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Discovery of a new class of 16-membered (2Z, 11Z)-3,11-di(aryl/naphthyl)-1,13-dioxa-5,9-dithia-2,12-diazacyclohexadeca-2,11-dienes as novel anti-tumor agents

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Abstract: A series of new 16-membered small macrocyclic compounds, (2Z,11Z)-3,11-di(aryl/naphthyl)-1,13-dioxa-5,9-dithia-2,12-diazacyclohexadeca-2,11-dienes (**1a-k**) were designed and developed by a simple and practical synthetic route from readily available substrates using simple organic transformations. Evaluation of *in vitro* anti-tumor activities on human triple negative breast cancer cells MDAMB-231 cell lines reveal that macrocycles (**1a**, **1f**, **1g**, **1i** and **1k)** are promising anti-tumor compounds as evidenced from inhibition of cell migration and proliferation, upregulation of anti-tumor genes p53, MDA7 and TRAIL. The anti-proliferative effect of macrocycles is specific to cancer cells but no cytotoxic effect on normal breast epithelial cells has been observed (MCF10A). The developed synthetic route is free from metals, protecting groups and air-free techniques. The structure of macrocycle (**1e**) is confirmed by single crystal XRD studies.

Keywords: Macrocycles, MDAMB-231 cells, p53, TRAIL, MDA7 & STAT-3 genes, Anti-tumor agents, Apoptosis.

Introduction

Design, synthesis and development of new anti-tumor agents has always been a challenging task for the scientific community as the cancer is a major public health problem in many parts of the world[1-4] particularly in developing countries increasingly, an adoption of cancer-associated lifestyle options including smoking, westernized diets and lack of adequate exercise.[5] Globally every 8th death is caused due to cancer.[6] Many of the available anti-tumor drugs have adverse effects on human health, therefore, there is a great need to develop new

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class of drugs for the treatment of cancer that are cost effective and chemically stable with low toxicity to normal cells.

Macrocyclic molecules played a key role in drug discovery as evidenced from natural products.[7] Among the macrocycles, the hetero-atoms incorporated macrocyclic molecules [7] might be the best option as they possess unusual characteristics such as i) structural complexity, regiogenicity, stereogenicity and rigidity, ii) potential hydrogen bonding to interact with biomolecules such as RNA, DNA, proteins (enzymes),[8,9] iii) high degree of structural pre-organization which leads to the interaction of the key functional groups across extended protein's active site and iv) 'drug-like' physico-chemical as well as pharmacokinetic properties.[10, 11] For instance, small macrocyclic molecules derived from natural products (**I**)[12] and many synthetic macrocycles (**II-IV**)[13, 14, 15] displayed apoptosis and 15-membered macrocycles (**V**)[16] exhibited the highest levels of cytotoxicity (Figure 1). Further, macrocyclic molecules exhibited various pharmacological properties which include anti-bacterial, anti-fungal, anti-tumor and anti-HIV activities.[17]

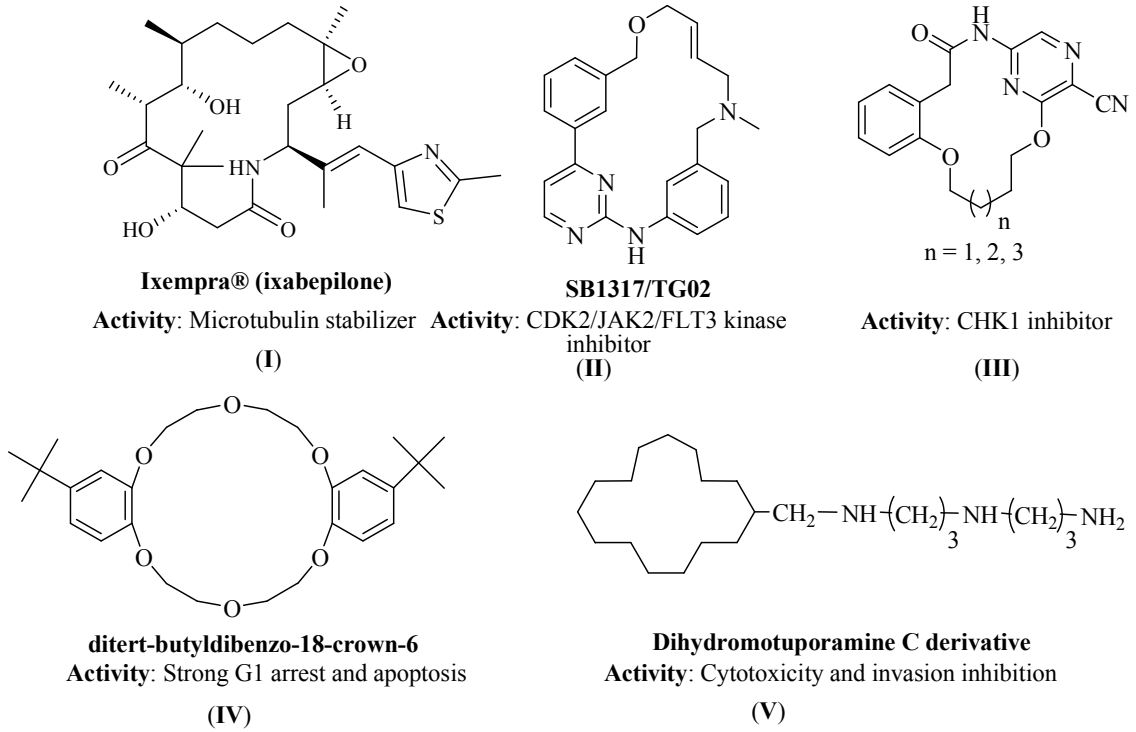

Figure 1. Representative examples of biologically active macrocyclic molecules.

However, disinclination to investigate and develop macrocycles as new drug candidates in the academic and pharmaceutical industry was observed owing to poor yields during

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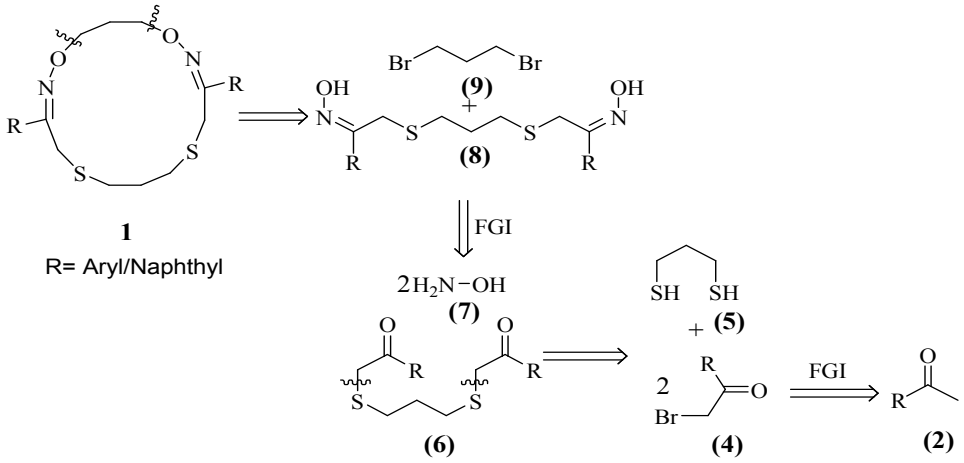
macrocyclization, difficulties in analog synthesis and unproductive side reactions during the joining of ends of a linear precursor molecule.[18] Later, metal template methods were developed,[19] but these methods were also associated with few disadvantages such as difficulties in choosing the correct template metal ion and its removal.[20] Other methods were non-template, but required high dilution conditions[21] which lead to huge organic waste generation along with low yields of desired product and also these methods require lengthy separations to remove oligomeric by-products. Later, ring closing metathesis,[22] Glaser–Eglinton–Hay-type sp–sp coupling[23] and head to tail macrocyclizations (Build/Couple/Pair strategies)[24] methods have emerged. But most of these methods suffer from one or more disadvantages as discussed above. Keeping in view of various pharmacological and biomedical applications and difficulties in the synthesis of macrocycles, we aimed to design and synthesize a new class of non-peptidic 16-membered macrocycles i.e. (2Z,11Z)-3,11-di(aryl/naphthyl)-1,13-dioxa-5,9-dithia-2,12-diazacyclohexadeca-2,11-dienes(**1**) from readily available substrates using simple organic transformations. The following factors formed the basis for the present design and synthesis: i) high probability of cyclic oxime-ethers in the synthesis of biologically active synthetic molecules[25] and ii) accessibility of diverse topologies[26] by the presence of sulfur atoms which were amiable to disrupt protein-protein interactions (PPIs).[27]

Retro-synthetic analysis: The retrosyntheic analysis of target 16-membered (2Z,11Z)-3,11-di(aryl/naphthyl)-1,13-dioxa-5,9-dithia-2,12-diazacyclohexadeca-2,11-dienes (**1**) is outlined in scheme 1.



