"Dibenzoylmethane derivatives for the treatment of childhood bone cancer"

Authors: Eman Al-melah, David Smith, Andrew McGown, Athar Aziz, J.H. Potgieter and Patricia Ragazzon.

Abstract

Childhood bone cancer though rare, has very limited treatment choices with poor survival rates and often involving amputation. We have developed a novel molecule, 2', 4'-dihydroxydithiondibenzoylmethane and tested it in hepatic, colon, lung and osteoblasts cancer cell lines. Thionylation of 2', 4'-dihydroxydibenzoylmethane has selectively targeted bone cancer cells, stopping the growth and leading to the death of those cancerous cells without affecting non-cancerous cells within the bone marrow or non-malignant cells.

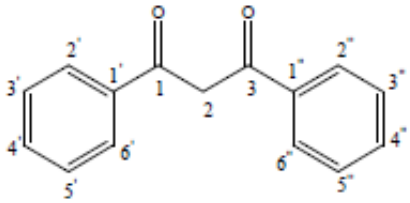
Introduction

Cancer in the bones can be either primary (initiated in the bones) or secondary (has spread to the bones)in nature. In primary bone cancer, osteosarcoma can affect any bone in the skeleton, though the femurs, tibia and humerus (arms and legs) are most commonly affected. Osteosarcoma accounts for about 3% of cancer in children, especially during early adolescence when bone growth is at its peak [1]. Ewing's sarcoma affects mainly teenagers and has an incidence of 34% of bone cancers. It appears mainly in the pelvis, thigh and shin areas, though this type of tumour can also occur in soft tissues (soft tissue sarcomas)[2]. Rare forms of bone cancer account for the remaining 6% of children with bone cancer [3]. Other primary bone cancers that affect mainly adults over 40 years old, are chrondrosarcoma in hips, legs, arms and shoulders; several variations of spindle cell sarcoma; and chordoma in lungs, nearby bones, lymph nodes, liver and skin. Secondary cancers can arise from primary tumours spreading to other tissues and the most prevalent tumours resulting from secondary bone cancer are prostate, breast, lung, kidney and thyroid.

As with any type of cancer, bone cancer can also be assessed in stages. For example, stage GX (also called stage 0) cannot be diagnosed, while low grade tumours will be divided in stages 1 and 2 and high grade tumours will be 3 and 4 [4]. Survival rate after treatment depends highly on a patient's age at the time of diagnosis, stage of the bone cancer and type of bone cancer. In teenagers with Ewing's sarcoma, survival rate after treatment ranges from 20 to 70% over 5 years but and is dependent on the site and size of the tumour [5-8].

Treatment generally involves some type of surgery, and the stage of the cancer will determine which type of surgery will be used. It can vary from removing only the area affected in the bone to amputation of the limb. Further treatment can include radiotherapy and chemotherapy [9-11]. Osteosarcomas can be treated with doxorubicin (a DNA intercalator), methotrexate (an antimetabolite) and cisplatin (a DNA alkylator). Ewing's sarcoma requires a mixture of drugs, including cocktail 1 consisting of vincristine (anti-mitotic agent), ifosfamide (DNA alkylator), doxorubicin, etoposide (Topoisomerase II inhibitor). This is followed by cocktail 2 made up of vincristine, ifosfamide and actinomycin D (DNA groove binder) and/or cocktail 3, which is a mixture of vincristine, actinomycin D and cyclophosphamide (DNA alkylator). Treatment of bone cancer with small molecules can be effective depending on the type of cancer and stage. However, there are side effects which affect the outcome for the patient. This can affect the rate of remission, produce delays in recovery times or cause long term effects such as fertility problems [12-17]. Biological therapies are currently being evaluated in clinical trials, with the aim to identify new molecules (generally antibodies or adjuvants) to encourage the immune system to attack the cancer cells by recruiting immune-modulators such as TNF (tumour necrosis factors) and INF (interferons)[18, 19].

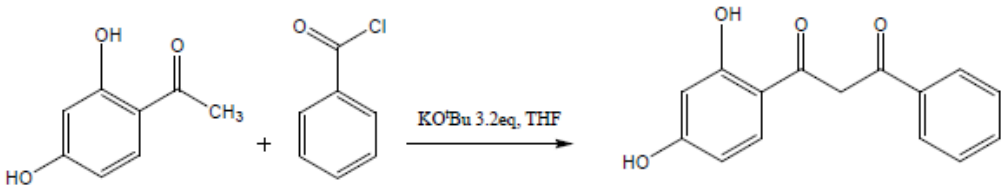
Dibenzoyl-methane derivatives (DBMD) (structure 1) have been found in tea genera and have been used in traditional medicine to treat several conditions, ranging from infection to rheumatic diseases, and as antioxidants. Their biological activity has not been completely elucidated but studies in other cell lines indicate activities in p53, Rb (retinoblastoma), and mitochondria [20-23].



Structure 1: dibenzoyl-methane.

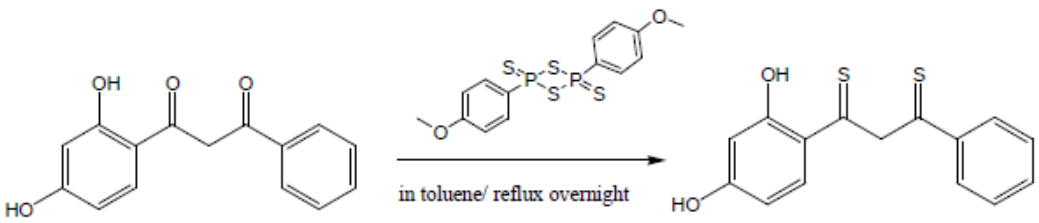
Generation of DBMD derivatives

We have employed the improved Baker-Venkatarman arrangement (Ares, Ragazzon) [25], (Scheme 1) to produce five dibenzoyl-methane derivatives: 2',4'-dihydroxy-dibenzoyl-methane (1), 2'-hydroxy-4'-amino-dibenzoyl-methane (2) , 3',4','-trimethoxy-dibenzoyl-methane (3) , 2',4'-dihydroxy-3''-methoxy-dibenzoyl-methane (4), and 2',4'-dihydroxy-4''-methoxy-dibenzoyl-methane (5).



