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Research Article

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Synthesis and Cytotoxic Evaluation of Bisphosphoramidates in A549 Human Lung Adenocarcinoma Cell Line

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ABSTRACT

Organophosphates compounds have important medical applications including anti-cancer and anti-viral activity. Particularly, phosphoramidates derivatives, characterized by the presence of a nitrogen-phosphorous-oxygen group (N-P=O), have demonstrated a selective cytotoxic effect against several cancer cells lines. Although the effect of several other phosphonates had been tested, phosphoramidate (bisphosphoramidates) have not been evaluated and their properties remain unexplored. Thus, the focus of this investigation was to synthetize a series of bisphosphoramidates to test their cytotoxic effect against the A549 human lung adenocarcinoma cell line. The synthesis of the title compounds was easily achieved adapting the synthetic route described by Kim et al. using three commercially available diamines. Once the compounds were purified and fully characterized, their cytotoxic activity was assessed using the MTT assay at a fixed concentration. From them, only one bisphosphoramidate derivative, named tetraphenyl propane diphosphoramidate (compound 5), was unknown and therefore was fully characterized by solution NMR spectroscopic techniques and mass spectrometry. The spectroscopic analyses of the rest of the compounds perfectly matched the previously reported data. Among the tested compounds, tetraphenyl ethylene diphosphoramidate (compound 4), showed the best cytotoxic effect against the cellular line with similar toxicity to that shown from the drug cisplatin. To the best of our knowledge this is the first time that the anti-cancer effect of the bisphosphoramidates is reported. We are currently synthetizing new bisphosphoramidates which anti-cancer activity would be evaluated against several cancer cell lines to determine their specificity.

Keywords: Bisphosphoramidates; Anti-proliferative; A549 cell line

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INTRODUCTION

Lung cancer has one of the highest death rates among different types cancer, where only 15.1% of patients diagnosed with non-small-cell-lung cancer, representing 85% of the total lung cancer, reach the 5 year survivor rate [1]. Unfortunately, the survival rate is drastically reduced by three thirds, that is to 4.2%, in those patients with metastasized lung cancer, and every year more than 1.5 million of new cases are diagnosed [1]. Thus, the dramatic low survivor rate and the increase number of patients with this type of cancer encourages development of new therapeutic compounds. Therefore, the discovery of new molecules and their *in vitro* evaluation represents an important step towards the discovery of new drug candidates for the treatment of this and other types of cancer. Among the myriad of new molecules tested *in vitro* as chemotherapeutic agents, we focused our investigation in the synthesis of organophosphorous compounds, particularly with phosphoramidates derivatives, which have a nitrogen-phosphorus-oxygen bounds (N-P=O) [2]. Because of their reactivity towards DNA, RNAs and some enzymes, phosphoramidates are widely used as anti-cancer drugs [3]. Several compounds containing the phosphoramidate group have shown their efficacy against several cancer lines *in*

vitro, while some of them are currently in phase I/II clinical trials [2]. Phosphoroamidates, are tipically used as pro-drugs where they are bound to different molecules that allow their release, promoting the alkylation of the DNA and causing irreversible damage, as it has been demonstrated with the naphthoquinone and benzimidazolequinone phosphorodiamidates derivatives [4]. Both compounds showed cytototoxic effect towards colon cancer cell lines, though the former showed less cytotoxic activity because of the poor release of the phosphoramidate compound.

