European Journal of Molecular & Clinical Medicine
ISSN 2515-8260 Volume 7, Issue 07, 2020

Design, Synthesis, Biological And In Silico Evaluation Of Phenylene (Bis) Hydrazone Derivatives Against Osteosarcoma Cancer

Pilli Govindaiah[1], Rakesh Kumar[2], Praveen Kumar Guttula[3], Mukesh Kumar Gupta[4], Jaya Prakash M[5]

[1]*School of Pharmaceutical sciences, Lovely Professional University, Phagwara, Panjab, 144411*
[2,4,5]*Department of Biomedical Sciences, National Institute of Technology, Rourkela, Odisha*
[3]*Department of Chemistry, National Institute of Technology, Rourkela, Odisha*
email: govindaiah.25372@lpu.co.in

ABSTRACT

Three sets of differentphenylene-bis(hydrazone) derivatives namely,Gc, Gd and Ge,were designed, synthesized and evaluated for their molecular properties andin vitro anticancer activity against human osteosarcoma MG-63 cells. All compounds showed potent anticancer activity against the MG63 cells with IC_{50}ranging from 18.27 to 21.68µM. Among three sets of compounds,Ge showed the most potent anticancer activity against osteosarcoma MG63 cells and was superior to standard anticancer reference drug,methotrexate (MTX). All compounds were characterized by spectroscopic studies (FT-IR, ^{1}H NMR, and^{13}C NMR). In silicomolecular properties and drug-like properties were predicted by using Osiris property explorer software. None of Gc and Ge set of compounds violated Lipinski's boundaries thereby suggesting good oral bioavailability. All the synthesized compounds possessed good pharmacokinetic properties in terms of absorption, distribution, metabolism and toxicity (ADMET). However, the GdSeries compoundswere predicted to be capableof crossing BBB due to their high lipophilicity. The Gc and Ge series of compounds showed good pharmacokinetic parameters within the acceptable range. All the synthesized drugs were predicted to have better pharmacokinetic properties thanthe MTX reference drug.Taken together, our study suggests that Ge series derivatives may be considered as lead drug molecules for possible anticancer applications to be useful against osteosarcoma.

Key words:Hydrazone,In silico, Molecular properties,Osteosarcoma, Cytotoxicity.

1. INTRODUCTION:

Cancer is a highly heterogeneous and complex disease caused by a number of etiological agents, many of which are unknown. No single targeting agents have been 100% successful in the treatment of cancer. Moreover, existing chemotherapeutic agents are becoming ineffective due to development of drug-resistance by the cancer cells,besides their side effects such as hepatotoxicity, myelotoxicity, neurotoxicity, pulmonary toxicity, urinary toxicity and cardiac toxicity upon their long-term use [Remesh, A., 2012]. Therefore, identification of causative agents and targeting newer molecules for the treatment of cancer has attracted wide

attention. Sarcoma is one of the cancers that arisefrom connective tissue such as bone, fat,muscle, and cartilage. Among allsarcomas, osteosarcoma isthe most frequently occurringbone cancers in children and young adults wherein bone growth occurs rapidly. It is a highly aggressive form of cancer and accounts for ~60% of all malignancies in children, adolescents and young adults[Vaidya*et al.,* 2020]. The osteosarcoma cells are usually resistance to apoptosis-inducing drugs including immunotherapy and hence, a number of newer drugs are under development for inducing cell death in osteosarcoma cells. Significant attempts are being made worldwide to use novel approaches for obtaining reliable and useful methods for treating this disease[Lindgren et. al., 2014.].

Hydrazone and their derivatives have recently gained attention in the recent past as highly active molecules for use as potential anticancer agents. They can be synthesized by the reaction between aromatic or aliphatic acid hydrazides (R-C=O)-NH-NH_2) and carbonyl compounds (i.e., aldehydes and ketones) and their derativeshave been shown to be capable ofinteracting with the active sites of tumor DNA [Vuda et al., 2016]. Hydrogen donors, acceptor groups,present on the drug moleculesincrease their interactions with targeting proteins or DNA to result in the inhibition of cell proliferation in the treatment of several diseases [Ren et al., 2014]. Bioactivity, based on hydrazone moiety, demonstrated that hydrazonecan form hydrogen bonds with ~~a~~ molecular target and, in addition, the N=C bond can process an additional reaction with the nucleophilic groups (i.e., NH_2, SH) in the target molecules to enhance their chemotherapeutic activity [Li et al., 2016].For example, pyrimidine bis-hydrazones were reported for the treatment of osteosarcoma as well as colon carcinoma cell lines [Amato et al., 2016]. Similarly, cyanoacetyl-hydrazoneswere also found to exhibit antitumor activity against cancer cell lines [Pillai et al., 2014]. Cyanocyclopentylidenehydrazide,Chloroacetylcyanohydrazide, and heterocyclic cyanohydrazide derivatives exhibited anticancer activity against SF-268- CNS cancer cell lines, NCI-H460- non-small cell lung cancer and MCF-7- breast adenocarcinoma [Mohareb et al., 2012; Mohareb and Mohamed 2010; Wardakhan et al., 2013]. On the other hand, salicylaldehyde benzoyl and Salicylaldehyde pyrazole hydrazone complexes were effective in anticancer therapeutics that inhibited the human adenocarcinoma cells. Salicylaldehyde pyrazole hydrazone was identified as one of the most efficient apoptosis inducers [Rodić et al., 2016]. Besides, hydrazone derivativesare also known to have other pharmacological applications such as antifungal, antimicrobial, anti-HIV (human immunodeficiency virus), antitubercular, antidiabetic, antimalarial, antidepressant, anti-panic and neuroprotective effects [Camacho et al., 2011; Ferreira et al., 2016; He at al., 2016; Imran et al., 2015;Kumar et al., 2010; MacKenzie et al., 2008; Neumann et al., 2014; Tatar et al., 2013; Vasantha et al., 2015].