Scheme 1: DBMD scaffold formation by reacting different analogues of 2-hydroxyacetophenone with benzoyl chloride and addition of potassium *tert*-butoxide.

Thionylation of 2',4'-dihydroxy-dibenzoyl-methane produced 2',4'-dihydroxy-dithio-dibenzoyl-methane (6), (Scheme 2).



Scheme 2: Thionylation of 2',4'-dihydroxy-dibenzoyl-methane using the Lawesson's reagent.

This paper describes the development a novel molecule, 2', 4'-dihydroxydithiondibenzoylmethane and derivatives thereof and highly promising results obtained in applying it against hepatic, colon, lung and osteoblasts cancer cell lines.

Materials and methods

A combination of compounds were obtained from various suppliers, e.g. **Alfa Aesar UK:** 3', 4', 5'-Trimethoxyacetophenone, benzoyl chloride, 3-Methoxybenzoyl chloride, Lawesson's Reagent. **TCI Tokyio Kasei:** 2', 4' –Dihydroxyacetophenone, 4-Methoxybenzoyl chloride. **Fluorochem:** Potassium tert-butoxide and Silica gel (60 Å). **Thermo Fisher Scientific:** 4-Amino-

2-hydroxyacetophenone, dry tetrahydrofuran, hydrochloric acid, toluene, ethyl acetate, petroleum ether, ethanol, hexane, dimethylsulfoxide, fetal bovine serum (GIBCO), RPMI-1640 (HyClone), 0.05% Trypsin-EDTA (GIBCO), sterile phosphate buffered saline. **VWR UK:** TLC Silica gel 60 F245 (Merck). **Cambridge isotope laboratories:** deuterated chloroform. **Sigma-Aldrich:** L-Glutamine 100x, MEM Non-Essential Amino acid 100x, penicillin-streptomycin 100x, chlorpromazine hydrochloride, MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) and Saos-2 cell line. **University of Salford Cell Bank:** HCT116 and A549 cell lines. **Cyprotex:** HepG2 cell line.

Synthesis of 2', 4'-dihydroxydibenzoylmethane (1)

2', 4'-dihydroxyacetophenone (1.0g, 6.57mmol) was dissolved in dry THF (tetrahydrofuran) (20ml) under argon added dropwise to a cooled mixture (0°C) of KO^{t}Bu (1.6equiv, 1.18g) in dry THF (50ml) under argon. The resulting white solution was left to stir at room temperature for 1 hour. The reaction mixture was then re-cooled to (0°C) before benzoyl chloride (1equiv, 0.762ml) was added dropwise, and the mixture was left to stir at room temperature for further 1 hour. The reaction mixture was cooled to (0°C) before KO^{t}Bu (1.6equiv, 1.18g) was added, and then the mixture was left to stir at room temperature for 15 min. Thereafter it was refluxed overnight with stirring. After 24h, TLC showed the presence of the product. The reaction mixture was then left to cool, and acidified water was added slowly until a light yellow precipitate was formed. The precipitate was separated by a Buchner funnel and left to dry for 5 min and then crystallized with ethanol to yield yellow needles. The TLC and NMR analysis showed the presence of starting materials, so further purification was required. Column chromatography using hexane: ethylacetate as eluent was carried out to purify the product and produced a pure solid. Yield: 51.2%. Yellow solid. Melting point: 158°C to 161°C. 1H NMR (CDCl3, ppm) δ 4.6 (s, 1H, H6'), δ 6.8 (s, 1H, H6), δ 3.4 (s, 1H, H10), δ 6.9-8.3 (m, 8H, Ar-H), δ 11.7 (s, 1H, H8'), δ 12.4(s, 1H, H8), 15.5(s, 1H, H7). IR (cm-1) 1741.42cm-1 (C=O), 3060.88CM-1(–OH stretch), MS m/z (relative intensity) 255.06(M-1). 13C NMR (CDCl3, ppm): δ 102.79 (C1), δ 158.05 (C2), δ 98.73(C3), δ 125.56(C4), δ 159.74(C5), δ 101.3(C6), δ 197.66(C7), δ 63.22(C8), δ 189.22(C9), δ 127.58(C10), δ 126.87(C11), δ 125.84(C12), δ 123.66(C13), δ 121.42(C14), δ 121.18(C15).

