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Synergistic effect of novel aspirin analogues in a colorectal cancer cell line

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Abstract
Studies show aspirin, a non-steroidal anti-inflammatory drug (NSAID) has potential to decrease incidence of, or mortality from a number of cancers including colorectal cancer (CRC). With emphasis on the treatment of CRC involving platinum compounds, oxaliplatin has been used in several combinations with other drugs. Unfortunately, these combinations do not improve overall survival and are accompanied with side effects that include gastrointestinal (GI), hematologic, neurologic toxicities, myopathy, and most recently interstitial lung disease, particularly fibrotic hypersensitivity pneumonitis, which has high mortality rates and long-term morbidity in survivors. The need of compounds that will reduce the doses of platinum compounds required for chemotherapy in order to reduce or alleviate these side effects is long overdue. This study investigates the synergistic effect if any, of novel aspirin analogues with platinum compounds cisplatin, oxaliplatin and carboplatin in order to lower doses needed of these platinum compounds and thus reduce or alleviate common debilitating side effects. MTT assay was used to assess cell viability and the CompuSyn software (Paramus, NJ, 2005) was used to calculate CI and DRI at ED_{50}, ED_{75} and ED_{90}. Oxaliplatin was found to exhibit synergistic effects when combined with *p*-aspirin (PN549), diaspirin (PN508) and *o*-thioaspirin (PN590). Although further investigations such as *in vivo* experiments will be needed to draw any conclusions, this study is a stepping-stone for platinum compounds and aspirin drug combinations in order to decrease doses needed for treatment and thus lessen or alleviate debilitating side effects.

Keywords: Colorectal cancer; Aspirin; Aspirin analogues; Oxaliplatin; DMSO

INTRODUCTION

The discovery of a positive effect of aspirin, a very cheap drug on colorectal cancer (CRC) [1] was a stepping-stone for the study of aspirin on various cancers. Evidence later emerged that indeed the daily intake of aspirin reduced mortality [2], incidence and metastasis of CRC [3]. These discoveries geared studies carried out on the effects caused by aspirins in different cancers [4-6]. The cell line primarily used in this study is SW480 CRC cell line (Figure 1), which has been well characterized by [7] and more recently genetically characterized [8]. The cell line was isolated from the primary adenocarcinoma in the colon of a 50-year-old Caucasian male [7,8]. The cells are polygonal in shape with microvilli on their cell surfaces when viewed under an electron microscope [7] with an intermediate growth rate [8]. They are also known to be hyperdiploid and a low producer of carcinoembryonic antigen (CEA)

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and are considered a good candidate for experimental studies in CRC research [7].

The SW480 cell line is DNA mismatch repair (MMR) proficient [9] and thus a good model for the study of changes involved in the progression of late CRC [10]. Semi quantitative analysis reveals the SW480 cell line to express low levels of cyclooxygenase (COX)-1 and undetectable levels of COX-2 [11,12], thus making it a good model for the study of mechanism of action of compounds that also follow COX-independent pathways. The cytotoxicity of aspirin and its analogues have been reported in various cancers [9,13,14].

The adaption of combined therapy using several active ingredients to produce a desired effect in the treatment of different diseases by both traditional and modern medical practitioners [15] dates back thousands of years when the Chinese and African herbalists used a combination of naturally occurring herbs to treat ailments [16,17]. One of the reasons behind the increased interest in the development of combination therapies can be attributed to the enhanced understanding that cancer, as a disease, involves the disruption of different molecular pathways, which are all connected and better tackled with the combined action of two or more drugs [18,19]. Another reason for the increased interest in combination therapy is the desire to achieve therapeutic effects at reduced doses, which will result in toxicity reduction and also delay or minimize the induction of drug resistance [20]. Drug combination therapy has increased in popularity for the treatment of complicated diseases such as cancer and AIDS [21].

Synergy can be defined as the greater effect for drugs in combination than in the simple additive effect produced by each drug individually. For example, 1+1 should be >2 for a synergistic effect while an antagonistic effect is less than an additive effect [15]. The term ‘combination index’ (CI) is used to quantitatively depict synergism (CI<1), additive effect (CI=1) or antagonism (CI>1) [22].