The potential of hydrazone derivatives encouraged us to design and synthesize new phenylene-bis(hydrazone) derivatives and investigate their potential for anticancer activity with preferred pharmacokinetic properties. Based on previous studies, in the present study, we synthesized nine different bis-hydrazone derivatives and analyzed by spectral, theoretical, and biological studies [Kumar *et at.* 2016; Kumar *et at.* 2017; Kumar *et at.* 2020].All the synthesized compounds were evaluated *in silico* using a rational approach employing a pipeline of computational cheminformatics namely, Lipinski Rule of Five for biodisposibility, bioactivity prediction for drug likeness and toxicity prediction by Osiris property explorer and, absorption, distribution, metabolism and toxicity (ADMET) predictions for pharmacokinetics. Finally, the anti-cancer property of the synthesized compounds was tested against Osteosarcoma MG63 cells. Methotrexate, a FDA-approved standard anti-cancer drug, was used as control for comparison.

2.RESULTS AND DISCUSSION

2.1. Chemistry:

Three different set of compounds (named Gc, Gd and Ge series) were synthesized, with different hydrazine starting precursor molecules. The Gc series of compounds were synthesizedby a condensation reaction between cyano Aceto hydrazide and (para/meta/ortho)-phenyldialdehyde (Scheme-I). Gd series of compounds weresynthesized with phenylhydrazineand (para/meta/ortho)-phenyldialdehyde (Scheme-II), and the Ge series of compounds was synthesized with benzoyl hydrazine and (para/meta/ortho)-phenyldialdehyde (Scheme-III) in ethanol at room temperature (0.5-3h) and obtained good yields. Spectral data for all compounds (IR, ^{1}H NMR, ^{13}C NMR) were in full agreement with the proposed structures. As an example, in the IR spectrumof compound Gc1, the C≡N stretching peak was observed at 2287 cm^{-1} and the N-H stretching peak was observed at 3195 cm^{-1}. The ^{1}H NMR spectrum of compound Gc1 exhibited four singlets at 11.93, 8.38, 4.24 and 8.36 ppm, which corresponds to the protons of N-H, C-H, and C-H_2, respectively. The hydrogens on the aromatic ring core showed two multiple peaks at 7.78 and 7.49 ppm, respectively. The ^{13}C NMR spectra exhibited a C=O signal at 165.45 ppm, C=N signal at 144.10 ppm and, three aromatic signals at 132.23, 130.23, and 129.22 ppm, CN signal at 116.52 ppm and CH_2 signal at 25.05 ppm.

2.2Molecular modeling and evaluation of pharmaco-kinetic properties:

The potential oral bioavailability of the synthesized compoundswas evaluated by using Lipinski's Rule of Five, which is commonly used in pharmaceutical chemistry for design and development of drugs having good oral bioavailability. All the newly synthesized compounds were confirmed to fulfill all the parameters of Lipinski's Rule of Five, which suggests that they may have good oral bioavailability [Pajouhesh and Lenz, 2005]. Biodisponsibility of compounds are based on physiochemical parameters such as polar surface area (PSA), molecular weight (MW), rotational bonds (RB), hydrogen bond acceptor (HBA), hydrogen bond donor (HBD), and LogP (clog P) values. The PSA is the important property for the absorption and transportation of the drug molecule whereas LogP value is the partition coefficient in the octanol-water system, which is used to find out the lipophilicity of the drug molecule and influences the absorption, bioavailability, metabolism and toxicity risks of a drug.As shown in Table 1, Gd series of compoundshad higher LogPvalues than Gc and Ge series of compounds and hence,Gd compounds are highly lipophilic. The higher lipophilicity of the drug moleculecauses poor absorption or permeation andenhance the probability of the binding with hydrophobic proteins or receptors other than target system, and therefore there is a possibility for toxicity to the normal cells [Shahraki et.al., 2017]. On the other hand, Gc and Ge series of compounds had desired LogP value of less than 5.0. Further, all the synthesized compounds, including standard MTX, had desired molecular weight of less than 500 and desired PSA value of less than 160. However, while synthesized compounds showed desired HBD of less than 5, HBA of less than 10 and one or less violations, MTX did not fulfillthese threedesired parametersin the Lipinski's Rule of Five, which might explain its poor bioavailability after oral administration. The details of the calculated biodisponsibilityparameters are shown in Table 1.

The data in Table2 show the drug-likeness, drug score and toxic properties of synthesizedcompounds and MTX. These properties were computed by using Osiris property software [http://www.organic-chemistry.org/prog/peo/], which computes drug likeliness by comparing structural features of compounds with structural features of 3300 marketed drugs and 15000 commercially available chemicals and calculating drug-likeliness as the sum of the score values of the fragments present in the molecules. We observed that,Gc series

compounds demonstrated a negative drug-likeness score probably due to the presence of cyano and carbonyl groups present in their structure. On the other hand, Gd and Ge series of compounds showed positive drug-likeliness, which indicates the presence of molecular fragments that are frequently present in marketed drugs. The Geseries compoundsnot only showed higher drug score, when compared with Gc and Ge series compounds, but also had the best drug score among the all synthesized molecules (Table 2). The drug score was calculated by combining the drug-likeness score, clogP, logS, MW and toxicity risks.The positive drug score of all the synthesized compounds indicates that thesecompoundshave pharmacophore moieties or active sites and they can be potential drug molecule [Swathi et al., 2013]. We also observed that all synthesized drugs, including MTX, did not had undesirable mutagenic, irritant or reproductive effects, measured based on a pre-computed set of the structural fragments. These compounds also did not had tumorigenic effect except Gd series of compounds which showed high risk of tumorigenicity similar to those reported for MTX.

Pharmacokinetic properties such as absorption, distribution, metabolism and excretion (ADME) are central to the suitability of an anticancer agent for use as drug because a large number of therapeutic agents fails in their ADME properties and hence could not be used as drugs. The ADME properties of the synthesized drugs were therefore evaluated in terms of human intestinal absorption(HIA), permiability to Madin-Darby Canine Kidney (MDCK)epithelial cells, human intestinal epithelial adenocarcinoma(Caco-2) cells, and skin, plasma protein binding and blood-brain barrier (BBB) penetration [Yu and Adedoyin 2003].As shown in Table3, the HIA scoresof Gd and Ge series of compoundswere more than 90% while those of Gc series compoundswas 69.38%. The intestine is the prime site of absorption for oral drugs and a positive HIA score indicates that the compounds can be absorbed and assimilated through the intestine. Thus, high HIA score in Gd and Ge series of compounds suggests that they have higher intestinal absorption than Gc compounds. Importantly, all the synthesized compounds showed higher HIA score than the standard reference MTX drug.