Scheme 1. Retrosynthetic analysis for the synthesis of (2Z,11Z)-3,11-di(aryl/naphthyl)-1,13-dioxa-5,9-dithia-2,12-diazacyclohexadeca-2,11-dienes (**1**).

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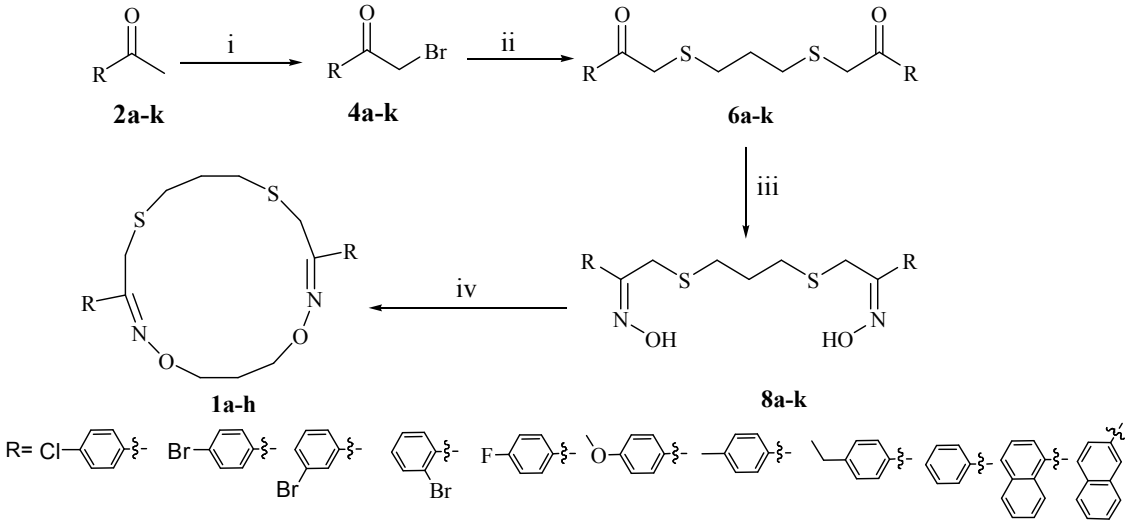
Herein, a simple, efficient and practical synthetic route is developed for the synthesis of structurally diverse non-peptidic 16-membered 2Z,11Z)-3,11-di(aryl/naphthyl)-1,13-dioxa-5,9-dithia-2,12-diazacyclohexadeca-2,11-dienes (**1**) (**1a-k**) from readily available substrates such as aralkyl ketones (2**a-k**), *N*-bromosuccinimide (**3**), 1,3-propanedithiol (**5**), $NH_2OH.HCl$ (7) and 1,3-dibromopropane (**9**) using simple organic transformations involving α-bromination, C-S bond construction, oxime formation followed by macrocyclization (C-O bond formation) as shown in scheme 2. Further, the synthesized macrocyclic molecules (**1a-k**) are well characterized by ^1H, ^{13}C NMR, HRMS and single crystal XRD studies and evaluation of their anti-tumor activity is duly undertaken.



Reaction conditions: (i) 10% (w/w) Silicagel, NBS (3), MeOH, reflux, 15-20 min; (ii) 1,3-propanedithiol (5), K_2CO_3, acetonitrile, 15-20°C, 85-95 min, (iii) $NH_2OH.HCl$ (7), NaOAc, reflux, 2 h; (iv) 1,3-dibromopropane (9), KOH in a mixture of H_2O-DMSO (1:5 ratio), 5-10°C, 25-35 min.

Scheme 2. Synthesis of (2Z,11Z)-3,11-di(aryl/naphthyl)-1,13-dioxa-5,9-dithia-2,12-diaza cyclohexadeca-2,11-dienes (**1**).

Results and Discussion

Chemistry

The synthetic route of title compounds i.e. (2Z,11Z)-3,11-di(aryl/naphthyl)-1,13-dioxa-5,9-dithia-2,12-diazacyclohexadeca-2,11-dienes (**1**) was accomplished via a four-step process as shown in scheme 2. Accordingly, first 2-bromo-1-(4-chlorophenyl)ethanone (**4a**) was prepared from 4-chloroacetophenone (**2a)** by α-bromination using NBS (**3**) in MeOH in the presence of silica gel under reflux condition in high yield (91%) within a short period of time.[28] The same procedure was applied for the preparation of other α-bromoaralkyl ketones

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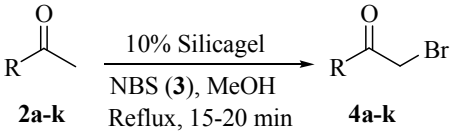
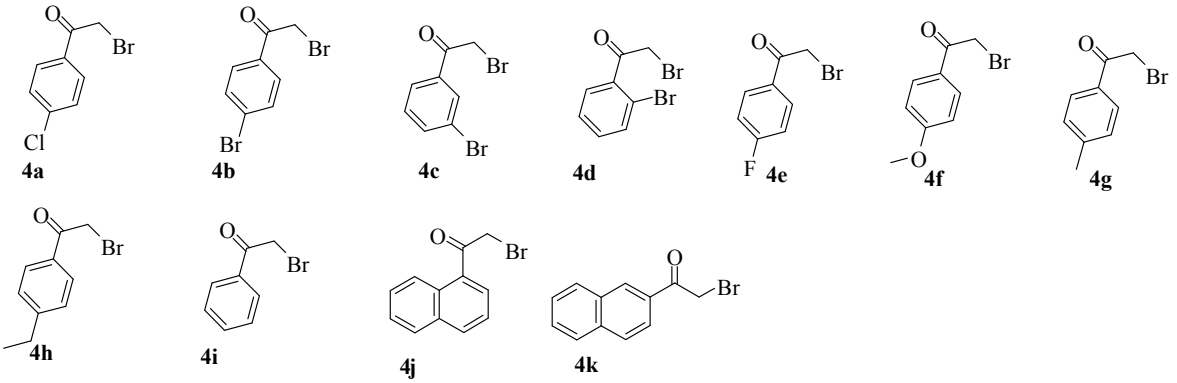
(**4b-k**) from various acetophenones and acetonaphthones (**2a-k**) and the obtained results were presented in scheme 3.

Scheme 3. Synthesis of α-bromoaralkyl ketones (**4**)[a,b]





[a]**Reagents and conditions:** Aralkyl ketones, **2a-k** (10.0 mmol), NBS (12.0 mmol) (**3**), 10% (w/w) Silica gel, MeOH, reflux, 15-20 min; [b]Isolated yields : **4a** (91%), **4b** (87%), **4c** (81%), **4d** (74%), **4e** (94%), **4f** (87%), **4g** (92%), **4h** (88%), **4i** (95%), **4j** (86%), **4k** (91%).

Later, the reaction conditions for the synthesis of 2,2'-(propane-1,3-diylbis(sulfanediyl))bis(1-(4-chlorophenyl)ethanone) (**6a**) were optimized. Towards this direction, firstly, 1, 3-propanedithiol was added drop wise to the α-bromo compound, 2-bromo-1-(4-chlorophenyl)ethanone (**4a**) in presence of Na_2CO_3 (3.0 equiv.) in ethanol at 20-25°C and obtained 45% yield of the product (**6a**). To improve the yield further, the effect of various solvents such as dichloromethane, methanol, acetonitrile, THF, DMF and DMSO was investigated and 42%, 54%, 65%, 49%, 58% and 53% yields of product (**6a**) were obtained respectively. Preliminary screening of solvent study revealed that acetonitrile was the best option for maximum conversion of product (**6a**). To improve the yield of the product (**6a**) further, various bases such as K_2CO_3 (3.0 equiv.), Et_3N (3.0 equiv.) and pyridine (3.0 equiv.) have been employed in acetonitrile which resulted in 85%, 65% and 55% yields of the product (**6a**) respectively. Finally, the effect of temperature on the course of C-S bond formation was also studied at various temperatures ranging from 5 to 25°C. The study indicated that 15-20°C was the optimum temperature for maximum yield of product **6a** (98%). With the help of optimized reaction conditions, the substrate scope was tested for the synthesis of various 2,2'-(propane-1,3-diylbis(sulfanediyl)-bis(1-aryl/naphthylethanone)derivatives (**6b-k**) using α-bromoketones (**4b-k**) as substrates and the obtained results were presented in scheme 4.