With emphasis on the treatment of CRC involving platinum compounds, oxaliplatin has been used in several combinations with other drugs. As far back as 1992, a study was published on the effects that resulted from the combination of oxaliplatin, folinic acid (leucovorin) and 5-FU with an objective response from 58% of the patients [23], later known as FOLFOX, which resulted in a high response rate in CRC patients [24,25]. Bevacizumab, a VEGF antagonist is also used in combination with FOLFOX in the treatment of metastatic CRC (mCRC) and has produced promising results [26]. EGFR antagonists, cetuximab or panitumumab have also produced improved response rates when used in combination with FOLFOX in patients harbouring the *KRAS* gene mutation [27]. Unfortunately, these combinations do not improve overall survival [26,28] and are accompanied with side effects that include gastrointestinal (GI), hematologic, neurologic toxicities and most recently interstitial lung disease, specifically fibrotic hypersensitivity pneumonitis, which has high mortality rates [29] and long-term morbidity in survivors [30]. Treatment with oxaliplatin is also associated with upregulation of myopathy-associated genes *Foxo3*, *MAFbx* and *Bnip3* [31].

Oxaliplatin, as part of a neoadjuvant chemotherapy regimen is administered to CRC patients in order to shrink tumours before surgery/main treatment [24,32]. However, resistance to oxaliplatin is an encroaching menace especially in cancers that harbour a mutation of the *TP53* gene of the tumour suppressor protein p53 [33]. P53 as a transcription factor acts as guard to the cell in response to stress signals and is responsible for the regulation of the cell cycle [34] and several metabolic enzymes, which are in turn responsible for drug metabolism [32,33]. For

example, mutations found in the *TP53* gene affect cytochrome P450, an enzyme involved in the metabolism of drugs such as oxaliplatin [32,35], thereby leading to resistance.

Other platinum compounds, namely, cisplatin and carboplatin are also used in cancer chemotherapy and present with a range of long-term side effects such as GI toxicity, hepatotoxicity and late-term ototoxicity.

This study aims to establish whether aspirin analogues when used in combination with platinum compounds will have an effect in either a synergistic, additive or antagonistic manner.

Terminologies and plots used in the interpretation of drug combination results.

An important plot found in both the CalcuSyn and CompuSyn software is the Fa-dose-reduction index (Fa-DRI) plot. As alluded before, one of the main reason for synergistic studies is to develop drug combinations with desired effects at reduced doses in order to decrease toxicity effects [21]. The dose-reduction-index, DRI>1 indicate favourable dose-reduction while DRI<1 indicate unfavourable dose-reduction. For example, (Table 1), Fa=0.5 means at 50% inhibition of cell proliferation. For a 50% inhibition of cell growth, 3.7 μM of drug 'A' and 4.2 μM of 'B' are required individually. However, when combined, CompuSyn software calculates the DRI value of A and B as 3.2163 and 3.5924 respectively. Thus, 3.2163-fold less 'A' plus 3.5924-fold less 'B' is required to achieve the same 50% inhibition at the chosen combination ratio (i.e., 3.7260 μM ÷ 3.2163 = 1.2 μM of drug 'A' plus 4.1938 μM ÷ 3.5924 = 1.2 μM of drug 'B'). CI values are interpreted as descriptive words or by symbols (Table 2).

This paper aims to investigate the cytotoxic effects of novel aspirin analogues, which include the *meta*- and *para*- isomers of aspirin and thioaspirin. It is also aimed to study if their cytotoxic effect against SW480 CRC cell line will result in a synergistic effect when in combination with DNA-damaging platinum compounds known to be used as chemotherapeutic agents by use of the CalcuSyn® and CompuSyn® software.

EXPERIMENTAL

Tissue culture. The SW480 CRC cell line (ECACC, Salisbury, UK) was cultured in Leibovitz's L-15 medium (11415-049, gibco® life technologies™, ThermoFisher Scientific) supplemented with 10% (v/v) FBS and 1% (v/v) penicillin-streptomycin. The cells were cultured at 37°C in a humidified incubator and passaged at approximately 80% confluency.

Combination assay using MTT and CompuSyn software. For the combination assay, SW480 cells were plated in 96-well plates at a density of 500 cells/well/100μl of medium and allowed to set overnight at 37°C and then treated the next day with serial dilutions of cisplatin, oxaliplatin, carboplatin and aspirin analogues for 72 h [36,37,38,39,40].