CaCo-2 is a human colon epithelial cell line, which express proteins and enzymes responsible for assimilation of drugs and therefore, is commonly used as a model system for assessing the human intestinal assimilation of drugs. The MDCK cells have shorter growth period than CaCo-2 cells, soMDCK cells are useful for assessing the rapid permeability of drug molecule [Kovačević et al., 2014]. The predicted CaCo-2 cell permeability of all compounds were found to be in the acceptable range, which helps the drug molecules in transportation to the intestine and thus, better intestinal absorption. Furthermore, the MDCK cell permeability of Ge1 and Ge2 compounds were found to be the highest and most favorable for clearance through the kidney cells. All compounds showed negative values of skin permeability, which suggests that these compounds may not have any skin permeability effect.

It was also observed that the Gd series of compounds are more prone to cross blood-brain barrier (BBB) and have an effecton central nervous system (CNS) [Khan et al., 2017]. Plasma protein binding also affects the protein stability in the blood and therefore, distribution to body compartments and half-life of the drug in the circulation. From the plasma protein binding data,the stay of the drug molecule in the body system and the renal clearance can be predicted [Nisha et al., 2016]. It was observed that theGc series compoundshad low plasma protein binding whereas Gd and Ge series compoundsshowedexcellent plasma protein binding ability. The details of the ADME properties are shown in Table 3.

2.3 Biological activity of the synthesized compounds on osteosarcoma MG63 cells:

The biological activity of the synthesized compounds wasassessed by estimating their ability to influence metabolism and induce cell death in osteosarcoma MG63 cells. The MTT assay, which assesses the metabolic activity of the cells, showed that the *in vitro* exposure of synthesized compounds to the osteosarcoma MG63 cells exhibiteddose-dependent cytotoxic effect (Figure 1).All the synthesized compounds were superior to the reference standard MTX drug in inducing cell death at a concentration as low as 5 µg/ml. Among all the compounds, Gc1 and Ge3 compounds showed the highest level of cytotoxicity when used at a concentration of 50 µg/ml. After 24 h of incubation with compounds, the cells showed detachment from petridishes and morphological changes in plasma membrane with visible vesicle formation, which increased with the increase in the concentration of the compounds (Figures 2-4) and were suggestive of drug-induced cell deaths. Table 4shows the IC_{50}values, drug concentration at which 50% decrease in cell growth is observed after 48 h of incubation in the presence of the drug, of all synthesized compounds and the MTX. Our results show that all compounds exhibited good inhibition activity against the osteocarcenomaMG63 cells. The IC_{50} values ranged from 18.27 to 21.68 µM with Ge1 and Ge3having the best IC_{50} values of18.27 and 18.20µM, respectively. The reference standard MTX exhibited IC_{50} value of 26.52µM. The mechanism by which the synthesized compounds induce cell death is yet to be determined. However, given that, the hydrogen donors of the hydrazones derivatives are capable of interacting with the active sites of DNA [Vuda et al., 2016], it can be presumed that the synthesized compounds may have interfered with the cell division by inhibiting DNA synthesis and/or chain termination. The enhanced activity may be due to an additional reaction with the nucleophilic groups in the target molecules to enhance their chemotherapeutic activity [Li et al.,2016;Amato et al., 2016].

3. CONCLUSION:

Synthesis of phenylene bis (hydrazone) derivatives (3 different sets of isomers) were described, and their molecularproperties and biological activity against osteosarcoma MG63 cellswere studied. Our results suggest that all the synthesized compoundshave good oral bioavailability and possessgoodpharmacokinetic properties in terms of absorption, distribution, metabolism and toxicity. The synthesized compounds also had potent anticancer activity against the osteosarcoma MG63 cells in a dose-dependent manner. Among all compounds, the Gd seriescompounds were predicted to have tumorigenic toxicity and had the highest BBB permeability. The Ge series of compounds showed the best results and were superior to standard reference MTX anticancer drug. Taken together, our study suggests that Ge series derivatives maybe consideredas lead drug molecules for possible anticancer applications to be useful against osteosarcoma.

4. EXPERIMENTAL SECTION:

4.1 Chemistry:

All reactions were carried out in a 100 ml RBF (round bottom flask) in nitrogen gas atmosphere. All the starting materials were purchased fromSigma Aldrich (MO, USA) and Himedia (Mumbai, India) and were used without further purifying. All solvents were reagent grade. The progress of thereaction was monitored by Thin Layer Chromatography (TLC) with ethyl acetate-hexane (1:1) mixture as eluent. Melting points of the compounds were recorded on a Digital Auto melting/ Boiling point apparatus (LABARD LIM-252) and were uncorrected. All NMR spectra wererecorded on (for 400 MHz ^{1}H NMR, 100 MHz for ^{13}C NMR) Bruker FT-NMR spectrometer and chemical shifts were expressed in δ units relevant

to Tetra methyl silane (TMS) signal as an internal source in DMSO-d^6. Signals were indicated as s (singlet), d (doublet), t (triplet) and, m (multiplet, when multiplicity complex) for ^{1}H NMR. Coupling constants, J was recorded in Hz. IR spectra were recordedon Shimadzu FT-IR spectrometer (IR affinity 1S W/L with quest ATR mode).

4.1.1 General method for the synthesis of the cyano-hydrazones (Gc):

Cyano-hydrazones (Gc) series compoundswere synthesizedby acondensation reactionbetween aromatic bis-aldehydes [phthaladehyde, isophthalaldehyde, terephthalaldehyde (1m.mol., 134mg)] and cyanoacetyl hydrazide (2mmol, 198mg) in 100 ml of ethanol solvent in a 250 ml round bottom flask (Scheme-I).The solution was stirred at room temperature for 1.5 h. The reaction was completed within 1.5h. (monitored by TLC), then the precipitate formed was collected by filtration and washedwith ice-cold ethanol. The compounds were further purified by washing with 50 ml of methanol solvent [Can et al., 2017].