Synthesis of 2'-hydroxy-4'-aminodibenzoylmethane (2)

2-hydroxy-4-Aminodibenzoylmethane (0.2g, 1.32mmol) was dissolved in dry THF (tetrahydrofuran) (8ml) under argon and added dropwise to a cooled mixture (0°C) of KO^{t}Bu (1.6equiv, 0.24g) in dry THF (15ml) under argon. The resulting strong yellowish solution was left to stir at room temperature for 1 hour. The reaction mixture was then re-cooled to (0°C) before benzoyl chloride (1equiv, 0.154ml) was added dropwise, and the mixture was left to stir at room temperature for further 1 hour. The reaction mixture was cooled to (0°C) before KO^{t}Bu (1.6equiv, 0.24g) was added and the mixture was left to stir at room temperature for 15 min. It was subsequently refluxed overnight with stirring. After 24h, TLC showed the presence of the product. The reaction mixture was then left to cool, and acidified water was added slowly until a brown yellowish precipitate was formed. The precipitate was separated by Buchner funnel and left to dry for 5 min before being crystallized with ethanol to yield yellow needles. The TLC and NMR analysis showed the presence of starting materials, so further purification was required. Column chromatography using petroleum ether: ethylacetate as eluent was carried out to purify the product and produced a pure solid. Yield: 46.3%. Yellow solid. Melting point: 110°C to 112°C. 1H NMR (CDCl3, ppm) δ 4.6 (s, 1H, H6'), δ 6.8 (s, 1H, H6), δ 5.3 (s, 2H, H10), δ 6.9-8.2 (m, 8H, Ar-H), δ 11.8 (s, 1H, H8'), δ 12.4(s, 1H, H8), 15.5(s, 1H, H7). IR (cm-1) 3496.3-3370.2cm-1 N−H stretch (primary amine), 1602.39 (C=O), MS m/z (relative intensity) 254.08 (M-1). 13C NMR (CDCl3, ppm): δ 92.13 (C1), δ

163.83(C2), δ 108.14(C3), δ 134.38(C4), δ 163.88(C5), δ 115.44(C6), δ 194.45(C7), δ 26.41(C8), δ 176.87(C9), δ 144.66(C10), δ 133.68(C11), δ 132.13(C12), δ 129.92(C13), δ 128.50(C14), δ 127.14(C15).

Synthesis of 3', 4', 5'-trimethoxydibenzoylmethane (3)

3', 4', 5'-Trimethoxyacetophenone (1g, 4.76mmol) was dissolved in dry THF (tetrahydrofuran) (20ml) under argon and added dropwise to a cooled mixture (0°C) of KO^{t}Bu (1.6equiv, 0.85g) in dry THF (50ml) under argon. The resulting brown yellowish solution was left to stir at room temperature for 1 hour. The reaction mixture was re-cooled to (0°C) before benzoyl chloride (1equiv, 0.55ml) was added dropwise, and the mixture was left to stir at room temperature for further 1 hour. The reaction mixture was cooled to (0°C) before KO^{t}Bu (1.6equiv, 0.85g) was added and the mixture was left to stir at room temperature for 15 min. It was then left to reflux overnight with stirring. After 24h, TLC showed the presence of the product. The reaction mixture was then left to cool, and acidified water was added slowly until yellow precipitate was formed. The precipitate was separated by a Buchner funnel and left to dry for 5 min and then crystallized with ethanol to yield yellow needles. The TLC and NMR analysis showed the presence of starting materials, so further purification was required. Column chromatography using hexane: ethyl acetate as eluent was carried out to purify the product and produced a pure solid. Yield: 51.0%. Brown solid. Melting point: 99℃ to 102℃. 1H NMR (CDCl3, ppm) δ 4.6 (s, 1H, H6'), δ 6.8 (s, 1H, H6), δ 3.9 (s, 9H, H9), 6.95-8.2 (m, 7H, Ar-H). IR (cm-1) 2834.71cm-1 (-OH stretch), 1583.56(C=O), MS m/z (relative intensity) 313.10 (M-1). 13C NMR (CDCl3, ppm): δ 152.88 (C1), δ 142.67 (C2), (C3), (C4), δ 106.36(C5), (C6), δ 193.54(C7), δ 64.90(C8), δ 12.16(C9), δ 134.45(C10), δ 134.08(C11), δ 130.48(C12), δ 129.01(C13), δ 128.06(C14), (C15), δ 60.18(C-CH_3O) δ 50.04(C-CH_3O) δ ??(C-CH_3O).

Synthesis of 2', 4'-dihydroxy-3''- methoxydibenzoylmethane (4)

2', 4' –Dihydroxyacetophenone (1.0g, 6.57mmol) was dissolved in dry THF (tetrahydrofuran) (20ml) under argon and added dropwise to a cooled mixture (0°C) of KO^{t}Bu (1.6equiv, 1.18g) in dry THF (50ml) under argon. The resulting white solution was left to stir at room temperature for 1 hour. The reaction mixture was re-cooled to (0°C) before 3-Methoxybenzoyl chloride (1equiv, 0.923ml) was added dropwise, and the mixture was left to stir at room temperature for further 1 hour. The reaction mixture was cooled to (0°C) before KO^{t}Bu (1.6equiv, 1.18g) was added and the mixture was left to stir at room temperature for 15 min. It was then left to reflux overnight with stirring. After 24h, TLC showed the presence of the product. The reaction mixture was then left to cool, and acidified water was added slowly until light yellow precipitate was formed. The precipitate was separated by a Buchner funnel and left to dry for 5 min before being crystallized with ethanol to yield yellow needles. The TLC and NMR analysis showed the presence of starting materials, so further purification was required. Column chromatography using petroleum ether: ethylacetate as eluent was carried out to purify the product and afford a pure solid. Yield: 47.1%. Yellow solid. Melting point: 110℃ to 112℃. 1H NMR (CDCl3, ppm) δ 4.6 (s, 1H, H6'), δ 6.8 (s, 1H, H6), δ 3.5 (s, 1H, H10), δ 3.9 (s, 3H, H2) 6.9-8 (m, 7H, Ar-H), δ 11.8 (s, 1H, H8'), δ 12.4(s, 1H, H8), 15.5(s, 1H, H7). IR (cm-1) 2834.71cm-1 (-OH stretch), 1583.56(C=O), MS m/z (relative intensity) 285.07 (M-1). 13C NMR (CDCl3, ppm): δ 113.15 (C1), δ 163.96 (C2), (C3), δ 113.15(C4), δ 164.22(C5), δ 119.21(C6), δ 194.72(C7), δ 92.50(C8), δ 177.36(C9), δ 134.92(C10), δ 120.51(C11), δ 119.21(C12), δ 114.55(C13), δ 129.75(C14), δ 130.24(C15), δ 55.53(C-CH_3O).

