SYNTHESIS, IDENTIFICATION AND IN VITRO ANTITUMOUR PRESCREENING TEST OF TRIPHENYLTIN BENZOATE TOWARDS A HUMAN CERVICAL CARCINOMA CELL LINE, HeLa

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ABSTRACT

In this study, triphenyltin benzoate was synthesized first, and followed by antitumor prescreening test of the compound towards a human cervical carcinoma cell line, HeLa. Three reaction steps were employed to obtain the compound needed, i.e. 1) synthesizing of tetraphenyltin compound via insitu phenilmagnesiumbromide Grignard reaction to tin(IV)chloride, 2) synthesizing triphenyltin chloride via redistribution reaction of tetraphenyltin to tin(IV) chloride without any solvent, the reaction completed depends on the temperature, in this case a good results was achieved at temperature 220 °C for 6 h, 3) finally, triphenyltin benzoate was produced through a methathetical reaction of triphenyltin chloride to an excess of sodium benzoate in ethanol. In vitro prescreening antitumour activity of the compound towards a human cervical tumour cell line, HeLa was carried out following an enzyme linked immunosorbent assays (ELISA). By this method, the test ended with good promising results. This indicates by the IC_{50} of 170 nM which is compared well to cisplatinum with IC_{50} 950 nM.

Keywords: *redistribution reaction, methathetical reaction, cell line, in vitro, antitumour.*

INTRODUCTION

Research on the use of compounds for inhibiting tumour cell lines are limited by knowledge of chemical properties, biological target, and negative impact of the compounds towards normal cells. So far, reaction mechanism of how the compounds tested could inhibit the tumour cells has not clearly been understood. However, the main target of the compounds tested could be DNA, whether they inhibit the DNA synthesis or stopped proliferation of tumour cell [1,2].

As well as tin based compounds, their mechanism for inhibiting tumour cell lines could be in a similar way to the general compounds. Therefore, the compounds tested should have a central atom that can react to biomolecular target (DNA), a good leaving group (anion) that bind weakly to the central atom, and an inert organic group to protect central atomic coordination zone [3-5]. Almost all of tin based antitumour tested have a similar unique properties explained above. Narayanan *et. al.* reported that tin(II) organic compounds are more reactive towards leukemia, P388 in vivo compare to tin(III) organic compound [6]. As reported by other researchers, that Tin(II) butyl compounds are found more reactive compared to cisplatinum, a clinically used drug for curing tumour [2,6-7].

Tranter *et. al.* reported that there is no antitumour activity difference between triphenyltin sulphinate ester and triphenyltin carboxylate ester derivatives [8]. It was also reported, that triphenyltin acetate is potent for antitumour agent as indicated by IC_{50} of 0.058 μg/mL (142 nM) towards a human tumour cervical carcinoma, HeLa compared to cisplatinum with IC_{50} of 950 nM [9]. Therefore, a further investigation was carried out to obtain a new antitumour agent with substitution of the acetate to benzoate, in the hope to increase activity of the compound obtained. Antitumour prescreening test towards the same tumour for triphenyltin benzoate compound was also employed following ELISA technique.

EXPERIMENTAL SECTION

Reagents

p-Bromobenzene, tin tetrachloride, magnesium, tetrahydrofuran (THF), benzene, chloroform, charcoal, petroleum ether (bp. 60-80 °C), and sodium benzoate. All reagents for antitumour prescreening test were provided by Queensland Institute of Medical Research (QIMR) Royal Brisbane Hospital Australia. The reagents for antitumour prescreening test are pyruvate, nicotinamide, penicillin, streptomycin, fetal calf serum, and 1,2-dimethoxy ethane (DME).

Instrumentations

Proton nmr spectra were recorded on a Hitachi FT-NMR R-1900 instrument operating at 200 MHz in deuteriochloroform (unless otherwise stated) and referenced to residual chloroform in the solvent (δ 7.24 ppm). Chemical shifts are expressed in δ (ppm) downfield from TMS. Carbon nmr spectra were obtained on the same instrument operating at 50.28

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MHz and referenced to the central resonance of deutoriochloroform (δ 77.00 ppm). NMR data were performed by Lab. Dasar Bersama Unair Surabaya.