Other strategies have been developed to promote the efficient release of the phosphoramidate bond, as it has been shown in the hypoxia mediated release in the 2-nitroimidazole phosphoramidate derivatives [4]. Hypoxia, is characteristic of solid tumours, and the capacity to release the phosphoramidate fragment of the prodrug under this conditions was assessed in H460 human non-small-cell lung cancer cell line maintained under N2 atmosphere for 4 hours. The compounds showed more cytotoxicity under hypoxia conditions when compared with normal conditions. Additionally, this strategy showed a 41% growth tumour inhibition with a significant increase in the survival rate, when used in co-therapy with gemcitabine in a Human Pancreatic Cancer Orthotopic Xenograft Model. Other strategies involving phosphoramidates as anti-cancer drugs are to bind them with nucleoside analogues. This approach is known as the ProTide approach, and has been highly successful in the discovery of compounds with anti-viral activity with two FDA drugs approved, so they have been tested as anti-cancer drugs [2,5]. In this context, the phosphoramidate derivatives help to deliver a phosphonate nucleotide analogue that can exert its effect. Moreover, the phosphoramidate group also helps to increase the liposolubility of the molecules and thereof enables the nucleotide to be delivered intracellularly. Among the different compounds tested, Thymectacin (or NB1011), a deoxyuridine phosporamidate pro-drug, was under phase I/II clinical trials for the treatment of colon cancer, although discontinuation of the trials was reported [6]. Nonetheless, given the potential of these compounds trough SAR structure optimization, new naphthyl phosphoramidates were tested against MB231 (breast cancer), PC-3 (prostate cancer) and T24 (bladder cancer), showing even better cytotoxic effect than the Thymectacin drug. [7] Currently, several other groups are interested in discovering new compounds using the ProTide approach, as new therapeutic agents to treat cancer. [3,6] Although some phosphoroamidates have been reported to have direct interactions with the DNA, there are few reports focused in the anti-cancer activity of these compounds and there are none reports of the use of the dimers molecules as anti-cancer agents. Thus, the aim of our investigation was to synthetize a series of bisphosphoramidates and to test their cytotoxic activity against the A549 cell line. Our preliminary results showed that the series of the bisphosphoramidates have indeed an anti-proliferative effect in A549 cells, opening the possibility of using these compounds against other cancer cell lines.

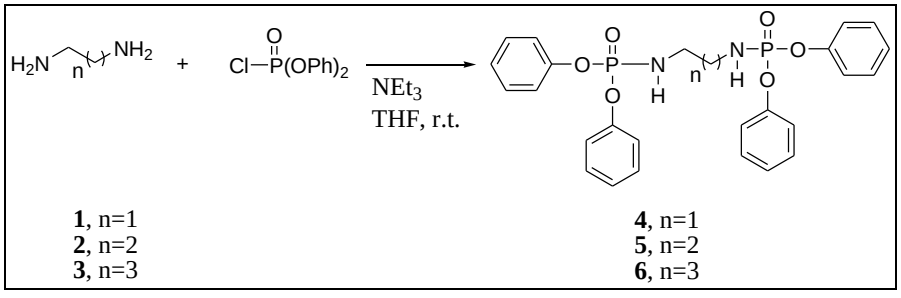
EXPERIMENTAL SECTION

Materials and Methods

All reagents were purchased from Sigma-Aldrich and used as received. Flash column chromatography was performed using Aldrich silica gel 230-400 mesh. 1H and 13C NMR data for all previously compounds were recorded at ambient temperature using Bruker Fourier300, Jeol Eclipse 300 and Bruker AV500 spectrometers. The FT-IR data were recorded with Bruker ATR in the 450-4000 cm^{-1} range. Melting points were determined using Fisher Johns melting point apparatus (uncorrected). High-resolution mass spectra were recorded on a JEOL AccuTOF JMS-T100LC mass spectrometer with TOF mass analyser.

Chemistry

For the synthesis, we used commercially available diamines (ethylenediamine, 1,3-diaminopropane and 1,4-diaminobutene) and diethyl cholorophosphate following the synthetic procedure previously described by Kim et al. [8] (Scheme 1).



1, n=1
2, n=2
3, n=3

4, n=1
5, n=2
6, n=3

Scheme 1: Synthesis of bisphosphoramidates

Cell Culture

A549 (ATCC® CCL-185™) cells were grown in Dulbecco's modified Eagle's medium (DMEM, GE Healthcare) containing 10% fetal bovine serum and 100 mg/ml of gentamicine, streptomycin and penicillin at 37°C under 5% CO_2. Cells were grown to 80% confluence.