4.1.1.1 (1,2-phenylenebis(metanylydene)bis(2-cyanoacetohydrazide) (Gc1).

White color solid, Yield: 79%, m.p:162-164^{0}C, FT-IR (KBr) $\bar{\nu}$/cm^{-1}: 3195(N-H), 3067(C-H), 2287(C≡N), 1663(C=O), 1596(C=N).^{1}H NMR (400 MHz, DMSO-d_6, δ ppm) 11.93 (s, 2H, NH), 8.38 (s, 2H, N=CH), 7.78 (m, 2H, aromatic CH), 7.49 (m, 2H, aromatic CH), 4.24 (s, 4H, CH_2) ^{13}C NMR (100 MHz, DMSO-d_6, δ ppm): 165.45, 144.10, 132.23, 130.23, 129.22, 116.52, 25.05.

4.1.1.2 (1,3-phenylenebis(metanylydene)bis(2-cyanoacetohydrazide) (Gc2).

White color solid, Yield: 70%, m.p:220-222^{0}C, FT-IR (KBr) $\bar{\nu}$/cm^{-1}: 3204(NH), 2958(CH), 2287(C≡N), 1663(C=O), 1596(C=N). ^{1}H NMR (400 MHz, DMSO-d_6, δ ppm) 11.91 (s, 2H, NH), 8.0 (m, 2H, N=CH), 7.76 (m, 2H, aromatic CH), 7.50 (m, 2H, aromatic CH), 4.24 (s, 4H, CH_2) ^{13}C NMR (100 MHz, DMSO-d_6, δ ppm): 165.39, 159.47, 147.48, 144.01, 134.79, 129.68, 128.75, 126.0, 116.52, 25.05.

4.1.1.3 (1,4-phenylenebis(metanylydene)bis(2-cyanoacetohydrazide) (Gc3).

White color solid Yield: 75%, m.p:258-260^{0}C FT-IR (KBr) $\bar{\nu}$/cm^{-1}: 3210(NH), 3085(CH), 2257(C≡N), 1673(C=O). ^{1}H NMR (400 MHz, DMSO-d_6, δ ppm) 11.90 (s, 2H, NH), 8.0 (s, 2H, N=CH), 7.78 (d, 4H, J=4.4Hz aromatic CH), 7.49 (m, 2H, aromatic CH), 4.24 (s, 4H, CH_2) ^{13}C NMR (100 MHz, DMSO-d_6, δ ppm): 165.39, 144.01, 135.63, 127.97, 116.51, 24.79.

4.1.2 General procedure for the synthesis of Phenyl-hydrazones (Gd):

The Gd series compounds were synthesizedby condensation reaction between aromatic bis aldehydes [phthaladehyde, isophthalaldehyde, terephthalaldehyde (1mmol, 134mg)] and phenyl hydrazine (2mmol, 196µl) in 100 ml of ethanol solvent in a 250 ml round bottom flask (Scheme-II). The solution was stirred at room temperature for 2 h. The reaction was completed within 2 h (monitored by TLC), then the precipitate formed was collected by filtration and washed with ice-cold ethanol. The compounds were further purified by washing with 50 ml of methanol solvent.

4.1.2.1(1, 2-[bis-(2-phenylhydrazino) methyl] benzene) (Gd1):

Yellow color solid, Yield: m.p:178-180^{o}C FT-IR (KBr) $\bar{\nu}$/cm^{-1}: 3303(NH), 3040(CH), 1596(C=N); ^{1}H NMR (400 MHz, DMSO-d_6, δ ppm): 10.48 (s, 2H, NH), 8.39(s, 2H, N=CH), 7.80 (t, 2H, J=4.4Hz aromatic CH), 7.31 (m, 2H, J=3.6Hz aromatic CH), 7.23 (t, 4H, J=8Hz aromatic CH) 7.09(d, 4H, J=4Hz), 6.75(d, 4H, J=7.2Hz), ^{13}C NMR (100 MHz, DMSO-d_6, δ ppm): 145.53, 135.47, 132.78, 129.53, 128.11, 127.03,119.24,112.49.

4.1.2.2 (1,3-[bis-(2-phenylhydrazino) methyl] benzene) (Gd2):

Light orange color solid, yield:85%, m.p:236-238^{o}C FT-IR (KBr) $\bar{\nu}$/cm^{-1}: 3313(NH), 3050(CH), 1569(C=N); ^{1}H NMR (400 MHz, DMSO-d$_6$, δ ppm): 10.41 (s, 2H, NH), 7.89 (s, 2H, N=CH), 7.84 (s, 1H, aromatic CH), 7.59 (dd, 2H, J_1=7.6Hz J_2=8Hz aromatic CH), 7.39 (t, 1H, J=7.6Hz aromatic CH) 7.23(t, 4H, J=7.6Hz), 7.09(d, 4H, J=8Hz), 6.76(t, 2H, J=7.2Hz): ^{13}C NMR (100 MHz, DMSO-d$_6$, δ ppm): 145.56, 136.57, 136.51, 129.50, 129.33, 125.27,123.39,119.17, 112.36.

4.1.2.3(1, 4-[bis-(2-phenylhydrazino) methyl] benzene) (Gd3):

Yellow color solid, Yield;75%, m.p:252-254^{o}C, FT-IR (KBr) $\bar{\nu}$/cm^{-1}: 3303(NH), 3050(CH), 1569(C=N);^{1}H NMR (400 MHz, DMSO-d$_6$, δ ppm): 10.45 (s, 2H, NH), 7.83 (s, 2H, N=CH), 7.63 (s, 4H, aromatic CH), 7.22 (t, 4H, J=7.6Hz aromatic CH), 7.06 (d, 4H, J=8Hz aromatic CH) 6.75(t, 2H, J=6.8Hz); ^{13}C NMR (100 MHz, DMSO-d$_6$, δ ppm): 145.41, 136.58, 136.62, 129.54, 126.23, 119.29,112.33.