Procedure

Synthesis of Tetraphenyltin

Magnesium (2.4 g) was placed in a three necked flask and stirred under nitrogen for 16 h. *p*-Bromobenzene (11g; 0.07 mol) in THF (150 mL) was added to this activated magnesium. The mixture was refluxed for 15 min. Tin tetrachloride (4.5 g; 0.018 mol) in benzene (50 mL) was then slowly added to the well stirred, cold (0 °C) Grignard reagent solution and refluxed for 1 h. The mass was filtered hot and the filtrate evaporated under vacuum. The solid product was recrystallized from chloroform to provide white needle crystals.

Synthesis of Triphenyltin Chloride

In a 50 ml flask equipped with an air condensor were placed tetraphenyltin (2 g; 0.5 mmol) and tin tetrachloride (0.42 g; 0.16 mmol). The flask was placed in an oil bath which was then slowly heated to 200 °C and maintained at that temperature for 1 h. After the mass had cooled, it was extracted with 50 ml of petroleum ether (bp. 60 – 80 °C), treated with a small amount of charcoal and filtered hot. On cooling, white needle crystals wre formed and recovered.

Synthesis of Triphenyltin Benzoate

This compound was prepared via the methatetical reaction between triphenyltin chloride (200 mg; 0.52 mmol) and the sodium salt of benzoic acid (75.38 g; 0.52 mmol) in ethanol (40 mL). The reaction was refluxed for 6 h. The product was precipitated along with sodium chloride and purified by recrystallization from ethanol to give fine white crystals.

In vitro Antitumour Prescreening Test

Antitumour testing was performed using facilities at the Queensland Institute of Medical Research (QIMR) Royal Brisbane Hospital Australia. The human tumour cell line HeLa was used for the in vitro assays. The cells were cultured at 37 °C in 5% CO_2/air in Roswell Park Memorial Institute Medium 1640 (referred to from now as culture medium) containing 1 mM pyruvate, 50 μM nicotinamide, 100 IU/mL penicillin, 100 μg/mL streptomycin, 3 mM HEPES and 5% fetal calf serum. Cells were seeded in 96 well microtitre plates, 2000 cells per 100 μL culture medium per 6 mm microtitre well. The cells were allowed to attach overnight.

A series of triphenyltin benzoate were dissolved in 1,2-dimethoxy ethane (DME) and diluted with culture medium to appropriate concentrations. Dilutions were such that the final concentration of 1,2-dimethoxy ethane was in each case below 5% and did not interfere with tests results. After overnight incubation, 5 dilutions of the compounds and 1 for control were plated out in triplicate. The plate were incubated for ca. 2.5 h, then the supernatant was decanted and replaced with no more than two drops of fresh culture medium (using a Pasteur pipette). The plates were incubated for ca. 2.5 d, the control by this time was nearly confluent. After 2.5 d incubation, 10 μl of [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazo-lium] (MTS) solution was added to the culture wells. Then, the plates were placed in an incubator and, after 45 mins, the formazen produced was assayed by measuring the optical density at dual wavelengths in the range 490 – 655 nm using a micro plate reader. The IC_{50} values were calculated from the graph of percent log surviving cells versus drug concentration.

RESULT AND DISCUSSION

Synthesis of Tetraphenyltin

By the method explained on the materials and method above, tetraphenyltin obtained is 5.9 g (80% yield), with melting point 225 – 226 °C. The compound observed is exactly tetraphenyltin as indicated by proton and carbon NMR data.

Synthesis of Triphenyltin Chloride

Triphenyltin chloride obtained by the method of redistribution reaction as indicated on materials and method above is 1.5 g (60% yield), melting point of 104 – 105 °C and confirmed by their proton and carbon NMR data.

Synthesis of Triphenyltin Benzoate

Triphenyltin benzoate is obtained by a methatetical reaction method, within this method the compound obtained around 198 mg (81.5% yield). The proton and carbon NMR data indicates that the compound is exactly triphenyltin benzoate.