Viability Analysis

For MTT [3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (Sigma Aldrich Chemicals)] reduction assay (Mosmann, 1983), cells (5000 cells/cm^2) were seeded on 96-well plates in 200 µl of culture media and incubated at 37°C in 5% CO_2. Under these conditions cell´s plates did not reach confluence in 96 h. Cells were treated with 100 µM of each of the Tetraphenyl ethylene diphosphoamidate (4), Tetraphenyl propane diphosphoamidate (5) and Tetraphenyl butane diphosphoamidate (6), and cisplatin (80 µM) and hydrogen peroxide (2%) as positive controls. Culture medium was aspirated after treatment and cells were analyzed in triplicates. Then, 40 µl of MTT labeling mixture (0.5 mg/ml) was added to each well, and the samples were incubated 4 h at 37°C in 5% CO_2. An isopropanol: HCl 0.4% solution was added to lyse the cells and to solubilize the colored crystals. The optical density of the samples was determined at 590 nm using an ELISA plate reader Varioskan.

RESULTS AND DISCUSSION

Tetraphenyl Ethylene Diphosphoramidate (Compound 4)

NMR spectra agreed perfectly to those reported by Kim [8].

Tetraphenyl propane diphosphoramidate (compound 5): Yield: 0.5 g (75%); white solid; mp 78-79°C; Rf = 0.21 (Hexane/AcOEt 1:1). 1H NMR (400 MHz, CDCl3): δ = 1.49-1.57 (m, 2H), 2.98-3.08 (m, 4H), 3.74-3.84 (m, 2H), 7.09-7.22 (m, 12H), 7.25-7.31 (m, 8H); 13C NMR (100 MHz, CDCl3): δ = 32.42 (1C), 38.05 (2C), 120.14 (8C), 124.86 (4C), 129.80 (8C), 150.79 (4C); 31P NMR (81 MHz, CDCl3): δ = 0.02 (s); FT-IR (cm^{-1}): 3225, 1187, 1105, 923, 753. HRMS (DART) $C_{27}H_{29}N_2O_6P_2$ m/z calc. 539.1422, found 539.1503 (Figures 1 and 2).

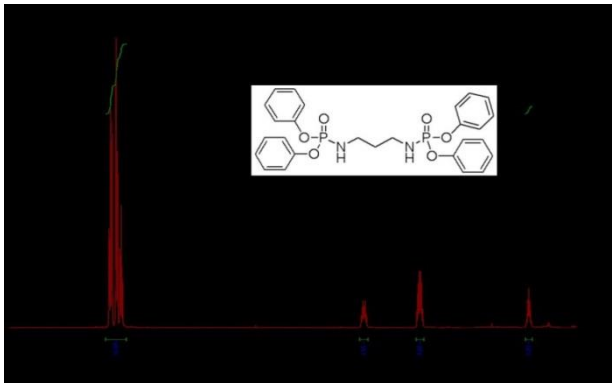


Figure 1: Spectrum 1H of 5

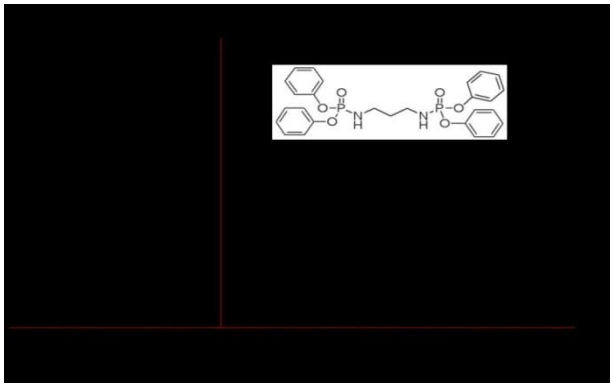
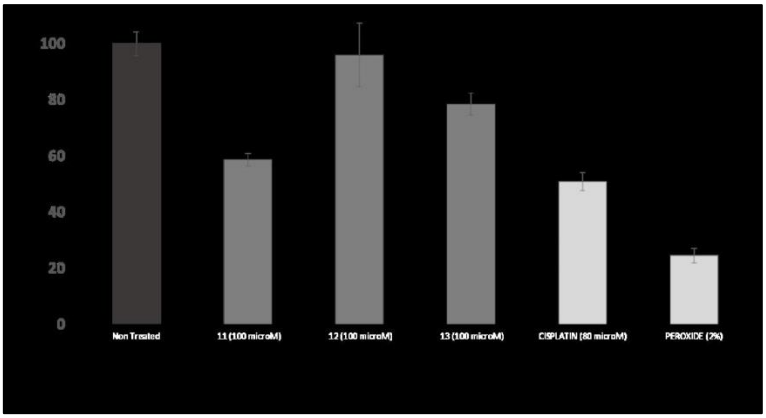


Figure 2: Spectrum 31P of 5

Tetraphenyl Butene Diphosphoramidate (Compound 6)

NMR spectra were in perfect agreement to those reported by Gholivand [9-11].