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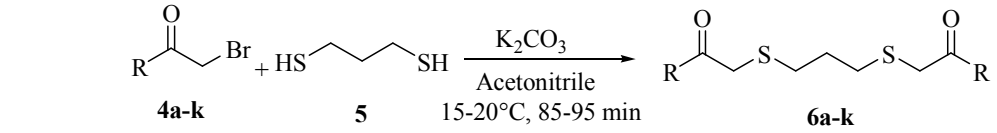
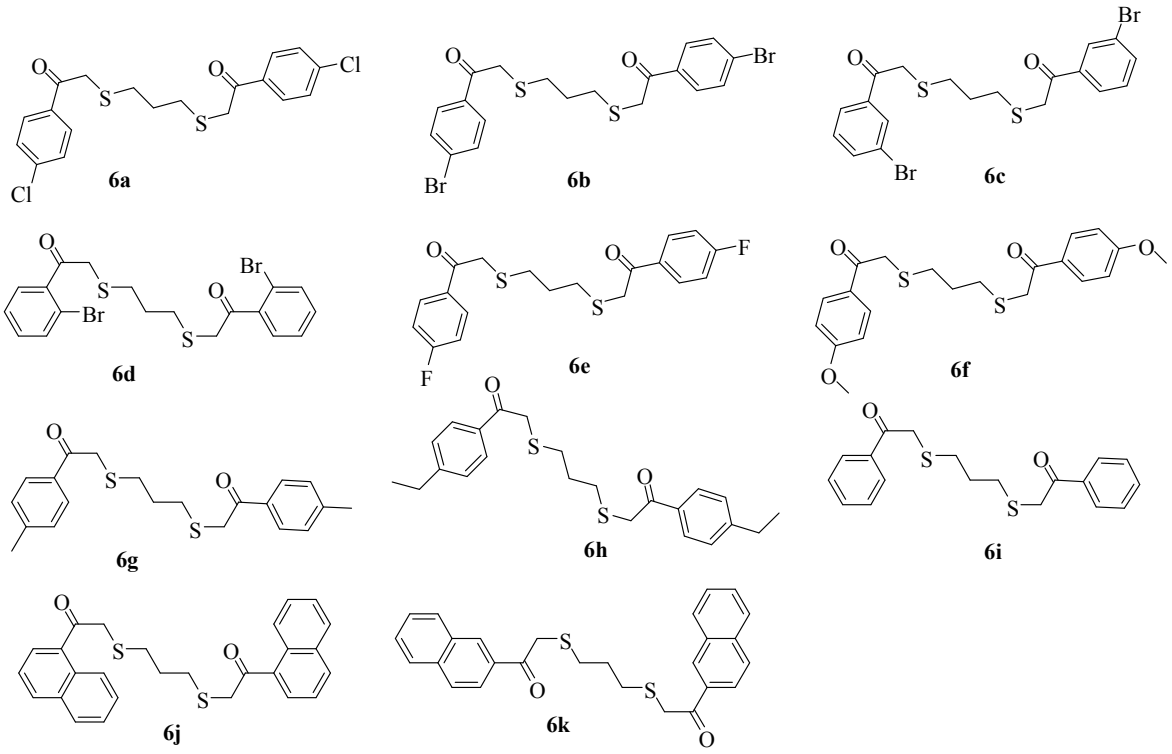
Scheme 4. Synthesis of various 2,2'-(propane-1,3-diylbis(sulfanediyl))bis(1-aryl/naphthyl ethanone) derivatives (**6**)[a,b]



4a-k **5** **6a-k**



6a **6b** **6c** **6d** **6e** **6f** **6g** **6h** **6i** **6j** **6k**

[a]**Reagents and conditions:** α-bromoaralkyl ketones, **4a-k** (10.0 mmol), 1,3-propanedithiol (5.0 mmol) (**5**), K_2CO_3, acetonitrile, 15-20°C, 85-95 min; [b]Isolated yields : **6a** (98%), **6b** (96%), **6c** (93%), **6d** (86%), **6e** (97%), **6f** (95%), **6g** (96%), **6h** (95**%**), **6i** (95%), **6j** (94%), **6k** (98%).

The third step was the oxime formation step which involves, the reaction between compound (6a) and $NH_2OH.HCl$ (**7**) in the presence of NaOAc in ethanol under reflux conditions[29] to produce the precursor of the pharmacophore, (1Z,1'Z)-1-(4-chlorophenyl)-2-(3-((Z)-2-(4-chlorophenyl)-2-(hydroxyimino)-ethylthio) propylthio) ethanone oxime (**8a**) in excellent yield (98%). The same procedure was followed for the preparation of (1Z,1'Z)-1-(ayl/naphthyl)-2-(3-((Z)-2-(ayl/naphthyl)-2-(hydroxyimino)-ethylthio)propylthio) ethanone oximes (**8b-k**) using various substrates, **6b-k** and the obtained results were presented in scheme 5.

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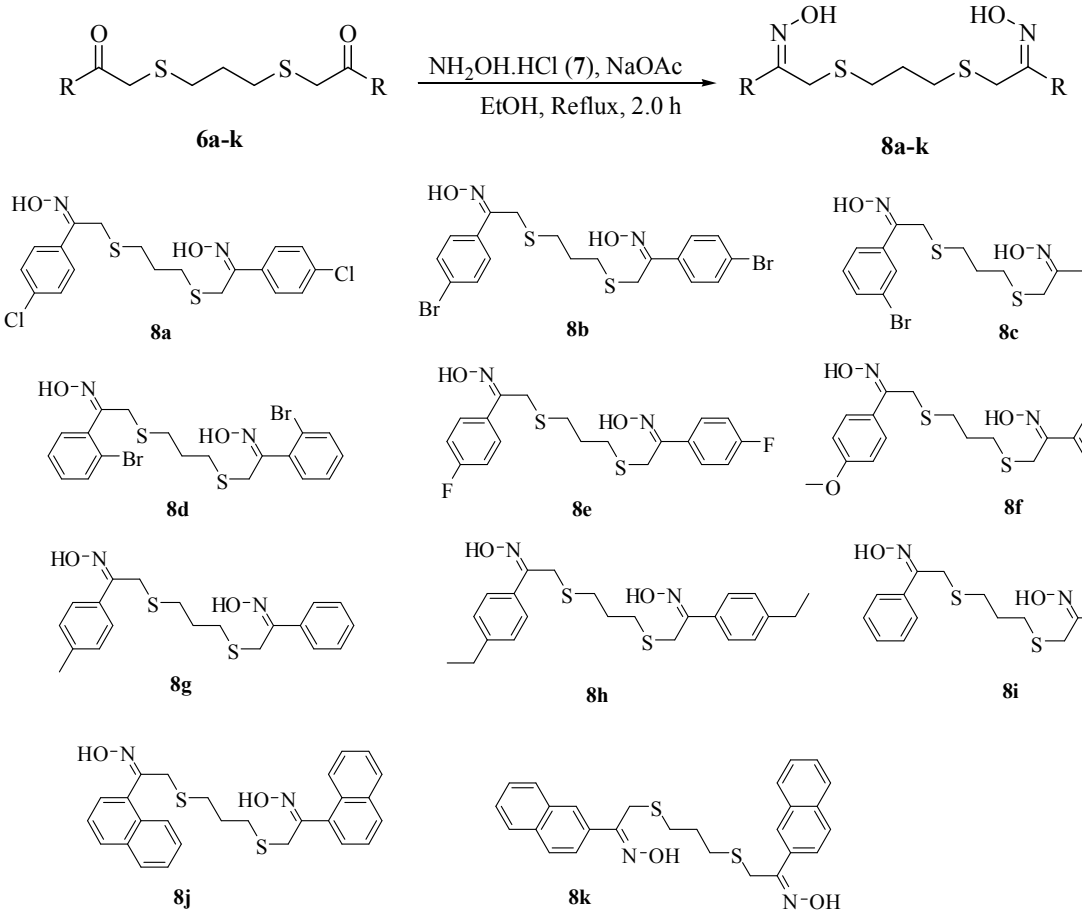
Scheme 5. Preparation of various (1Z, 1'Z)-1-(aryl/naphthyl)-2-(3-((Z)-2-(aryl/naphthyl)-2-(hydroxyimino) ethylthio) propylthio) ethanone oxime (**8**)[a,b]



[a]**Reagents and conditions:** Substrates, **6a-k** (10.0 mmol), $NH_2OH.HCl$ (40.0 mmol) (**7**), NaOAc (30.0 mmol w. r. t. $NH_2OH.HCl$), ethanol, reflux, 2h; [b]Isolated yields : **8a** (98%), **8b** (97%), **8c** (95%), **8d** (88%), **8e** (98%), **8f** (97%), **8g** (98%), **8h** (96**%**), **8i** (97%), **8j** (96%), **8k** (98%).