Synthesis of 2', 4'-dihydroxy-4''-methoxydibenzoylmethane (5)

2', 4' –Dihydroxyacetophenone (1.0g, 6.57mmol) was dissolved in dry THF (tetrahydrofuran) (20ml) under argon and added dropwise to a cooled mixture (0°C) of KO^{t}Bu (1.6equiv, 1.18g)

in dry THF (50ml) under argon. The resulting white solution was left to stir at room temperature for 1 hour. The reaction mixture was re-cooled to (0°C) before 4-metoxybenzoyl chloride (1equiv, 0.89ml) was added dropwise, and the mixture was left to stir at room temperature for further 1 hour. The mixture was cooled to (0°C) before KO^{t}Bu (1.6equiv, 1.18g) and then was left to stir at room temperature for 15. It was left to reflux overnight with stirring. After 24h, TLC showed the presence of product. The reaction mixture was then left to cool, and acidified water was added slowly until a light yellow precipitate was formed. The precipitate was separated by a Buchner funnel, left to dry for 5 min and then crystallized with ethanol to yield yellow needles. The TLC and NMR analysis showed the presence of starting materials, so further purification was required. Column chromatography using hexane: ethylacetate as eluent was carried out to purify the product and produced a pure solid. Yield: 51.3%. Yellow solid. Melting point: 137°C to 141°C. 1H NMR (CDCl3, ppm) δ 4.6 (s, 1H, H6'), δ 6.75 (s, 1H, H6), δ 3.5 (s, 1H, H10), δ 3.9 (s, 3H, H3) 6.8-8.3 (m, 7H, Ar-H), δ 11.8 (s, 1H, H8'), δ 12.4(s, 1H, H8), 15.5(s, 1H, H7). IR (cm-1) 3475.03cm-1 (−OH stretch), 1606.08(C=O), MS m/z (relative intensity): 285.07 (M-1). 1C NMR (CDCl3, ppm): δ 102.24 (C1), δ 164.16 (C2), δ 108.07(C3), δ 133.69(C4), δ 166.96(C5), δ 113.77(C6), δ 202.68(C7), δ 55.40(C8), δ ??(C9), δ 122.91(C10), δ 131.30(C11), δ 112.82(C12), δ 26.33(C-CH_3O).

Synthesis of 2', 4'-dihydroxydithiondibenzoylmethane: thionylation of the carbonyl groups of 2', 4'-dihydroxydibenzoylmethane (6)

Lawesson's reagent (0.6equiv) was added to Benzoyl-(2', 4'-dihydroxy benzoyl)-methane (0.5g, 1.95mmol) which was dissolved in toluene (25ml). The reaction was then refluxed with stirring overnight. After 24h TLC showed the presence of the product. The solvent was then evaporated and the residue purified by preparative TLC using petroleum ether: ethylacetate as eluent to yield a red solid. Yield: 60.7%. Red solid. Melting point: 165°C to 167°C. 1H NMR (CDCl3, TMS, ppm) δ 3.3 (s, 1H, H6'), δ 5.3 (s, 2H, H10), δ 6.9-8.5 (m, 8H, Ar-H). IR (cm-1) 3246.42cm-1(−OH stretch), 3065.79cm-1 (C−H stretch), MS m/z (relative intensity): 575.07 (2M-1). 1C NMR (CDCl3, ppm): δ 102.79 (C1), δ 158.05 (C2), δ 98.73(C3), δ 125.56(C4), δ 159.74(C5), δ 101.3(C6), δ 197.66(C7), δ 63.22(C8), δ 189.22(C9), δ 127.58(C10), δ 126.87(C11), δ 125.84(C12), δ 123.66(C13), δ 121.42(C14), δ 121.18(C15).

Cell maintenance:

Cells were grown as adherent monolayer culture in 75cm^2 flasks in RPMI 1640 medium supplemented with 10% heat inactivated fetal bovine serum (FBS), 1% v/v nonessential amino acids (NEAA), 2mM L-glutamine and 115 units/mL of penicillin G, and 115 µg/mL of streptomycin at 37 °C under a humidified atmosphere containing 5% CO_2 and 95% air.

MTT and IC_{50} determination:

The six compounds were tested at a range of concentrations (range, 0-100 µM) and cell death percentage was determined by the colorimetric MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] micro-culture assay. Cells were detached from the 75cm^2 flasks (at a confluence of 70%) by trypsinisation, seeded in 100µL aliquots into 96-well clear micro-culture plates. Cell densities of 3.0 × 10^3 cells/well for 72 hours of incubation was chosen in order to ensure exponential growth of untreated controls throughout the experiment. Cells were allowed to adhere into the 96-well micro-culture plate for 24 hours prior to dosing. Stock solutions of the test compounds in DMSO were appropriately diluted in complete culture media to make up the required concentrations, and then added in 25µL aliquots into the 96-well micro-culture plate. Cells were exposed to the test compounds for 72 hours. Plates were maintained at 37°C in a humidified atmosphere containing 95% air and 5% CO_2. At the end of the incubation period, 30µL/well MTT solution in phosphate-buffer

(PBS) (3 mg/mL) were added, and then incubated for further 3 hours. After the end of the incubation, the supernatants containing the medium and MTT were removed and the formazan crystals formed by viable cells were dissolved in 100µL of DMSO per well. Optical densities at λ = 540 nm were measured with LUMIistar Omega multi-mode plate reader. The colorimetric MTT assay was used to determine the cell death percentage at serial diluted concentrations of the tested compounds and the IC_{50} (the concentration at which 50% of cell growth was inhibited as compared to the control wells which did not contain any drug) was determined from a dose response curve using OriginPro 9.1 data analysis and graphing software.