4.1.3 General procedure for the synthesis of Benzoyl-hydrazones (Ge):

The compounds were synthesized by the reaction between aromatic bis aldehydes [phthaladehyde, isophthalaldehyde, terephthalaldehyde (1mmol, 134mg)] and benzoyl hydrazide (2mmol, 272mg) in 100 ml of ethanol solvent in a 250 ml round bottom flask(Scheme-III). The solution was stirred at room temperature for 1.2 hrs. The reaction was completed within 1.2 h (monitored by TLC), then the precipitate formed was collected by filtration and washed with ice-cold ethanol. The compounds were further purified by washing with 50 ml of methanol solvent.

4.1.3.1(1, 2-[phenylenebis(methanylidene)] di(benzohydrazide) (Ge1):

White colour solid, yield:68%, m.p:176-178^{o}C, FT-IR (KBr) $\bar{\nu}$/cm^{-1}: 3237(NH), 3050(CH), 1643(C=O) 1560(C=N);^{1}H NMR (400 MHz, DMSO-d$_6$, δ ppm): 11.92 (s, 2H, NH), 8.56 (s, 2H, N=CH), 8.25 (d, 2H, aromatic CH), 7.93 (d, 4H, J=7.2Hz aromatic CH), 7.78 (d, 4H, J=7.6Hz aromatic CH) 7.58(m, 2H), 7.31 (m, 2H, J=3.6Hz aromatic CH);^{13}C NMR (100 MHz, DMSO-d$_6$, δ ppm): 164.66, 146.51, 134.28, 133.65, 132.22, 129.80,129.82, 128.48, 128.10.

4.1.3.2 (1, 3[phenylenebis(methanylidene)] di(benzohydrazide) (Ge2):

White colour solid, yield:78%, m.p:240-242^{o}C, FT-IR (KBr) $\bar{\nu}$/cm^{-1}: 3213(NH), 3012(CH), 1638(C=O), 1586(C=N);^{1}H NMR (400 MHz, DMSO-d$_6$, δ ppm): 11.98 (s, 2H, NH), 8.51 (s, 2H, N=CH), 8.13 (s, 1H, aromatic CH), 7.93 (d, 4H, J=7.2Hz aromatic CH), 7.78 (d, 4H, J=7.6Hz aromatic CH) 7.58(m, 7H); ^{13}C NMR (100 MHz, DMSO-d$_6$, δ ppm): 163.66, 147.51, 135.28, 133.65, 132.22, 129.80,129.22, 128.88, 128.00, 125.21.

4.1.3.3 (1, 4-[phenylenebis(methanylidene)] di(benzohydrazide) (Ge3):

White colour solid, yield:82%, m.p:268-270^{o}C, FT-IR (KBr) $\bar{\nu}$/cm^{-1}: 3237(NH), 3050(CH), 1643(C=O), 1554(C=N);^{1}H NMR (400 MHz, DMSO-d$_6$, δ ppm): 11.94 (s, 2H, NH), 8.49 (s, 2H, N=CH), 7.93 (d, 4H, J=7.2Hz aromatic CH), 7.83 (s, 4H, aromatic CH), 7.56 (m, 6H, aromatic CH); ^{13}C NMR (100 MHz, DMSO-d$_6$, δ ppm): 163.53, 147.36, 136.12, 133.70, 132.18, 128.88, 128.86,128.18, 128.00.

4.2 Evaluation of Pharmacokinetic profiles of the synthesized compounds:

All the synthesized compunds were evaluated for their pharmacokinetic profiles by*in silico* methods for predicting their molecular properties.

4.2.1 Lipinski Rule of Five:

To evaluate the potential oral bioavailability of the synthesized compounds, we examined the molecular structures of the above compounds by computational methods to confirm the Lipinski's Rule of Five [Lipinski et al., 2001]. This rule states that, the orally active compounds should have the molecular weight (MW) ≤ 500g/mol, a ClogP ≤ 5, hydrogen bond acceptors (HBA) ≤ 10, hydrogen bond donors (HBD) ≤ 5, and polar surface area ≤ 140A^2[34]. Osiris property explorer software was used to determine the molecular properties of these compounds in the scope of Lipinski Rule of Five.

4.2.2 Drug-likeness and Toxicity:

The drug-likeness and the drug score factors were obtained for all the synthesized compounds by using Osiris property software. The molecular structures were drawn in Chem Draw Ultra 12.0 and saved in structure data format (SDF) format and then uploaded into Osiris Property Explorer software.Thedrug score value was used to predict the potency of the drug molecule. The standard drug,Methotrexate, showed negative drug score due to its toxicity and low bioavailability. Further, we studied various possible toxicities like mutagenicity, tumorigenicity, reproductive effect, and irritant effects for thecompounds [Kalita et al., 2017].

4.2.3 ADME Profile:

The term ADME refers to absorption, distribution, metabolism, and excretion.These four progressions associated with the pharmacokinetic profile of substances are interrelating with living organisms [Hassan et al., 2013]. This ADME properties prediction provides valuable data to select better preclinical candidates. These pharmacokinetic profiles were evaluated by using PreADMET server, the molecular structures of the compounds were drawn online and submitted for ADME properties. The absorption properties such as HIA, MDCK cell permeability, Skin permeability and CaCo-2 cell permeability, distribution parameters, Plasma Protein Binding and Blood-Brain Barrier penetration were estimated.