Then, the attention was focused on macrocyclization step to synthesize the title compound, (2Z,11Z)-3,11-bis(4-chlorophenyl)-1,13-dioxa-5,9-dithia-2,12-diazacyclohexadeca-2,11-diene (**1a**) by the reaction of pharmacophore, (1Z,1'Z)-1-(4-chlorophenyl)-2-(3-((Z)-2-(4-chlorophenyl)-2-(hydroxyimino)-ethylthio)propylthio)ethanoneoxime (**8a**) with linker i.e. 1,3-dibromopropane (**9**)**.** Towards this direction, initially, a reaction was carried out by using the substrate, **8a** and 1,3-dibromopropane (**9**) in MeOH in the presence of NaOH at 20-25°C and lower yield (15%) of product, **1a** was obtained. To improve the yield, the reaction conditions for the preparation of target 16-membered macrocycle **1a** have been optimized using different solvents and bases. Initially, the effect of solvent or mixtures of solvents was studied for their impact on the course of macrocyclization. It was found that the solvents like MeOH, EtOH, acetonitrile, DMSO and DMF were provided 15%, 20%, 10%, 50% and 35% yields, respectively.

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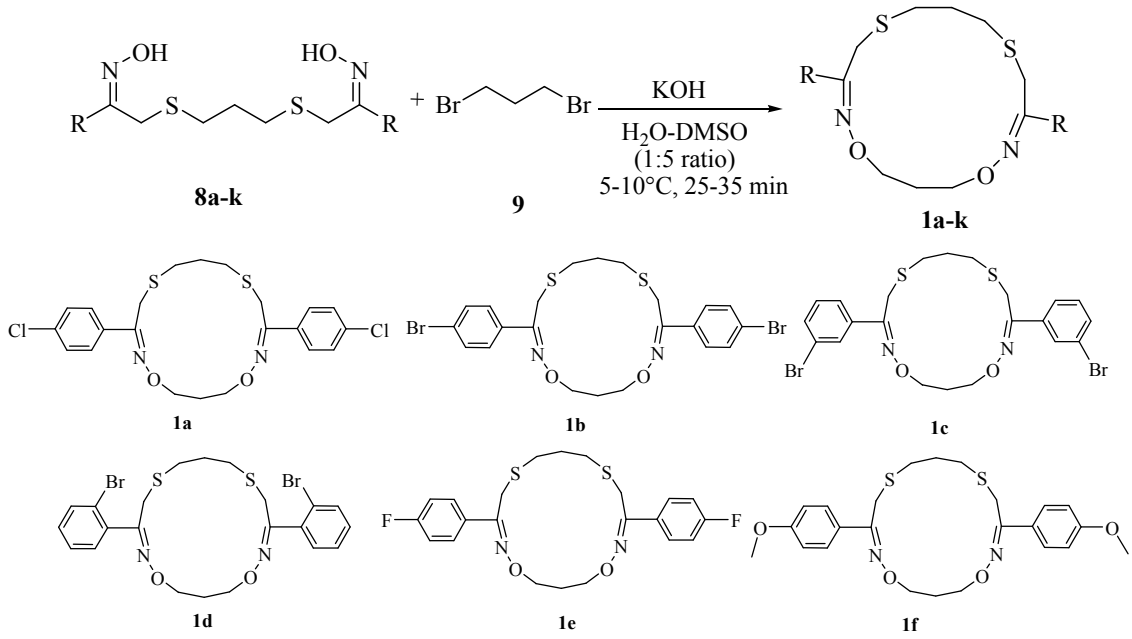
From this study, it was found that DMSO provided considerable yield (50%) of the product **1a**. For the improvement of the yield of the product **1a** further, the effect of mixture of solvents was investigated. Towards this direction, a mixture of H_2O and DMSO was selected and the study revealed that 1:5 ratio of H_2O and DMSO provided good yield (75%) of product **1a**. Further, the effect of temperature was also studied and found that 5-10°C was the optimum temperature for maximum yield of product **6a** (86%). The effect of various bases such as Na_2CO_3, K_2CO_3, NaOH, KOH, Et_3N and pyridine on the course of macrocyclization was studied. From this study, it was concluded that KOH was the best in obtaining maximum isolated yield (94%) of the desired product **1a** in presence of 1:5 ratio of H_2O and DMSO at 5-10°C. Encouraged by the productive results the same procedure was applied for the synthesis of a series of novel non-peptidic 16-membered (2Z,11Z)-3,11-di(aryl/dinaphthyl)-1,13-dioxa-5,9-dithia-2,12-diazacyclohexadeca-2,11-diene derivatives (**1b-k**) from various oximes (**8b-k**) and 1,3-dibromopropane (**9**) and the yields obtained were shown in scheme 6.

The synthesized compounds were characterized by ^{1}H-NMR, ^{13}C-NMR and HRMS data and the structure of one of the macrocyclic compound, (2Z,11Z)-3,11-bis(4-fluorophenyl)-1,13-dioxa-5,9-dithia-2,12-diazacyclohexadeca-2,11-diene (**1e**) was confirmed by single crystal X-ray diffraction studies as shown in figure.2.

Scheme 6. Synthesis of (2Z, 11Z)-3,11-di(aryl/dinaphthyl)-1,13-dioxa-5,9-dithia-2,12-diazacyclohexadeca-2,11-diene derivatives (**1**)[a,b,c].



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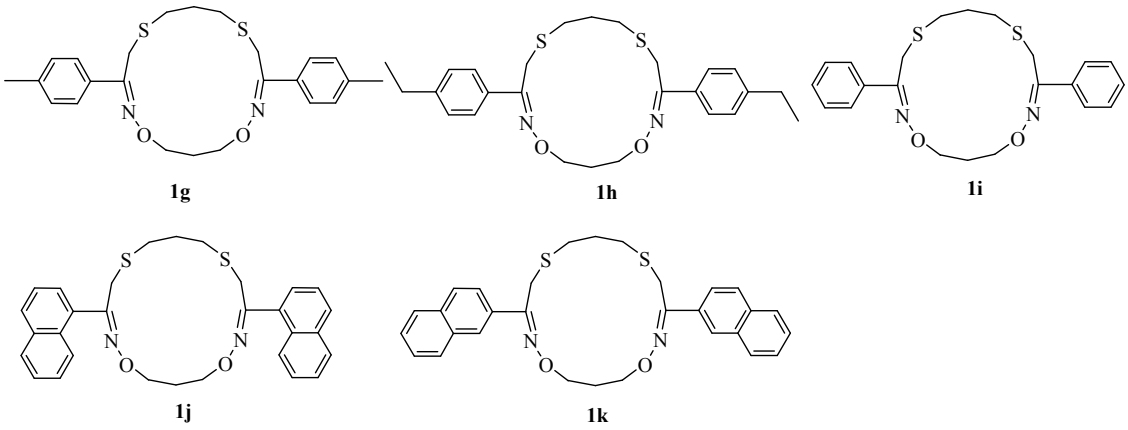

1g **1h** **1i**

1j **1k**

[a]**Reagents and conditions:** Substrates, **8a-k** (10.0 mmol), 1,3-dibromopropane (10.0 mmol) (**9**), KOH (20.0 mmol) in a mixture of H_2O-DMSO (1:5 ratio), 5-10°C, 25-35 min; [b]Isolated yields : **1a** (94%), **1b** (94%), **1c** (91%), **1d** (86%), **1e** (95%), **1f** (92%), **1g** (94%), **1h** (94%), **1i** (95%), **1j** (89%), **1k** (95%); [c]Overall Yield: (**%**): **82,76, 65, 48, 85, 74, 81, 75, 83, 69** and **83**.

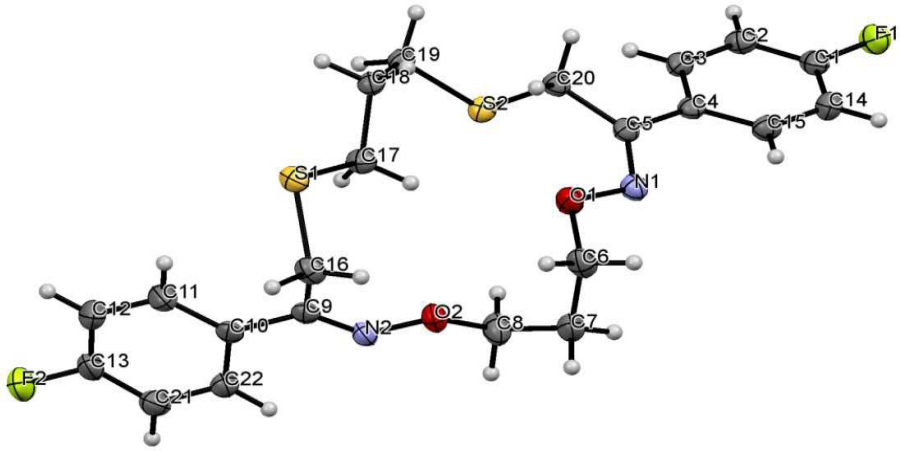

Figure 2. ORTEP view of (2Z, 11Z)-3,11-bis(4-fluorophenyl)-1,13-dioxa-5,9-dithia-2,12-diazacyclohexadeca-2,11-diene (**1e**).