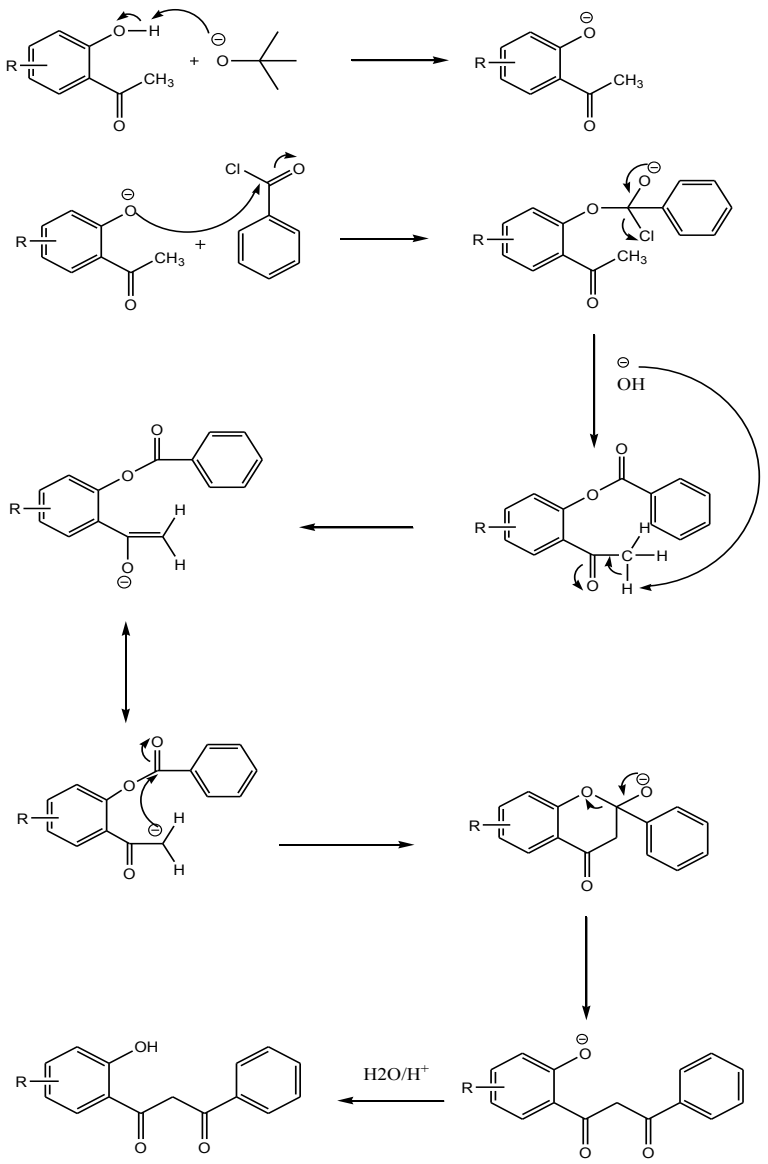
Caspase assay:

2', 4'-dihydroxydithiondibenzoylmethane was tested at a range of concentrations (range, 0-5-10 and 20 µM) and caspase activity was determined by using an ApotoxGlo® (Promega) assay. Cells were detached from the 75cm^2 flasks (at a confluence of 70%) by trypsinisation, and seeded in 100µL aliquots into 96-well clear micro-culture plates. Cell densities of 5, 4 and 3.0 × 10^3 cells/well were incubated for 24, 48 and 72 hours, respectively. Cells were allowed to adhere to the 96-well micro-culture plate for 24 hours prior to dosing. Stock solutions of the test compounds in DMSO were appropriately diluted in complete culture media to make up the required concentrations, and then added in 25µL aliquots into the 96-well micro-culture plates. Cells were exposed to the test compounds for 24, 48 and 72 hours. Plates were maintained at 37°C in a humidified atmosphere containing 95% air and 5% CO_2. At the end of the incubation period 20µL/well of the part 1 ApotoxGlo® was added and after 30 minutes of incubation at 37ºC cell viability was read at 404 excitation / 520 emission with LUMIistar Omega multi-mode plate reader. Caspase assay was performed by adding part 2 of the ApotoxGlo® as 100µL/well and incubating for 30 minutes at room temperature. The luminescence was read using the same plate reader in luminescence mode. The data was analysed and normalised.