4.3 Biological activity of the synthesized compounds against osteosarcoma MG63 cells:

The biologicalactivity of the synthesizedcompounds on osteosarcoma MG63 cellswere determined by treating the osteosarcoma cells with the synthesized compounds and estimating their metabolic activity using MTT assay. Standard MTX drug was used as positive control for comparison. Briefly, the osteosarcoma MG 63 cells (HiMedia AL007) were cultivated in high glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 0.2 mM L-Glutamine, 1% (v/v) Non-essential amino acids, 0.05 mM 2-mercaptoethanol, 100 U/ml penicillin, and 100 mg/ml streptomycin at 37 °C in a humidified atmosphere of 5% CO_2 in air. Experiments were conducted in triplicates in 200 µl of complete mediumwithout (negative control) or with 5, 10, 25 and 50 µg/ml of Gc1, Gc2, Gc3, Gd1, Gd2, Gd3, Ge1, Ge2, Ge3 or MTX (positive control) compounds in 96-well multiplatesfor 24 and 48 h. After 24 h of treatment, cells were examinedfor qualitative and quantitative assessment of cytotoxic activity based on optical microscopy and MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide, a tetrazole) assay, respectively[Carfi et al., 2007]. Briefly, 100 µl of MTT reagent was added to each well of 96well multiplate and incubated for 4 h in CO_2 incubator. Subsequently, the MTT solution was discarded and 100 µl of DMSO was added to each well.Acidic isopropanol (150 µl) was then added to each well to stop the reaction. The optical densities (O.D.)of the plates weremeasuredat 570 nm (Thermo Fisher, USA). The percentage of cell death was calculated as: (O.D. of treated group well-O.D. of the blankDMSO well)/O.D. of negative control multiplied by 100. Experiments were replicated three times and mean values were analyzed statistically by ANOVA after arc sine

transformation of the percent data. IC_{50} of the drug molecules were calculated as concentration of the drug required to inhibit the cell proliferation by 50% after 42h incubation, as determined by MTT assay, using statistical software GraphPad Prism version 5.00.

Supporting Information:

FT-IR spectra, ^{1}H and ^{13}C NMR spectraof the compounds areavailable in the supporting information.

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List of Figures and Tables

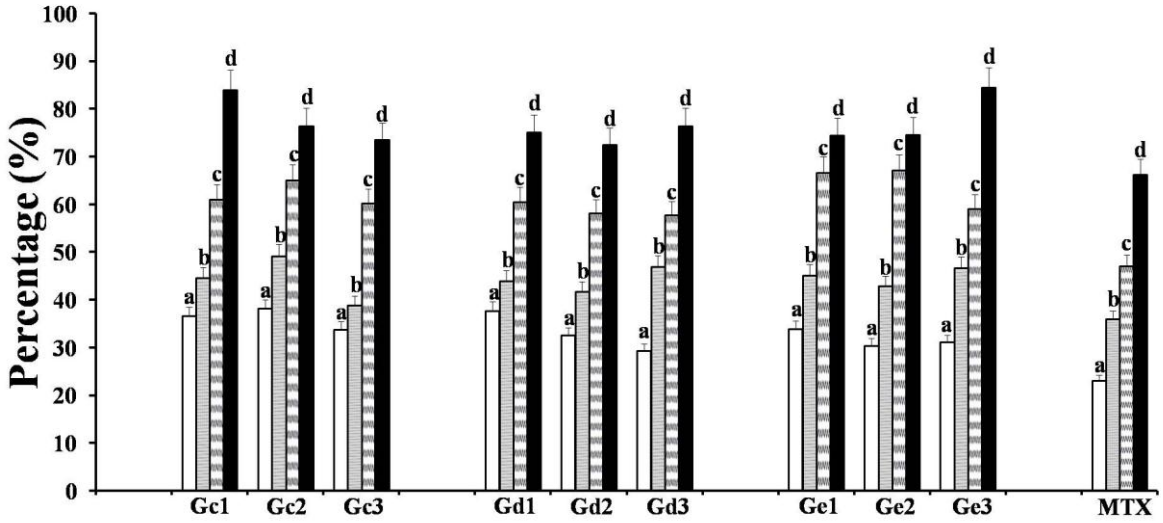


Figure 1. Percentage cell death induced by Gc, Gd and Gd series of compounds on human osteosarcoma cells. Cells were treated 5 (Bar 1), 10 (Bar 2), 25 (Bar 3), 50 (Bar 3) µg/ml concentration of compounds for 24h and analyzed by MTT assay. Bars with different superscripts (a,b,c,d) within each treatment group differ significantly ($p<0.05$)

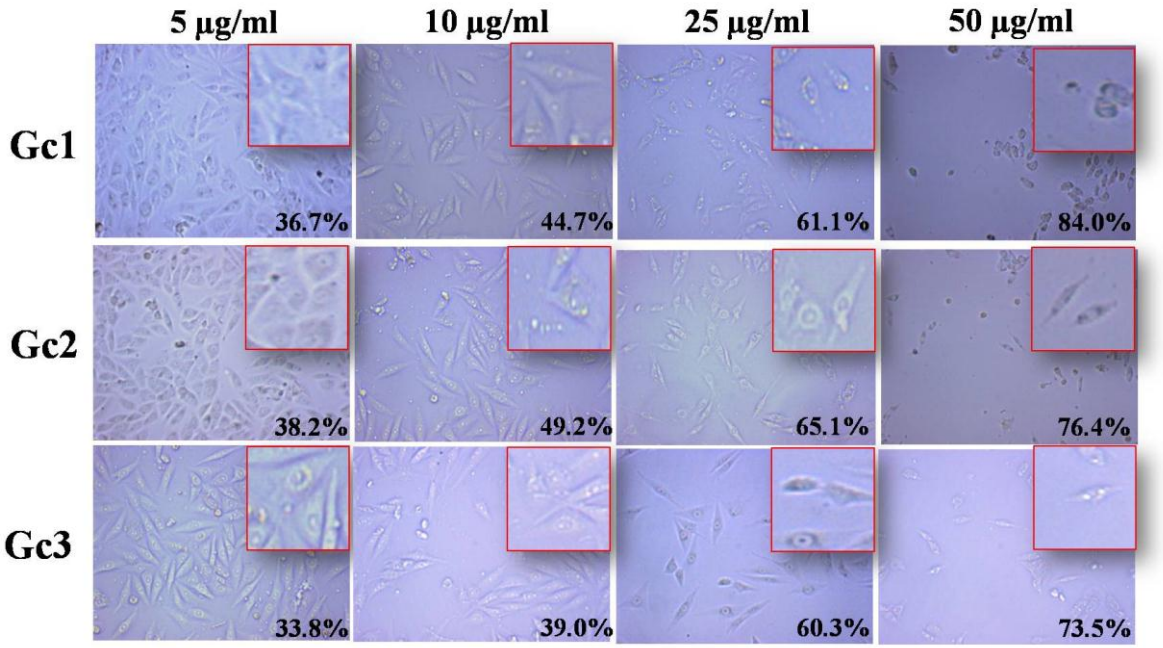


Figure 2: Anti-cancer activity of Gc series of compounds. Human osteosarcoma cells, treated without (Control) or with Gc 1, Gc 2 or Gc 3 compounds (5, 10, 25, 50 µg/ml), were