Anti-tumor activity studies

The anti-tumor activity of macrocyclic compounds (**1a-k**) was evaluated *in-vitro* using triple negative breast cancer cells (TNBC) MDAMB-231. The anti-tumor effects of macrocyclic compounds were evaluated using standard anti-tumor assays, including wound healing assays (**Figure 3**) and colonogenic assay (**Figure 4**), depicting effect on cancer cells metastatic and cell division potential, respectively. For understanding the underlying molecular mechanism involved in anti-tumor effects of macrocyclic compounds (**1a-k**), transcription levels of key anti-tumor genes was measured by quantitative (q) RT-PCR (**Figure 5**). Fragmentation of genomic DNA representing induction of apoptosis was analyzed by DNA laddering (**Figure**

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6) in agarose gel electrophoresis. The cytotoxic effects of these compounds (**1a-k**) were measured on normal breast epithelial cells (MCF10A) (**Figure 7**).

Macrocycles inhibit migration of cancer cells

Tumor cells possess the property of cell migration and invasion that can be measured by standard wound healing assay measuring effect of anti-tumor drugs on migration cells in cancer cell line ex-vivo.[30] The inhibitory effect of macrocyclic compounds was evaluated in comparison to known anti-cancer drug 17-N-Allylamino-17-demthoxygeldanamycin (17AAG). The macrocyclic compounds **1a, 1f, 1g** and **1i** strongly reduced migration of TNBC MDAMB-231 and healing of the wound delayed (**Figure 3**), compounds **1b, 1e** and **1j** showed negligible effects on cell migration, while compound **1k** had moderate effect on migration of cancer cells (**Figure 3**). These results suggested that macrocycles **1a, 1f, 1g, 1i** and **1k** are anti-tumor in their nature.

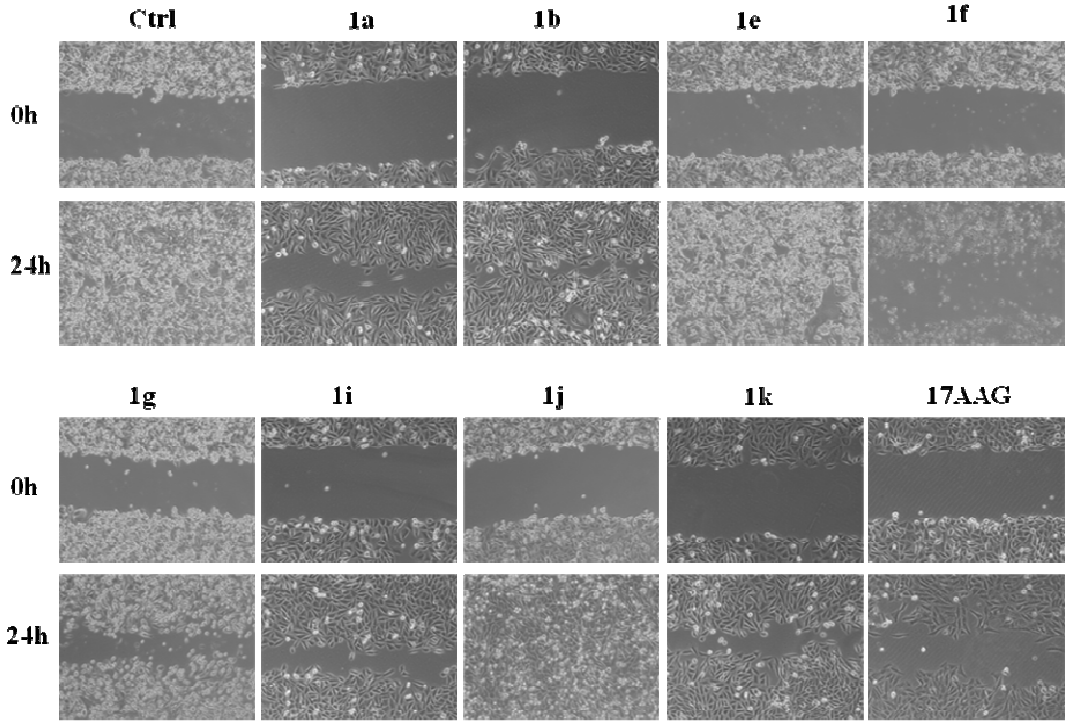

Figure 3: Effect of macrocyclic compounds in cell migration: Macrocyclic compounds (**1a, 1b, 1e, 1f, 1g, 1i, 1j** and **1k**) differentially inhibit the migration of TNBC MDAMB-231 in the wound. Compounds **1a, 1f, 1g, 1i and 1k** possess substantial anti-proliferative properties.

Macrocycles hampers proliferation of cancer cells

The cell division potential of cancer cells was measured by colonogenic assay besides measuring the effect of anti-tumor drug on survival and proliferation of cancer cells in ex-

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vivo system. The anti-proliferative effect of macrocyclic compounds (**1a**, **1b**, **1e**, **1f**, **1g**, **1i**, **1j** and **1k)** was evaluated on MDAMB-231 cells. Except 1b and 1j all the tested macrocycles possess varying degree of anti-proliferative potential (**Figure 4**), compared with positive controls 17AAG and hydroxyl urea (HU). Macrocycles **1a**, **1e**, **1f**, **1g**, **1i** and **1k** effectively inhibited colonogenic survival of MDAMB-231 cells. Similar results were observed with IMR32 cells (data not shown). In comparison to macrocycles **1e, 1f, 1g** and **1k**, macrocycles **1b** and **1j** showed least response on colonogenic survival of cancer cells, while macrocycles **1a** and **1i** had intermediate effect. These results suggest that macrocycles **1a**, **1e**, **1f**, **1g**, **1i** and **1k** are anti-tumor in their nature and the results are identical to wound healing assay.

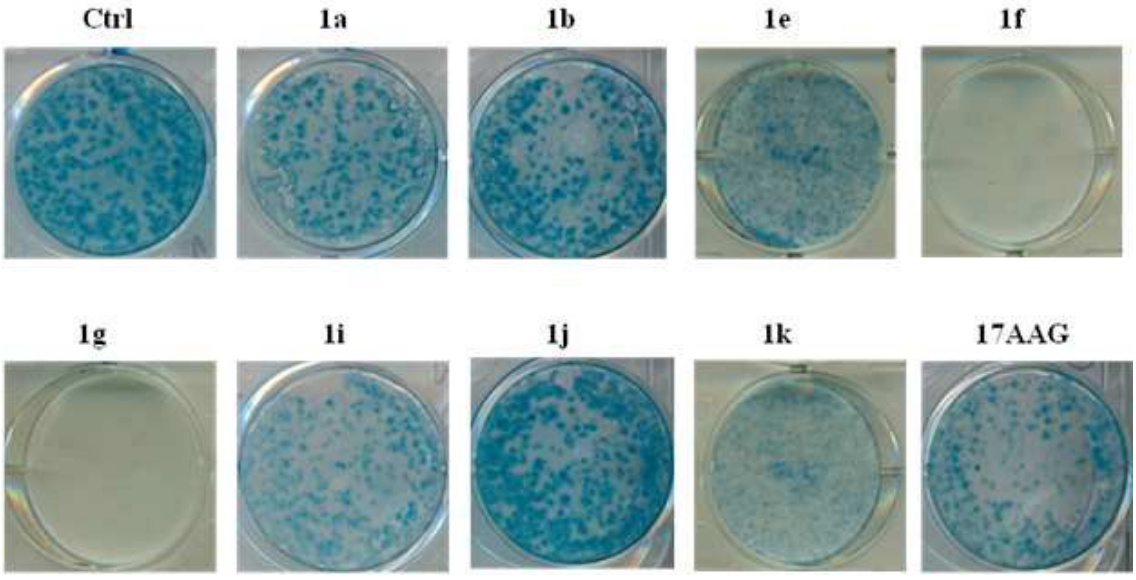

Figure 4: Effect of macrocyclic compounds on colonogenic survival of tumor-cells: Macrocyclic compounds (**1a**, **1b**, **1e**, **1f**, **1g**, **1i**, **1j** and **1k)** possess differential effect on colonogenic survival and proliferation of MDAMB-231 cells. Compounds **1e**, **1f**, **1g**, and **1k** substantially inhibited colonogenic survival of tumor cells.