Results:

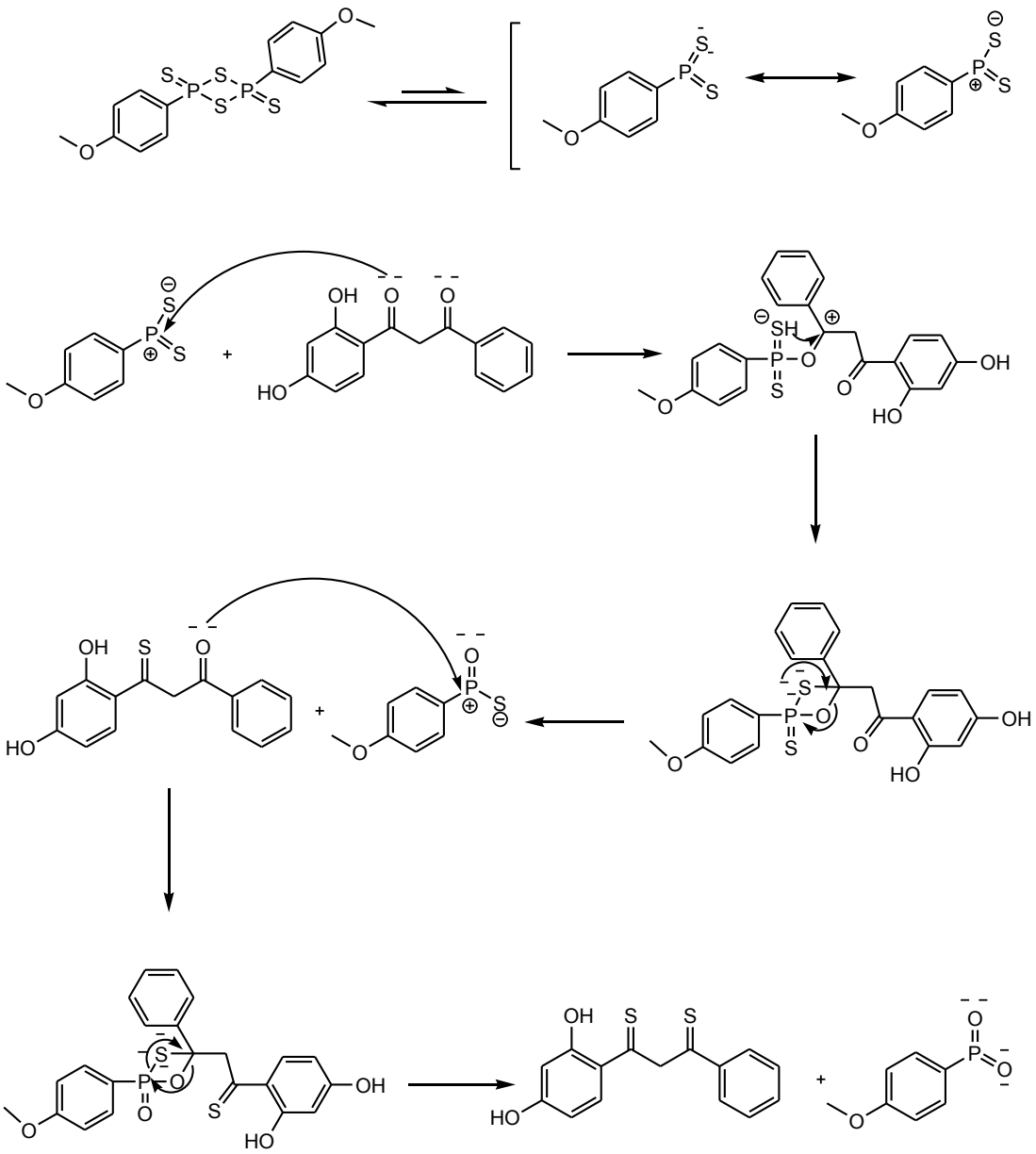
Chemical synthesis.

A new family of dibenzoyl-methane derivatives were synthesised employing the Baker-Venkatarman rearrangement of acetophenones with benzoyl chloride in the presence of potassium tert-butoxide. The 2-acetoxyacetophenones reacted in a straightforward manner with benzoyl chloride in the presence of KOtBu (acting as the condensing agent) via the formation of enolate, followed by the acyl transfer as shown in scheme 2:



Scheme 2: Elucidation of the reaction mechanism of the synthesis of dibenzoylmethane derivatives.

To obtain thionylated derivatives that could provide different characteristics, we synthesised 2', 4'-dihydroxydithiodibenzoylmethane, by reacting the benzoylmethane with the Lawesson's reagent as shown in scheme 3.



Scheme 3: Schematic elucidation of the reaction mechanism of the synthesis of 2', 4'-dihydroxydithiodibenzoylmethane.

The reactions proceeded without any problem, and compounds were purified using flash chromatography and then fully characterised (Accurae MS, NMR and IR). The two carbonyl oxygens on the dibenzoyl-methane derivatives are known to form hydrogen bonding. This was observed in the proton NMR at higher than 10ppm.

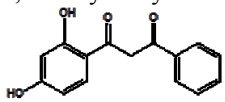
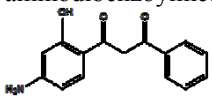
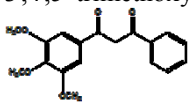
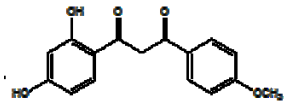
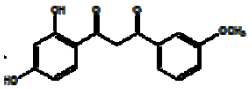
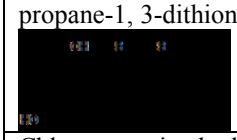
Biological activity

Saos-2 (Sarcoma osteogenic**)** is a bone cancer cell line derived from the primary osteosarcoma of an 11-year-old Caucasian girl. This cancer cell line possesses several osteoblastic features that makes it extremely useful as a permanent line of human osteoblast-like cells for drug discovery and as a source of bone-related molecules [24].