In vitro Antitumour Testing

A human cervical cell line, HeLa was used for in vitro antitumour prescreening test. The ELISA was employed in order to obtain the activity of the compound. Data obtained were listed on Table 1.

In this study, triphenyltin benzoate was synthesized via three step reactions as can be seen on Figure 1.

First step of reaction on Figure 1 reveals the formation of tetraphenyltin. Reaction of *p*-bromobenze-

Table 1. Antitumour activity data of triphenyltin benzoate

Dilution of ml/100 ml	0	2	4	6	8	10
Test I : 1	0.764	0.657	0.420	0.290	0.092	0.069
2	0.771	0.655	0.424	0.292	0.093	0.071
3	0.768	0.658	0.422	0.292	0.095	0.068
Mean	0.767	0.656	0.422	0.291	0.093	0.069
SD x 10^{-3}	3.5	1.5	2.0	1.1	1.5	1.5
Log % surviving cells	2.00	1.93	1.74	1.58	1.08	0.95
Test II: 1	0.363	0.312	0.207	0.131	0.062	0.044
2	0.370	0.318	0.210	0.133	0.059	0.039
3	0.368	0.316	0.209	0.132	0.063	0.042
Mean	0.367	0.315	0.208	0.132	0.061	0.042
SD x 10^{-3}	3.6	3.0	1.5	1.0	2.1	2.5
Log % surviving cells	2.00	1.92	1.75	1.56	1.23	1.08
Test III: 1	0.304	0.255	0.167	0.103	0.034	0.028
2	0.315	0.260	0.170	0.106	0.032	0.028
3	0.312	0.259	0.169	0.105	0.032	0.025
Mean	0.310	0.258	0.168	0.105	0.032	0.025
SD x 10^{-3}	2.6	2.6	1.5	1.5	2.5	3.0
Log % surviving cell	2.00	1.92	1.73	1.53	1.00	0.90

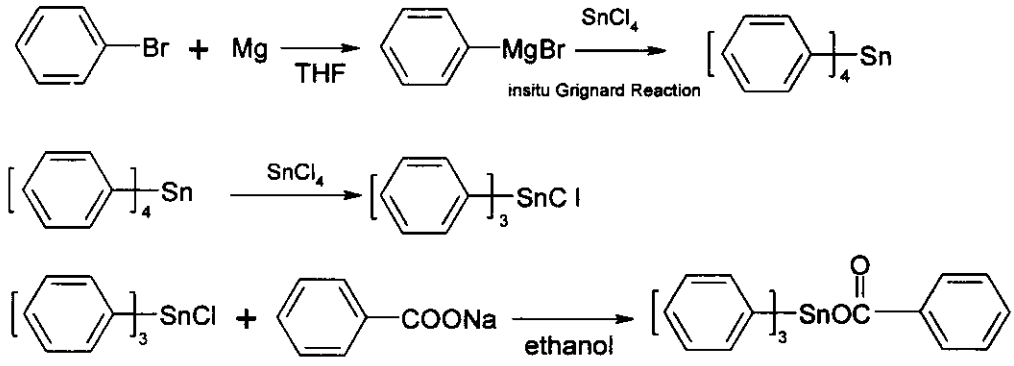


Figure 1. Reaction scheme for synthesizing triphenyltin benzoate

na dissolved in THF and activated magnesium turning with a catalytic amount of iodine produced a Grignard reagent of *p*-bromomagnesiumbenzene. The reaction was then left for 15 min at room temperature. Addition of tin tetrachloride dissolved in benzene to this Grignard reagent and refluxed for 1 h. The crude material obtained was filtered and evaporated under vacuum to give a crude product which on recrystallization from chloroform gave tetraphenyltin. By this method tetraphenyltin obtained extremely pure as supported by proton and carbon NMR data (Table 2).