Macrocyclic compounds induces transcription of anti-tumor genes

The anti-tumor agents are known to induce array of tumor suppressor genes such as p53, TRAIL, MDA7, STAT-3 etc. These genes exert anti-tumor activity by induction of apoptosis through variety of mechanisms, upregulation of tumor suppressor protein have direct impact on tumor cells survival.[31] Transcriptional upregulation of p53, TRAIL and MDA7 was observed for MDAMB-231 cells, treated with macrocyclic compounds **1a**, **1i**, **1f**, **1g** and **1k.** Compounds **1b** and **1j** did not show significant induction of anti-tumor genes. Previous studies clearly establish the role of TRAIL mediated induction of caspase dependent apoptotic cell death of tumor cells.[32, 33] Macrocycles compounds **1a**, **1f**, **1g**, **1i** and **1k**,

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upregulate transcription of TRAIL might be leading to activation of caspase dependent apoptotic cell death of MDAMB-231 cells. Macrocycles **1a**, **1i** and **1k** synergistically induce transcripts for TRAIL, MDA7 and p53 (**Figure 5**) genes, higher than observed with compounds **1f** and **1g**. The tumor suppressor gene p53 regulates transcription of TRAIL[34], also regulates MDA7 activities.[35]. The MDA7 is an anti-cancer gene, which can be induced by retroviral mediated restriction of tumor growth, both *in vivo* and *in vitro* [35, 36]. Among 8 tested macrocycles, compounds **1a**, **1f**, **1g**, **1i** and **1k** induced transcription of MDA7. Both MDA7 and TRAIL belong to IL-10 family of cytokines which are known to induce apoptosis in tumor cells in caspase-3 dependent manner that has been considered as model genes for studying anti-tumor activities and gene therapy.[37] Earlier report suggests that MDA7 induces apoptotic cells death of tumor cells in type –I interferon dependent manner,[31b] however, we have not found any induction of IFN-β gene (data not shown). The study suggests that anti-cancer activities might be interferon independent. Further it establishes that the down regulation of pro-cancer gene STAT3 concurrently elevates TRAIL mediated-induction of cell death [38] and two compounds **1a** and **1i** follow similar trend[38] (**Figure 5**). Induction of STAT3 in **1f**, **1g** and **1k** treatment groups might be antagonizing TRAIL effect and that might be the reason for the absence of DNA laddering in groups treated with these compounds (**Figure 6**).

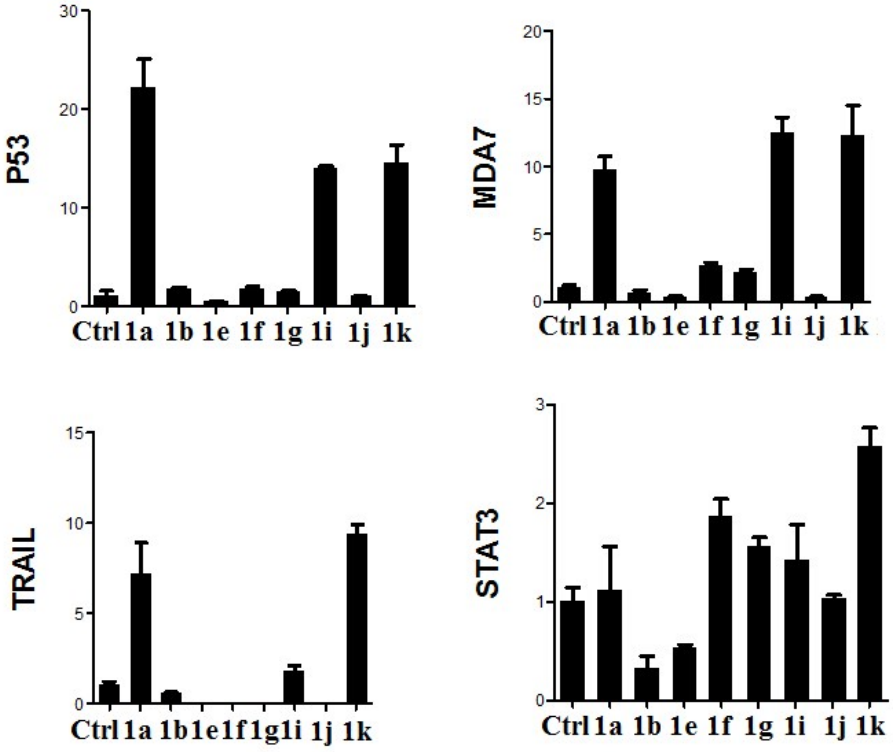

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Figure 5: Effect of macrocyclic compounds on expression of anti-tumor genes: Macrocyclic compounds **1a**, **1i** and **1k** robustly induced the transcription of p53, MDA7 and TRAIL, while **1f** and **1g** have moderate effect, however **1b** and **1j** fail to induce anti-tumor genes. Expression of genes is represented in fold change where as compared solvent only treated control groups and were measured after 24hrs treatment with respective compounds.

Macrocycles induces apoptosis in tumor cells

Among the investigated macrocyclic compounds (**1a**, **1b**, **1e**, **1f**, **1g**, **1i**, **1j** and **1k**), only two compounds **1a** and **1i** effectively induced DNA laddering in MDAMB-231 cells (**Figure 6**). The ladder like impression of genomic DNA in agarose gel represents fragmentation of DNA into nucleosome monomers (consisting of 146bp DNA) and oligomers, a caspase-3 dependent cellular event, observed during induction of apoptosis. These results suggest that compounds **1a** and **1i** induces caspase-3 mediated activation of apoptosis.

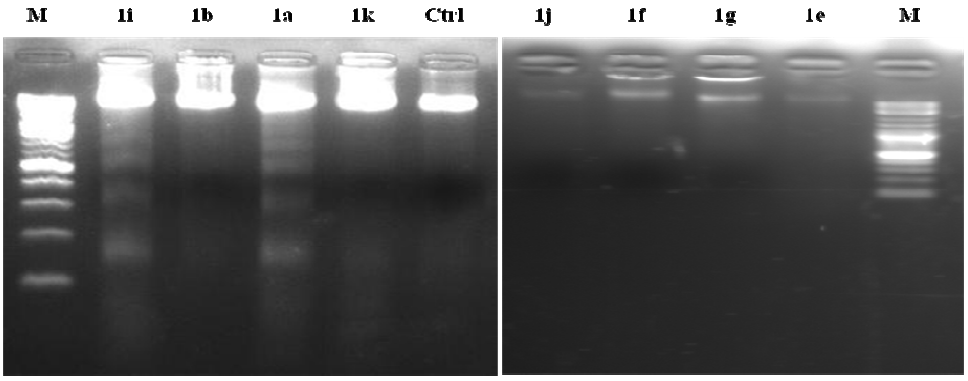

Figure 6: Effect of macrocyclic compounds on integrity of genomic DNA: Macrocyclic compounds **1a** and **1i** induce genomic DNA fragmentation in MDAMB-231 cells as evidenced from observed DNA ladder like impression of genomic DNA in agarose gel.

Macrocyclic compounds have no observed cytotoxic effect on normal breast cells.

None of the investigated macrocyclic compounds **1a**, **1b**, **1e**, **1f**, **1g**, **1i**, **1j** and **1k** exerted cytotoxic effect on breast epithelia cells (MCF10A) (**Figure 7**) were observed till 72 hours. These results suggest that macrocyclic compounds **1a**, **1f**, **1g**, **1i**, and **1k**, which restrict proliferation of tumor cells (**Figure 4**) have no cytotoxic effect on primary cells and can be considered for further validation in animal system.

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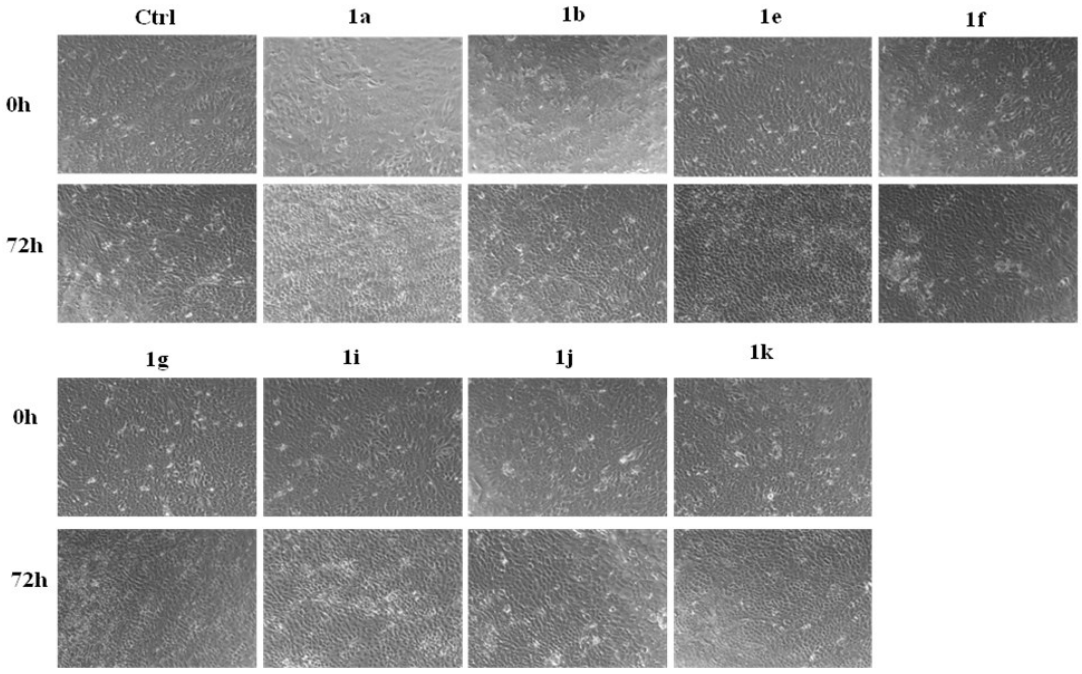

Figure 7: *Cytotoxic activity studies on primary human breast MCF10A cells:* Investigated macrocyclic compounds **1a**, **1b**, **1e**, **1f**, **1g**, **1i**, **1j** and **1k** have no cytotoxic effect on normal human breast epithelial cells (MCF10A).