Dissolution of platinum compounds, cisplatin, oxaliplatin and carboplatin with DMSO was avoided because DMSO reacts with platinum complexes thereby decreasing their cytotoxic effect [41]. It has been found out that the nucleophilic sulphur in DMSO reacts with platinum complexes thereby displacing their ligands, which result in a structure change, and thus making the compounds unstable in DMSO [42,43]. Due to these facts, cisplatin was dissolved using PBS while both oxaliplatin and carboplatin were dissolved using water [41]. Cell viability was measured by 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) reduction assay [44] with modifications [45]. Combination drug treatments were performed by pairing cisplatin, oxaliplatin or carboplatin with each of the aspirin analogues in a constant ratio design based on their IC_{50}. A

mixture of the two drugs was made at 2-fold and then serially diluted to 1-fold, 0.5-fold, 0.25-fold and 0.125-fold. After 24 h of seeding, the culture medium was then replaced with medium containing combinations of aspirin analogues and platinum compounds for 72 h. The medium was then aspirated from each well and cells washed once with fresh medium to completely remove drugs and replaced with 300 µl of MTT reagent (0.5 mg/ml). Cells were incubated at 37°C for 3 h after which time the medium was aspirated and replaced with 200 µl dimethyl sulfoxide (DMSO). The plates were further incubated at 37°C for 30 min at which time the conversion of MTT into formazan crystals by living cells was measured by recording changes in the absorbance at 540 nm in a microplate reader (Microplate Reader Thermo Multiskan Ascent 96 & 384). Plates were protected from light throughout this procedure. All assays were performed in duplicates, three independent times (*n=3*).

Doses and fraction of cells affected (Fa) of individual and combinations of compounds were fed into the CompuSyn software (Paramus, NJ., 2005), which calculated combination index (CI) and dose reduction index (DRI) at ED_{50}, ED_{75} and ED_{90}. This produces multiple drug dose-effect calculations using the Median Effect methods described by Chou and Tatalay [22]. The CI is the quantitative measure of the degree of drug interaction in terms of additive effect (CI = 1), synergism (CI < 1), or antagonism (CI > 1) while the DRI is the measure of favourable dose reduction when two drugs are used in combination.

RESULTS

The standard curve for the MTT assay was determined to make sure that the concentration of formazan is directly proportional to the absorbance at 540 nm. Linear regression analysis shows "r-square" value as 0.99, which indicates the assay to be a good fit and thus rendering it ideal for purpose (Figure 2).

Platinum compounds in combination with aspirin analogues were used to treat SW480 CRC cells for 72 h. The cytotoxic effect and Fa were then calculated and fed into the CompuSyn software where the CI was determined at ED_{50}, ED_{75} and ED_{90}. Against the colorectal cell line, SW480, synergistic effect was observed with cisplatin and *m*-aspirin [1:400] at ED_{90}; cisplatin and *p*-aspirin [1:250] at ED_{50}, ED_{75} and ED_{90}; cisplatin and diaspirin [1:17.5] at ED_{50}, ED_{75} and ED_{90}. Oxaliplatin, the platinum compound of interest showed synergistic effects with *p*-aspirin [1:100] and diaspirin [1:15] at ED_{50} and ED_{75}. Carboplatin only had a favourable synergistic effect with *p*-aspirin [1:50] at ED_{75} and ED_{90} (Table 3).

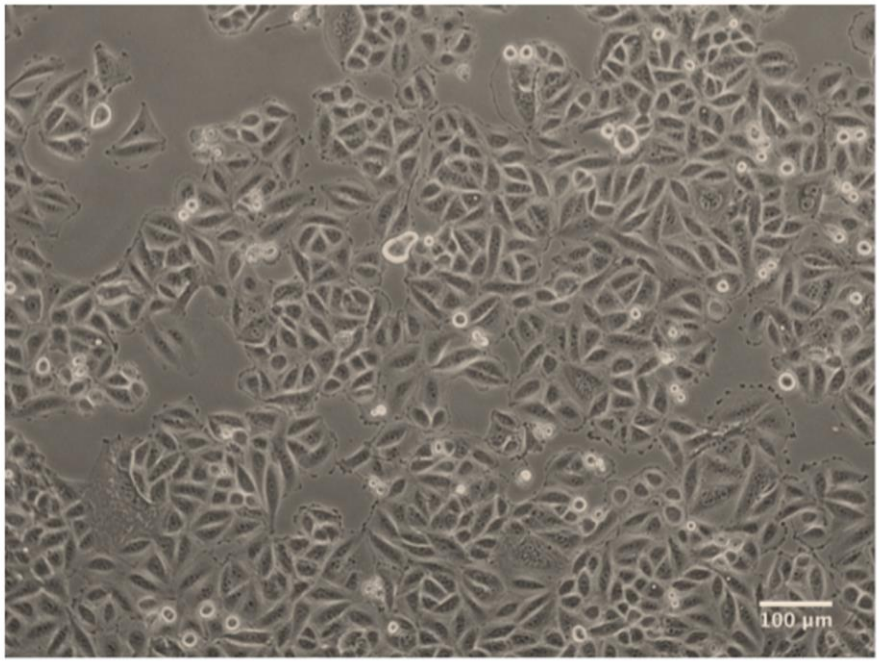

Figure 1. Morphology of SW480 CRC cell line (100X magnification).

