hybrid [37] and indomethacin-macrolide hybrid [38] have been designed and synthesized according to this strategy. These novel hybrids increased the anti-inflammatory effects of indomethacin significantly.

Since MTX is widely used in the treatment of breast and ovarian cancers [22], this newly synthesized hybrid of indomethacin-linker-MTX was tested against MCF-7 and HeLa cell lines which were derived respectively from those organs.

Different linkers were used for drugs conjugation including: 1) Amide or peptide linkers such as amino acids or diamines which are protease sensitive and rapidly cleaved by lysosomal or extracellular enzymes while they have high plasma stability; 2) Esters linkers which are poorly stable in blood circulation [39]; 3) Phosphodiester linkers [40]; 4)

Table 1. IC_{50} values of synthesized hybrids and indomethacin against Hela and MCF-7 cell lines

	IC_{50}(µm)	
Compounds	*HeLa*	*MCF-7*
Indomethacin	100	94
Hybrid A	92	80
Hybrid B	16	10

Disulfide linkers [41]. In addition, it is reported that linkers with different structures have various biological activity. When the number of methylene group in the structure of diamine linkers reduced the cytotoxic activity would be increased [21]. Thus to improve the cytotoxic activity of proposed hybrid B, we decided to use 1, 6-diaminohexane as a cleavable linker instead of 1, 12- diaminododecane.

In both hybrids were formed drug-liker amid bond and molecular weight hybrids were more than 500, the same peptide compounds (42). Thus, we will expect that these compounds get inside the cells, like peptides.

As it has been shown in Table 1, hybrid B had higher activity than indomethacin and MTX in both cell lines in a concentration dependent manner; although it's inhibitory activity was more significant against MCF-7 than HeLa cell line.

CONCLUSION

In conclusion, two novel hybrids of indomethacin with two different linkers were synthesized. The biological activity was measured against two different cell lines. The results showed that both hybrids were more cytotoxic than indomethacin and MTX alone. This study suggests novel hybrid drugs with improved bioactivity which could be lead compounds for further optimizations in future studies.

CONFLICT OF INTEREST

The authors declare that this research does not have any conflict of interest with anyone or any institute.

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