Conclusion

We designed and synthesized a new class of small macrocyclic molecules, (2Z,11Z)-3,11-di(aryl/naphthyl)-1,13-dioxa-5,9-dithia-2,12-diazacyclohexadeca-2,11-dienes (**1a-k**) from readily available substrates using simple organic transformations. These macrocycles were evaluated for their anti-tumor effect on triple negative breast cancer cell line, MDAMB-231. Compounds **1a, 1f, 1g, 1i** and **1k** restrict cell invasion, compounds **1e, 1f, 1g,** and **1k** restrict cell proliferation and colonogenic survival, compounds **1a, 1i** and **1k** induces TRAIL, MDA7 and p53 anti-tumor genes. None of the tested compound was found to be cytotoxic for normal breast epithelial cells. Further, it would be of great interest to evaluate these macrocyclic compounds in animal system, especially the compounds **1a** and **1i** to establish its effect under physiological condition. Other notable advantages are (i) accessibility of diverse topologies due to presence of two 'S' atoms that are amiable to disrupt protein-protein interactions (PPIs) and (ii) the presence of pharmacophore cyclic oxime-ether functionalities (C=N-O-) which are suitable for further 'FG' transformations holds the potential to access lead analogs other than the present reported macrocyclic molecules.

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Materials and methods

General information

All commercially available starting materials, reagents and solvents were purchased from Sigma Aldrich, Acros organics and Merck were used without further purification. Melting points of various obtained products were determined and uncorrected. NMR spectra were recorded on a Varian, Jeol/AL and Bruker 400 MHz. Chemical shifts were expressed in parts per million (ppm), coupling constants were expressed in Hertz (Hz). Splitting patterns describe apparent multiplicities and were designated as s (singlet), br s (broad singlet), d (doublet), t (triplet), q (quartet), quintet, m (multiplet). High-resolution mass spectra (MS) and compound purity data were acquired on a Waters LCT premier XE TOF HRMS and single quadrupole system equipped with electro spray ionization (ESI) source and Exactive orbitrap mass spectrometer (Thermo Scientific). Single crystal XRD analysis was carried out on a Bruker Apex ii diffractometer with CCD detector MoKα radiation (λ = 0.71069 Å) operating ω-2θ scanning mode at room temperature. Thin-layer chromatography was performed on 0.25 mm Merck silica gel plates and visualized with UV light. Column chromatography was performed on silica gel. The structures of isolated compounds were elucidated on the basis of spectroscopic data (^{1}H &^{13}C-NMR, HRMS) and single crystal XRD analysis.

Cell culture

Human triple negative breast cancer (TNBC) MDAMB231-cells (ATCC; cat no. HTB-26™) and neuroblastoma cell, IMR-32 were grown in 10% fetal bovine supplemented (FBS) (GIBCO, Life technologies, USA) complete Dulbecco's modified Eagles medium (cDMEM) (Sigma Aldrich, USA). The primary human breast epithelial cells (MCF10) were cultured in MEBM (Lonza/clonetics MEGM Kit cat# CC-3150), supplemented with 100 ng/ml cholera toxin, 2 ml/liter bovine pituitary extract (cat# CC4009G), 0.5 ml/liter gentamicin sulfate (cat# 4081G), 0.5 ml/liter recombinant growth factor human (cat# CC-4017G), recombinant insulin human (cat# CC-4021G), 0.5ml/liter hypo cortisone (cat# CC-4031G). All the culture were maintained at 37°C in humidified chamber (Thermo Fisher Scientific USA), supplied with supplied with 5% CO_2. For subculture and seeding, TNBC MDAMB-231, IMR-32 cells were harvested with 0.01% trypsin-EDTA and MCF10A with 0.05% trypsin-EDTA, setting the cells density as per experimental requirements.

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Wound healing assay

For wound healing assay TNBC MDAMB-231 cells were seeded in six well plate, at density of 1 x 10^6 cells/well in cDMEM. The 95% monolayer of cells carefully wounded using 20-200 µl micro tips. Cells were washed with DMEM for removing cell debris. Wounded monolayer of cells was treated with **1a**, **1b**, **1e**, **1f**, **1g**, **1i**, **1j** and **1k** (5µM each) for 24 hrs. 17AAG (5 µM) and solvent only treated cells were used as positive and negative control. Migration of cells into wounded area was captured by phase contrast microscope (Zeiss Axiovert model) at 0 hr and 24 hrs time points.

Colonogenic assay

The anti-proliferative effect of macrocycles (**1a**, **1b**, **1e**, **1f**, **1g**, **1i**, **1j** and **1k**) were tested by this assay. TNBC MDAMB-231 cells were seeded in six well plates at density of 1 x 10^3 cells/well and left in culture for 10 days for formation of colonies. Than cells were treated with test macrocyclic compounds **1a**, **1b**, **1e**, **1f**, **1g**, **1i**, **1j** and **1k** (5µM each), positive control 17AAG (5µM) or hydroxyl urea (1mM) for 24 hrs. Followed by cells were washed with 1X-PBS and fixed using 70:30 solution of ethanol acetic acid for 10 minutes. Fixed cells were stained with 1% methylene blue for 15 min. Extra stain was drained by washing with water. Dried plates were scanned for representation of effect of macrocycles on colonogenic survival of cells.

Quantitative-PCR analysis

The MDAMB-231 cells were seeded in six well plate at density of 0.2 x 10^6. Next day, cells were treated with macrocycles **1a, 1b, 1e, 1f, 1g, 1i, 1j** and **1k** (5µM each) for 24 hrs. The cells were washed with 1X-PBS and total RNA was extracted TRIzol reagent (Invitrogen USA) and first strand cDNA was synthesized for 1µg RNA, using iScript-cDNA synthesis kit (BIORAD USA), according to manufacturers instruction. Relative quantification of anti-tumor genes (p53, MDA7, and TRAIL) and transcription factor STAT3 was performed with SYBR green master mix, using 18S as housekeeping control. Following, initial denaturation step at 95°C for 10 min, 40 cycles of PCR amplification were performed with denaturation for 15 sec and annealing at 60°C for 1 min. In order to confirm amplification as single specific product, melt curve analysis was done as 95°C for 15 sec, 60°C for 1 min, 95°C for 15 sec. The sequences of primer used in RNA quantification is given is **Table 1.**

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Table 1: Primer used for quantitative PCR

Primer	Direction	Sequence	Size bp	Amplicon
S18	Forward	ATCACCATTATGCAGAATCCACG	23	93 bp
	Reverse	GACCTGGCTGTATTTTCCATCC	22	
P53	Forward	GCCATCTACAAGCAGTCACAG	21	143 bp
	Reverse	TCATCCAAATACTCCACACGC	21	
TRAIL	Forward	AGCAATGCCACTTTTGGAGT	20	120 bp
	Reverse	TTCACAGTGCTCCTGCAGTC	20	
STAT3	Forward	TTTGTCAGCGATGGAGTACG	20	168 bp
	Reverse	TGTTGACGGGTCTGAAGTTG	20	
MDA7	Forward	GAGGAACACGAGACTGAGAG	20	116bp
	Reverse	TCCAGAGAAGCAGGGTAAAAC	21	

DNA fragmentation assay

Human TNBC MDAMB-231 and IMR32 cells were seeded in 6 well culture dish at density of 0.2×10^6 cells/well. Next day cells were treated with macrocyclic compounds **1a**, **1b**, **1e**, **1f**, **1g**, **1i**, **1j** and **1k.** Followed to 48 hrs incubation, cells were harvested by trypsin-EDTA and pellet down at 200g for 2 min. Cells washed with 1X-PBS and re-suspended lysis buffer (10 mM Tris-Cl: pH 8.0, 10 mM: EDTA, 0.5% Triton X-100) and nuclei pellet was collected at 13000g for 20 min at 4°C. Obtained nuclei pellet, re-suspended in lysis buffer and treated DNAse-free RNAse (0.1 mg/ml) for 1 h at 37°C. Then proteinase K (0.2 mg/ml) and SDS (final concentration of 1%) were added and a resultant mixture was incubated further for 2 h at 50°C. DNA from these samples was extracted using phenol chloroform isoamyl alcohol (25:24:1). DNA precipitation was done using 3.5 volume of 100% ethanol and 0.1 volume of CH_3COOK (pH 5.2) at -80°C. The DNA pellet was resuspended in 40µl of MQ and equal quantity of DNA was analyzed in 2% agarose gel.