We tested all six compounds on HepG2 (children liver cancer), HCT116 (adult colon cancer), A549 (adult lung cancer) and Saos2 (children osteoclast) cell lines for 72 hours (Table 1). 2',4'-dihydroxybenzoylmethane (1), 3',4', 5'-trimethoxybenzoylmethane (3) and 2',4'-dihydroxy – 3'' methoxy benzoylmethane (4) showed similar activity on all the cell lines with 4-10 (µM) for IC_{50} at 72 hours of incubation. The amino substituent in 2'- hydroxyl-4'-

aminobenzoylmethane (2) produced loss of activity by a factor of 5 times on HepG2, HCT116 and A549 with near 20 (µM) for IC_{50} at 72 hours of incubation. However, it showed a clear preference for Saos-2. From this group with hydroxyl and methoxy substitutions, 2',4'-dihydroxy – 4'' methoxy benzoylmethane (4) presented high activity at around 4 (µM) for IC_{50} at 72 hours of incubation, but no selectivity on the cell lines.
From our six compounds, the tyonilated 1-(2', 4'-dihydroxyphenyl)-3-phenyl-propane-1, 3-dithion (6) showed poor activity on HepG2 and A549 (IC_{50} of 33.43 and 43.54 µM respectively), moderate activity on HCT116 (IC_{50} of 13.17 43.54 µM) and a strong selection and activity on Saos-2 (IC_{50} of 4.33 µM). We observed this compound was between 3 and 10 times more selective for the children osteoclast cell line.

Table 1: IC_{50} of DBM derivatives against HepG2, HCT116, A549 and SAOS-2 human cancer cells after 72h.

Compound	Inhibitory concentration, IC_{50} (µM) / 72h			
	HepG2 cell line	HCT116 cell line	A549 cell line	Saos-2 cell line
2', 4'-dihydroxydibenzoylmethane (1) 	4.88 ± 1.19	5.32 ± 0.70	4.68 ± 0.88	6.49 ± 0.83
2'-hydroxy-4'-aminodibenzoylmethane(2) 	19.69 ± 0.83	13.90 ± 2.95	18.30 ± 30	5.44 ± 1.18
3',4',5'-trimethoxydibenzoylmethane (3) 	3.92 ± 0.45	5.35 ± 0.47	4.25 ± 0.65	6.73 ± 0.55
2', 4'-dihydroxy-3"-methoxydibenzoylmethane (4) 	9.31 ± 1.34	5.55 ± 0.65	6.61 ± 0.56	3.95 ± 0.46
2', 4'-dihydroxy-4"-methoxydibenzoylmethane (5) 	3.96 ± 1.04	2.82 ± 0.86	2.83 ± 0.47	3.56 ± 0.27
1-(2', 4'-dihydroxyphenyl)-3-phenyl-propane-1, 3-dithion (6) 	33.43 ± 2.88	13.17 ± 0.72	43.54 ± 3.13	4.33 ± 0.64
Chlorpromazine hydrochloride (CPZ) Positive control	7.62 ± 0.67	7.89 ± 1.00	10.84 ± 1.54	5.07 ± 0.7

Apoptosis is a form of programmed cell death and is caspase dependant, which is a safe guard mechanism protecting the organism while sacrificing the individual cell,

(http://cshperspectives.cshlp.org/content/5/6/a008672.full). Some drugs, such as doxorubicin and dactinomycin, are activators of the caspase pathway (http://pubs.acs.org/doi/abs/10.1021/mp070002r). However, other pathways of cell death are also possible in which caspases are not activated., These can include: autophagy, paraptosis, mitotic catastrophe, and the descriptive model of apoptosis-like and necrosis (http://clincancerres.aacrjournals.org/content/11/9/3155.abstract), (http://www.ncbi.nlm.nih.gov/books/NBK6197/). Drugs with cytotoxic properties can trigger necrosis as a death stimuli, with features such as disrupted cell membrane, destruction of organelles, swelling and then shrinking of the cell and with the toxic waste being dispersed within the surrounding cells (http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4075070/), (http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3867948/). These caspase independent mechanisms can be produced by reactive oxygen species produced by an external stimuli in the mitochondria (http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3823134/). This mechanism can be activated by molecules interacting with the T cell / interferon / Toll-like / TNF superfamily receptors (http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4343053/) and various anticancer molecules such as ionomycin (http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1905487/), and catechins from green tea, (http://onlinelibrary.wiley.com/doi/10.1111/j.1349-7006.2008.01046.x/full). In a bid to identify a mode of action, we employed the ApoToxGlo® (Promega) assay for assessing the mode of action. The caspase assay is composed of a substrate (proluminescent caspase-3/7 DEVD-aminoluciferin) which is cleaved and the free aminoluciferin is reacted by the luciferase, producing a luminescent signal proportional to the caspase-3/7 activity. (http://www.promega.com/~/media/Files/Resources/Protocols/Technical%20Manuals/101/ApoTox-Glo%20Triplex%20Assay%20Protocol.pdf). We tested 1-(2', 4'-dihydroxyphenyl)-3-phenyl-propane-1, 3-dithion (6) at 5, 10 and 20 µM for 24, 48 and 72 hours and normalised the data to the vehicle control dimethylsulfoxide. The results indicate 1-(2', 4'-dihydroxyphenyl)-3-phenyl-propane-1, 3-dithion (6) decreased the caspase activity steadily as the concentration was increased (see Table 2). At an exposure of 24 hours the caspase activity was reduced between 30-40 %, at 48 hours it was reduced around 50% and for a longer exposure of 72 hours the activity was similar to the 1 day exposure as the level of activity was around 60%.

Table 2: Caspase activity for 1-(2', 4'-dihydroxyphenyl)-3-phenyl-propane-1, 3-dithion.