observed under bright field microscope for cytological changes after 24h of treatment. Numerical value shows the mean percentage deathof cells as estimated by MTT assay.Insets show the magnified region of the images for cytological changes. Bar 100 µm.

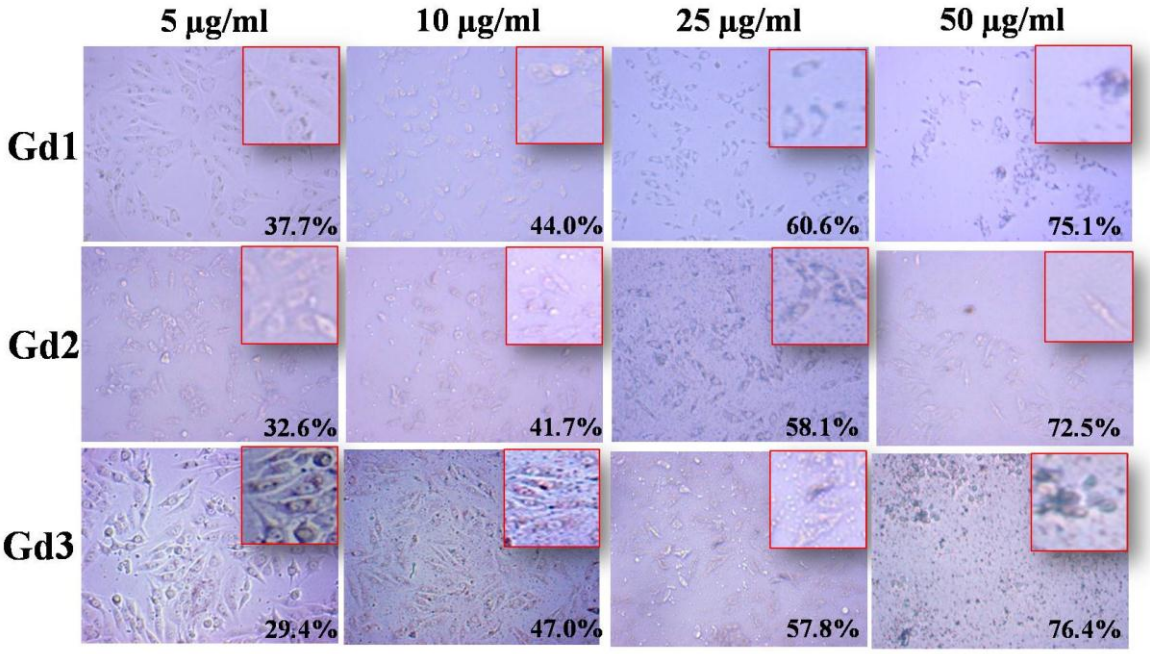


Figure 3. Anti-cancer activity of Gd series of compounds. Human osteosarcoma cells, treated without (Control) or with Gd 1, Gd 2 or Gd 3 compounds (5, 10, 25, 50 µg/ml), were observed under bright field microscope for cytological changes after 24h of treatment. Numerical value shows the mean percentage death of cells as estimated by MTT assay.Insets show the magnified region of the images for cytological changes. Bar 100 µm.

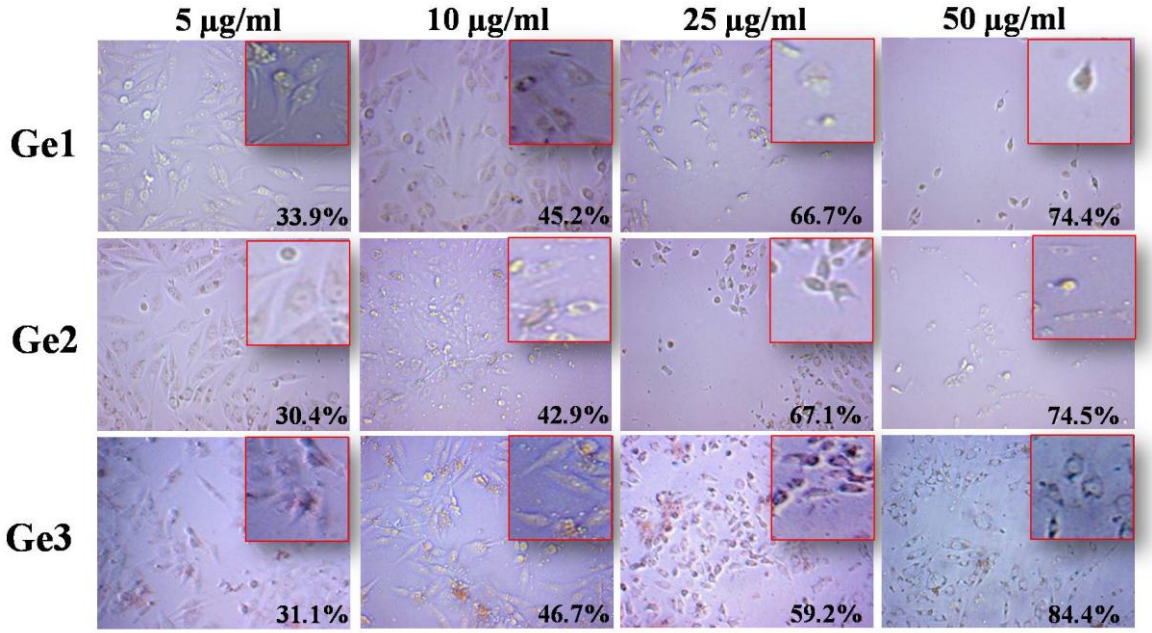


Figure 4. Anti-cancer activity of Ge series of compounds. Human osteosarcoma cells, treated without (Control) or with Ge 1, Ge 2 or Ge 3 compounds (5, 10, 25, 50 µg/ml), were observed under bright field microscope for cytological changes after 24h of treatment.