Bisphosphoramidates reduce proliferation in A549 cells. We evaluated the cytotoxic effect of the tetraphenyl ethylene diphosphoamidate 4, tetraphenyl propane diphosphoamidate 5 and tetraphenyl butane diphosphoamidate 6 using the MTT assay in the A549 human lung adenocarcinoma cell line. After 24 h, a decrease in cell viability was found when cells were treated with both compounds 4 and 6; however, no effect was detected when using compound 5 (Figure 3).



Note: Cell viability was determined by a MTT assay. Mean viability percentage from non-treated cells at time zero (NT). Proliferation rates were measured after 24 h. Mean values and standard errors (±S.E.) from at least three independent experiments performed in triplicates are shown. *P>0.05 between non-treated and treated cells. **P>0.005

Figure 3: A549 cells (4000 cells/cm^2; seeded 24 h before the treatment starting point) were treated as indicated: NT-non treated, 4, 5 or 6 at same concentration range and cisplatin and hydrogen peroxide as positive and negative control respectively

The two active compounds 4 and 6, decreased the cell viability significantly when compared with non-trated cells (p>0.001 and p>0.05, respectively), but compound 4 showed the best effect, similar to that obtained using the well-known drug cisplatin (Figure 3). Regarding the mechanism of cytotoxicity, it seems possible that the compounds could release the phosphoramidate anion as a result of enzymatic interaction, or through intracellular chemical reduction as it has been reported with the Quinone Phosphordiamidate Prodrugs [12]. It is possible that the enzymatic reduction is mediated by the NAD(P) H:quinone oxidoreductase, DT-diaphorase (DTD), which is highly expressed in lung, breast, colon, and liver carcinomas.

However, considering that DT-diaphorase shows a higher affinity towards drugs containing a quinone core, is possible that other enzymes or mechanisms might be involved in the release of the phosphordiamidate drug and needs further investigation. According with the structure of the molecules, the tetraphenyl propane diphosphoramidate and Tetraphenyl butene diphosphoramidate have strong intra and inter molecular interactions C-H---O=P or P=O---H-N for both of them. These interactions might give more stability to the structure hindering the release of the phosphoramidate anion. Importantly this type of strong interactions have been previously reported with N,N'-bis(O,O'-diarilfosforomidatos) compounds [10,11]. On the contrary, the Tetraphenyl ethylene diphosphoramidate molecule will have weaker intra and inter molecular interactions facilitating the release of the anion and increasing cytotoxicity, although structural and electronic analysis of the compound are currently investigated. Another factor that might increase the cytotoxicity of compound 4 is its weight. The ethylene derivative has the lower molecular weight 524.126 g/mol which according with the Lipinski rule of five will be more likely to be membrane permeable increasing the concentration intracellularly and facilitating the liberation of the phosphorodiamidate anion [13]. Nonetheless several experiments need to be perform to demonstrate that indeed the release of the phosphoramidate anion is directly associated with toxicity and under which circumstances it is favored.

CONCLUSION

We have found an anti-proliferative activity of two biphosphoramidates against the A549 human lung adenocarcinoma cell line. Among the three compounds tested, the tetraphenyl ethylene diphosphoramidate 4 showed the highest anti-proliferative effect, measured by the percentage of decreased of cell viability at a fixed concentration. Even though our results are preliminary, we are currently evaluated the anti-proliferative effect of the compounds in other cells lines and synthetized new biphosphoramidates. To the best of our knowledge this is the first time that these compounds have been tested against tumour cells, opening the possibility of designing new biphosphoramidates base upon of this N-P=O functional group.

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