Compound (µM)	Caspase activity, normalised to DMSO / Saos-2 cell line		
	24 hours	48 hours	72 hours
5	77.21 ± 0.35	49.85 ±2.91	66.58 ± 30.83
10	69.07 ± 4.27	48.32 ± 5.37	61.26 ± 6.90
20	59.23 ± 15.15	40.54 ± 11.16	53.40 ± 0.71

From the caspase studies we can assume the mode of action is related to primary necrosis, characterised by caspase independent death, loss of proliferation, membrane permeabilisation and cell shrinkage amongst some of the features (http://www.nature.com/onc/journal/v27/n50/pdf/onc2008311a.pdf).

Conclusion

Treating cancer in children aggressively is the best option for continued health, despite the risk that side effects can arise years later. Sometimes sequels from surgery can also diminish quality of life afterwards. Producing new selective and effective anticancer agents has become a goal for many labs which are now focusing their resources in this direction to achieve that aim.

Nature has provided us with some of the best anticancer drugs, so one can take this as an indication of where to focus attention in the quest for new drugs to treat cancer. In this paper we used classic organic reactions to produce a family of dibenzoyl-methane derivatives with promising anti-cancer activities. Using established organic methods, we produced 6 compounds that were tested for anticancer activity on different cell lines including hepatic, colon, lung and osteoblast cancers. Though all compounds showed some degree of activity against the cancer cell lines, 1-(2', 4'-dihydroxyphenyl)-3-phenyl-propane-1, 3-dithion (6) produced the most promising and interesting results. This novel compound, tested on different types of cancer cells, has shown to be very selective for the osteoblasts, something that could lead to the deduction that this compound would produce less side effects related to gastro-intestinal problems. This property can be exploited in formulation studies, as well as following it up for chemical modifications to produce a subsequent family of dithionated dibenzoyl-methane compounds. We concluded that primary necrosis is the mode of action responsible for the successful action against cancer cells..

References In the references, all the authors of a paper have to be listed, and the use of the abbreviation "et al." often used in the text section is not allowable in the references section.

1. AmericanCancerChildhoodOrganisation, *Adult childhood cancer*, 2015.
2. CancerResearchUK, *About cancer types*, 2015.
3. Cancer.Org, *Bone Cancer, Statistics*, A.C. Society, Editor 2015.
4. CancerResearchUK, *Bone Cancer Stages and Treatments*, 2015.
5. Plantaz D, et al., *Long term follow-up after chilhood cancer.* Rev Pract, 2014. **64**(9): p.
6. CancerResearchUK, *Bone Cancer Stages, Treatment and Outlook*, 2015.
7. BCRTOrgUK, *Teenage Bone Cancer*, 2015.
8. Escalas C, et al., *Long-term effects of radiation on the spine - Results of a cohort of symptomatic survivors of childhood and review of the literature.* Bull Cancer, 2015.
9. SarcomaUK, *Sarcomas*, 2015.
10. Yoon JH, et al., *A study of docetaxel and irinotecan in children and young adults with recurrent or refractory Ewing sarcoma family of tumors.* BMC Cancer, 2014. **14**: p. 622.
11. Shalaby MS, et al., *The long-term outcome of patients diagnosed with sacrococcygeal teratoma in childhood. A study of a national cohort.* Arch Dis Child, 2014. **99**(11): p. 4.
12. CancerResearchUK, *What is new in bone cancer*, 2015.
13. NHS, *Bone Cancer*, 2015.
14. Cancer.Org, *Bone Cancer Treatments and News*, 2015, American Cancer Association.
15. Ferrari S, et al., *Nonmetastatic osteosarcoma of the extremity. Neoadjuvant chemotherapy with methotrexate, cisplatin, doxorubicin and ifosfamide. An Italian Sarcoma Group study (ISG/OS-Oss).* Tumori, 2014. **100**(6): p. 7.
16. van Maldegem AM, et al., *Etoposide and carbo-or cisplatin combination therapy in refractory or relapsed Ewing sarcoma: a large retrospective study.* Pediatr Blood Cancer, 2015. **62**(1): p. 4.

17. Le Deley MC, et al., *Cyclophosphamide compared with ifosfamide in consolidation treatment of standard-risk Ewing sarcoma: results of the randomized noninferiority Euro-EWING99-R1 trial.* J Clin Oncol, 2014. **32**(23): p. 8.
18. MDAndersonCancerCentre, *Types of bone cancer*, 2015.
19. McDowell KA, et al., *NK cell-based immunotherapies in Pediatric Oncology.* J Pediatr Hematol Oncol, 2015. **37**(2): p. 4.
20. Sukumari-Ramesh, S., et al., *DIETARY PHYTOCHEMICALS INDUCE p53- AND CASPASE-INDEPENDENT CELL DEATH IN HUMAN NEUROBLASTOMA CELLS.* Int J Dev Neurosci, 2011. **29**(7).
21. Nami Kim, et al., *Dibenzoylmethane Exerts Metabolic Activity through Regulation of AMP-Activated Protein Kinase (AMPK)-Mediated Glucose Uptake and Adipogenesis Pathways.* PLOS one, 2015.
22. Jackson KM, et al., *Dibenzoylmethane induces cell cycle deregulation in human prostate cancer cells.* Cancer Lett, 2002. **178**(2): p. 4.
23. Huang, M.T., et al., *Effect of dietary curcumin and dibenzoylmethane on formation of 7,12-dimethylbenz[a]anthracene-induced mammary tumors and lymphomas/leukemias in Sencar mice.* Carcinogenesis, 1998. **19**(9): p. 3.
24. Rodan SB, et al., *Characterization of a human osteosarcoma cell line (Saos-2) with osteoblastic properties.* Cancer Res. , 1987. **47**(18): p. 5.
25. Ragazzon, P., et al., *The characterisation of flavone-DNA isoform interactions as a basis for anticancer drug development.* Anticancer Res, 2009. **29**: p. 10.