Numerical value shows the mean percentage death of cells as estimated by MTT assay.Insets show the magnified region of the images for cytological changes. Bar 100 µm.

Table 1: Theoretical oral biodisponsibility (Lipinski Rule of Five)

Compound	**PSA (A^2)**	**MW (Da)**	**HBA**	**HBD**	**Log (p)**	**No. of violations**
Gc1	130.51	296.29	8	2	-0.31	0
Gc2	130.51	296.29	8	2	-0.27	0
Gc3	130.51	296.29	8	2	-0.25	0
Gd1	48.78	314.00	4	2	7.54	1
Gd2	48.78	314.00	4	2	7.57	1
Gd3	48.78	314.00	4	2	7.59	1
Ge1	88.92	370.00	6	2	4.26	0
Ge2	88.92	370.00	6	2	4.21	0
Ge3	88.92	370.00	6	2	4.24	0
MTX	210.55	454.45	13	7	-1.97	2

PSA: Polar Surface Area; MW: Molecular Weight; HBA: Hydrogen Bond Acceptors; HBD: Hydrogen Bond Donor.

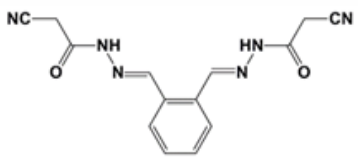
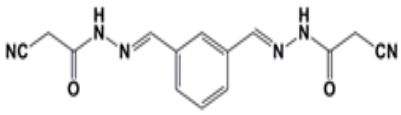
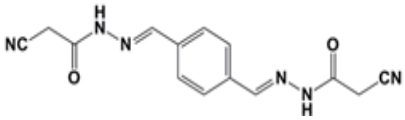
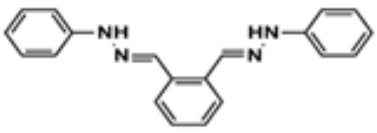
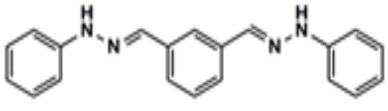
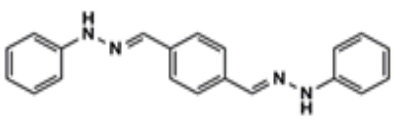
Table 2: Drug-likeness and toxic properties of hydrazone derivatives

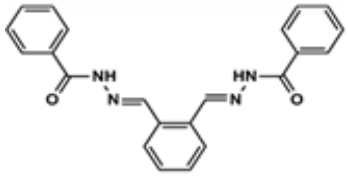
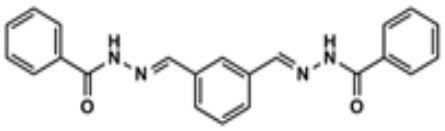
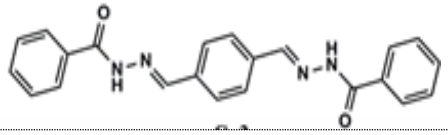
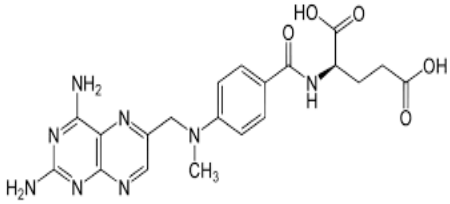
Compound	**Drug likeness**	**Drug Score**	**Mutagenic**	**Tumorigenic**	**Irritant**	**Reproductive Effect**
Gc1	-2.86	0.41	No	No	No	No
Gc2	- 2.86	0.41	No	No	No	No
Gc3	-2.86	0.41	No	No	No	No
Gd1	2.42	0.22	No	**High**	No	No
Gd2	2.42	0.22	No	**High**	No	No
Gd3	2.42	0.22	No	**High**	No	No
Ge1	4.46	0.46	No	No	No	No
Ge2	4.46	0.46	No	No	No	No
Ge3	4.46	0.46	No	No	No	No
MTX	-7.58	0.21	No	**High**	No	No

Table 3:Calculated ADME Properties

Compound	Absorption				Distribution	
	Human Intestinal Absorption (%)	CaCo-2 Cell Permeability (nm/s)	MDCK Cell Permeability (nm/s)	Skin Permeability (logkp,cm/hr)	Plasma Protein Binding (%)	Blood-Brain Barrier Penetration (c.brain/c.blood)
Gc1	69.38	18.37	9.74	-3.56	59.17	0.0461
Gc2	69.38	10.73	2.36	-3.56	51.05	0.0110
Gc3	69.38	18.15	4.74	-3.56	56.94	0.0089
Gd1	94.38	31.10	0.70	-1.87	94.07	6.2616
Gd2	94.38	51.67	1.26	-1.87	94.48	6.1425
Gd3	94.38	54.46	0.85	-1.87	91.95	5.5754
Ge1	94.71	21.24	33.38	-2.53	97.75	0.5181
Ge2	94.71	20.86	44.41	-2.54	96.91	0.1404
Ge3	94.71	21.21	0.95	-2.55	97.65	0.1270
MTX	35.71	18.88	2.44	-4.63	44.49	0.0402

Table 4: Half-maximal inhibitory concentration (IC_{50}) values of chemically synthesized compounds against MG 63 cell lines.

Compound	**Structure**	**IC_{50} (µM)**
Gc1		20.29
Gc2		19.21
Gc3		20.73
Gd1		20.61
Gd2		21.68
Gd3		19.91

Ge1		18.27
Ge2		18.71
Ge3		18.20
MTX		26.52