As can be seen from Table 2, the ^{1}H nmr spectrum showed three peaks. The proton in *para* position appeared at δ 7.76 ppm which is located at upper field. On the other hand, the protons in the *ortho* and *meta* position were observed at δ 7.49 and 7.63 ppm, respectively. Supported by ^{13}C nmr data, there were 4 peaks of carbon observed, i.e. four carbon in the *ipso*, *ortho*, *meta*, and *para* positions appear at δ 137.98, 137.35, 128.74, and 129,17 ppm, respectively. Based on ^{1}H and ^{13}C nmr data assignment, it can be concluded that the compound produced on the first step reaction on Figure 1 is tetraphenyltin.

The second step of reaction on Figure 1 is synthesizing triphenyltin chloride. This reaction was carried out by cleaving one aryl group of tetraphenyltin through a Kocheskov redistribution reaction. In this experiment, reaction of tetraphenyltin and tin(IV) chloride (3:1 mole ratio) occur at temperature between 200 – 220 °C for 4 h. Product of this reaction was then crystallized using petroleum ether (bp. 60 – 80 °C). The crystals obtained are triphenyltin chloride as indicated by NMR data (Table 3).

Table 2. NMR data of tetraphenyltin compound

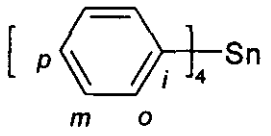
Compound	^{1}H NMR Spectra (ppm)	^{13}C NMR Spectra (ppm)
	7.49 (8H, AA'MM'X, H_o) 7.63 (8H, AA'MM'X, H_m) 7.76 (4H, AA'MM'X, H_p) *i = ipso, o = ortho, m = metha,* and *p = para*	C_i = 137.98 C_o = 137.35 C_m = 128.74 C_p = 129.17

Table 3. NMR data of triphenyltin chloride compound

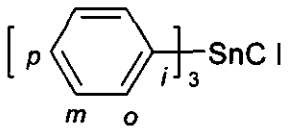
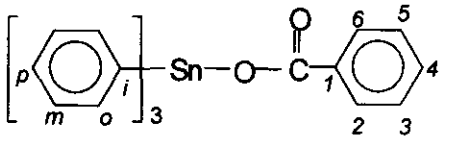
Compound	^{1}H NMR Spectra (ppm)	^{13}C NMR Spectra (ppm)
	7.46 (8H, AA'MM'X, H_o) 7.54 (8H, AA'MM'X, H_m) 7.72 (4H, AA'MM'X, H_p) *i = ipso, o = ortho, m = metha,* and *p = para*	C_i = 137.36 C_o = 136.20 C_m = 129.21 C_p = 130.42

Table 4. NMR data of triphenyltin benzoate compound

Compound	^{13}C spectra (ppm)	
	C_i = 138.24	C_1 = 130.37
	C_o = 136.81	$C_{2,6}$ = 130.06
	C_m = 128.81	$C_{3,5}$ = 128.07
	C_p = 130.86	C_4 = 132.59
		COO = 172.79
	^{1}H spectra (ppm)	
	7.85 (3H, AA'MM'X, H_p)	
	7.54 (6H, AA'MM'X, H_m)	
	7.48 (6H, AA'MM'X, H_o)	
	8.19 (2H, AA'MM'X, $H_{2,6}$)	
	7.80 (2H, AA'MM'X, $H_{3,5}$)	
	7.39 (1H, AA'MM'X, H_4)	

From Table 3, it can be seen that replacement of one aromatic substituent by chloride decreases the ^{1}H resonance. All protons in *ortho*, *meta*, and *para* position appear at δ 7.46, 7.54, and 7.72 ppm, respectively. A similar trend were also observed for carbons resonance in *ipso* and *ortho* positions which were appeared at δ 137.36 and 136.20 ppm, respectively. However, for carbons in *meta* and *para* positions were appeared at δ 129.21 and 130.42, respectively which were increased compare to the parent compound tetraphenyltin. On the basis of these ^{1}H and ^{13}C nmr assignment, the compound obtained was triphenyltin chloride. This result was also in line with ^{13}C nmr data observed by Wharf for the compound, i.e for carbons resonance in *ipso*, *ortho*, *meta*, and *para* were appeared at δ 137.39, 136.18, 129.18, and 130.51, respectively [10].