Cell cytotoxicity assay

The cytotoxic effect of newly synthesized macrocyclic compounds **1a, 1b, 1e, 1f, 1g, 1i, 1j** and **1k** was analyzed on human breast epithelial cells MCF10A. Cells were seeded in 24 well plates at density of 0.5×10^6 cells/well, cultured till >90% cell density and treated with macrocyclic compounds **1a**, **1b**, **1e**, **1f**, **1g**, **1i**, **1j** and **1k** (200 µM each) for 24hrs. Cells micrographs were taken before and after treatment at 0h and 24h time points using phase contrast microscopy.

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General experimental procedure

Synthesis of 2-bromo-1-(4-chlorophenyl)ethanone (4a): In a 100-mL two necked round-bottom flask, 1-(4-chlorophenyl)ethanone (**2a**) (10.0 mmol), *N*-bromosuccinimide **(3)** (12.0 mmol), 10% (w/w) Silica gel in methanol (20 mL) were stirred under reflux conditions for 19 min. The progress of the reaction was monitored by TLC. After completion of the reaction, the stirring was stopped and cooled to RT. The solvent was removed under reduced pressure using rotary evaporator. To the crude product dichloromethane and distilled water were added. The organic layer was collected and again washed with water (2x50 mL) twice. The layers were separated and the organic layer was collected and it was dried using anhydrous Na_2SO_4. Again, the solvent was evaporated using rotary evaporator. The crude was purified over column chromatography using Silica gel (99:1 ratio of n-hexane and EtOAc) and pure product **3a** was obtained in 91% yield. The same experimental procedure was adopted for the preparation of other α-bromoketones (**4b-k**).

Synthesis of 2,2'-(propane-1,3-diylbis(sulfanediyl))bis(1-(4-chlorophenyl)ethanone (6a): In a 100-mL two necked round bottom flask, 2-bromo-1-(4-chlorophenyl)ethanone (**4a**) [10.0 mmol] in acetonitrile (15 mL) was taken. The temperature of the reaction mixture was cooled to 15-20^{o}C and then K_2CO_3 (30 mmol) was added. Then, the propane-1,3-dithiol **(5)**[5.0 mmol] was taken in acetonitrile (15 mL) and it was added drop wise using dropping funnel at 15-20^{o}C. The reaction mixture was stirred for 90 min at the same temperature up to the disappearance of the substrate (**4a**). The completion of the reaction was confirmed by TLC. Then, the reaction mass was filtered and then the filtrate was concentrated under reduced pressure. The obtained crude product (**6a**) was purified by column chromatography using silica gel (4:1 ratio of n-hexane and ethyl acetate). The same experimental procedure was adopted for the preparation of other 2,2'-(propane-1,3-diylbis(sulfanediyl))bis(1-aryl/naphthyl)ethanone derivatives (**6b-k**).

Synthesis of (1Z,1'Z)-1-(4-chlorophenyl)-2-(3-((Z)-2-(4-chlorophenyl)-2-(hydroxyimino)ethylthio)propylthio)ethanoneoxime (8a): In a 100-mL two necked round bottom flask, substrate (**6a**) [10.0 mmol] and hydroxyl ammonium chloride ($NH_2OH.HCl$) (**7**) [40.0 mmol] were dissolved in ethanol (30 mL) and then NaOAc (30.0 mmol w.r.t $NH_2OH.HCl$) was added. Then, the reaction mixture was stirred at reflux temperature for 2 h. The progress of the reaction was monitored by TLC. After completion of the reaction, reaction mixture was cooled to RT and then filtered, washed thoroughly with ethanol. The filtrate was collected and the solvent was removed under reduced pressure. To the crude

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reaction mass, water (50 mL) was added and the product (**8a**) was extracted with EtOAc (2x25 mL). Then organic layer was collected and washed with water (2x25 mL) twice. Again, the organic layer was collected and dried over anhydrous Na_2SO_4 and the solvent was removed under reduced pressure. The crude product (**8a**) was recrystallized from the ethanol (10 mL) and n-hexane (30 mL) mixture. The same experimental procedure was followed for the preparation of other ketoxime derivatives (**8b-k**).

Synthesis of (2Z,11Z)-3,11-bis(4-chlorophenyl)-1,13-dioxa-5,9-dithia-2,12-diazacyclohexadeca-2,11-diene (**1a**)**:** In a 100-mL two necked round bottom flask, compound **8a** (10.0 mmol) was dissolved in DMSO (15 mL). The reaction mass was cooled to 5 to 10°C. At this temperature, aqueous KOH solution (20.0 mmol of KOH was dissolved in 5 mL of water) was added slowly and stirred at 5 to 10°C for 15 min. TLC was checked to confirm the disappearance of substrate (it convert to dipotassium salt form). Then, 1,3-dibromopropane (**9**) [10.0 mmol] was taken in 10 mL of DMSO and was added slowly at 5 to 10°C and the reaction mass was stirred at the same temperature for 10 min. The reaction progress was monitored by TLC. After completion of the reaction, the reaction mixture was quenched in crushed Ice (100 mL) and then the product (**1a**) was extracted with diethyl ether (2x50 mL) twice and the solvent was evaporated under reduced pressure. The obtained crude product (**1a**) was purified by column chromatography using silica gel (99:1 ratio of n-hexane and ethyl acetate). The same experimental procedure has been followed for the preparation of other macrocyclic compounds (**1b-k**). The synthesized compounds were characterized by ^{1}H, ^{13}C NMR and HRMS spectral data and the structure of compound **1e** was confirmed by single crystal XRD data.

Supporting information:

Physical and spectral characterization data and single crystal XRD analysis data are available at

Single crystal XRD data:

Crystallographic data of macrocycle **1e** was deposited in CCDC, 12 Union Road, Cambridge CB2 1EZ, UK, **CCDC 1046587**. The detailed information and copies can be obtained free of charge at http://www.ccdc.cam.ac.uk.

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Discovery of a new class of 16-membered (2Z,11Z)-3,11-di(aryl/naphthyl)-1,13-dioxa-5,9-dithia-2,12-diazacyclohexadeca-2,11-dienes as novel anti-tumor agents

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Abstract: A series of new 16-membered macrocyclic compounds were synthesized from readily available substrates using simple organic transformations. Evaluation of in vitro anti-tumor activities on MDAMB-231 cell lines revealed that macrocycles (1a, 1f, 1g, 1i and 1k) are promising anti-tumor compounds, evidenced from inhibition of cell migration and proliferation, upregulation of anti-tumor genes p53, MDA7 and TRAIL.

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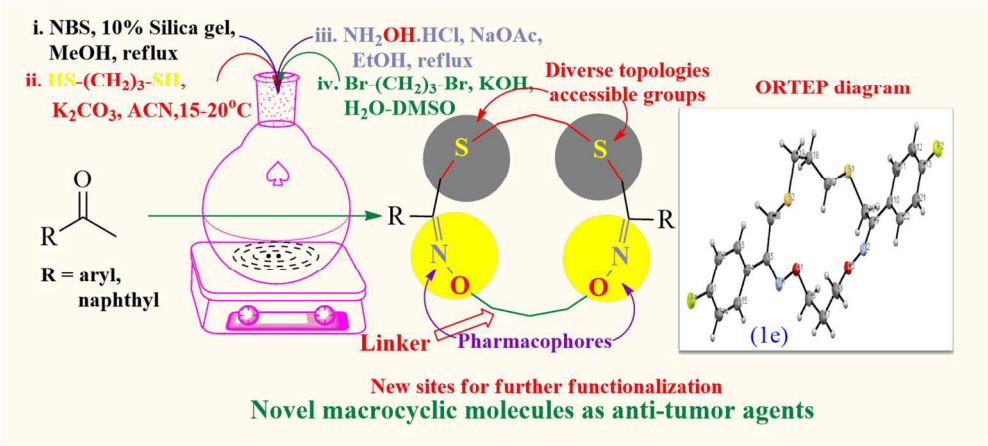

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