The third step of reaction on Figure 1 is formation of triphenyltin benzoate, the target compound. This reaction is a methatetical type reaction, within reaction of triphenyltin chloride to an excess of sodium salt of benzoic acid on ethanol. The reaction is refluxed for 6 h. The negative center of the sodium benzoic will attack positive center of triphenyltin chloride and leading to cleavage of chloride ion. The product was then recrystalized using ethanol to yield fine crystals. The nmr data (Table 4) indicates that the compound is triphenyiltin benzoate, the target compound needed.

This compound was first prepared by Gilman and Eish by refluxing triphenyltin iodide and sodium benzoate in ethanol [11]. Frankel *et al* have also synthesized this compound via a two phase reaction. Triphenyltin chloride dissolved in an inert organic solvent (i.e. DCM) was reacted with benzoic acid suspended in water and neutralized to phenolpthalein with sodium hydoxide [12]. However, there were no nmr data gained by these two group researchers. As can be seen from Tabel 4, it was a significance different of ^{1}H and ^{13}C nmr data compared to triphenyltin chloride. The ^{1}H nmr spectra indicates 6 peaks in which three of them originate from benzoate species as a substitution of chloride occurred. A similar trend was also observed for ^{13}C nmr data in which there was a peak appeared at δ 172.79 which was characteristic for carboxylate (COO) fucntional group.

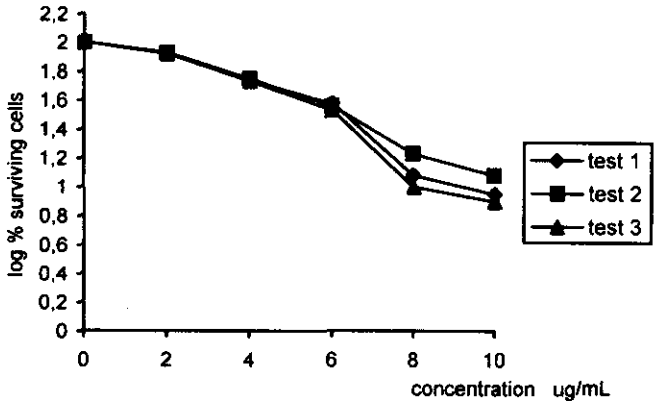


Figure 2. Graph of log % surviving cells vs. concentration

Table 5. IC_{50} of triphenyltin benzoate towards, HeLa

Antitumour Towards HeLa	IC_{50} ($\mu g.mL^{-1}$)
Test 1	0.081
Test 2	0.085
Test 3	0.078
Mean	0.081
SD x 10^{-3}	3.378

Antitumour Prescreening Test

The antitumour activity of triphenyltin benzoate towards a human cervical carcinoma cell line, HeLa is indicated by IC_{50} value, in which 50% of the cells were killed by the compound. The IC_{50} values were derived from the graph of log % surviving cells versus compound dose (Figure 2).

The IC_{50} of the compound is listed on Table 5. Data on Table 5 reveals that triphenyltin benzoate is very toxic with IC_{50} = 170 nM. Based on this IC_{50} value, triphenyltin benzoate was obtained more toxic than cisplatinum with IC_{50} = 950 nM [9]. This great toxicity is probably due to the compound that can be hydrolyzed in the molecular biological system to form a cation of $(triphenyltin)^+$ in which this moiety is believed to have toxic activity properties [8].

CONCLUSION

Triphenyltin benzoate could be prepared via three step reactions, including: (1) formation of tetraphenyltin through insitu Grignard reaction; (2) redistribution reaction to form triphenyltin chloride and, (3) synthesizing of triphenyltin benzoate through a methatetical reaction of triphenyltin chloride to an excess of sodium salt of benzoic acid in ethanol.

Antitumour prescreening test of triphenyltin benzoate towards a cervical carcinoma cell line, HeLa resulted in a promising result. This was indicated by IC_{50} of 170 nM, which is below the IC_{50} of cisplatinum (950 nM).

Based on antitumour prescreening data against a cervical carcinoma cell line, HeLa, triphenyltin benzoate is more toxic than cisplatinum, an antitumour agent clinically used, therefore, a further investigation of in vivo antitumour of the compound should be carried out.

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