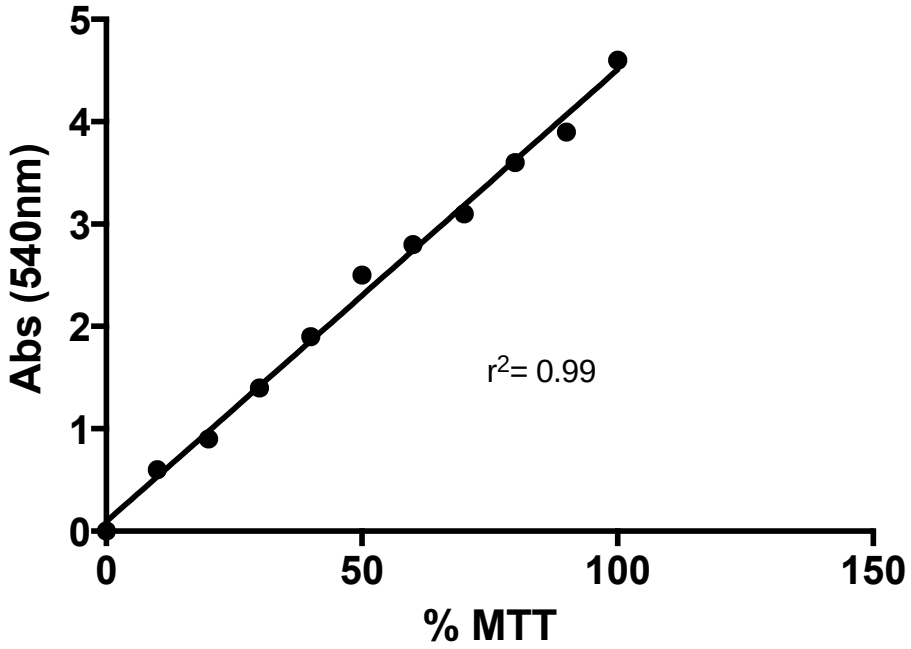

Figure 2. Standard curve for MTT solution.

Table 1. Example of a DRI Data for Drug Combination involving drugs A and B.

Fa	Dose A	Dose B	DRI A	DRI B
0.5	3.7260	4.1938	3.2163	3.5924

Table 2. Description and Interpretation of CI values [Adapted from (Bijnsdorp *et al.*, 2011, Chou, 2006)].

Combination Index (CI)	Graded Symbols	Interpretation	Simplified CI values and their interpretation
<0.1	+++++	very strong synergism	Synergism (+)
0.1-0.3	++++	strong synergism	
0.3-0.7	+++	synergism	
0.7-0.85	++	moderate synergism	
0.85-0.9	+	slight synergism	
0.9-1.1	*	nearly additive	Additive (*)
1.1-1.2	−	slight antagonism	Antagonism (-)
1.2-1.45	−−	moderate antagonism	
1.45-3.3	−−−	antagonism	
3.3-10	−−−−	strong antagonism	
>10	−−−−−	very strong antagonism	

Table 3. Summary of drug combination CI values in SW480 CRC cell line.

Compounds	Cisplatin (CP)			Oxaliplatin (OX)			Carboplatin (CB)		
	CI values at								
	ED_{50}	ED_{75}	ED_{90}	ED_{50}	ED_{75}	ED_{90}	ED_{50}	ED_{75}	ED_{90}
aspirin (Kilari et al., 2019)	+	+	+	+	+	+	-	-	-
m-aspirin (PN548)	*	*	+	*	*	-	*	-	-
p-aspirin (PN549)	+	+	+	+	+	*	*	+	+
diaspirin (PN508)	+	+	+	+	+	*	-	-	+
o-thioaspirin (PN590)	*	*	*	*	*	+	-	-	*
m-thioaspirin (PN591)	-	-	-	*	*	-	-	-	-
p-thioaspirin (PN592)	-	-	*	*	-	-	*	-	-

Drug combinations with CI values at effective dose for 50% kill of the cell population (ED_{50}), 75% of the population (ED_{75}) and 90% of the population (ED_{90})

DISCUSSION

The mutational status of *TP53* gene in SW480 CRC [9,43] and p53 dependence for sensitivity to oxaliplatin indicate that cancers made up of such cell lines will present reduced sensitivity to platinum compounds and thus cytotoxicity reduced [32,40,46,47] in such cancers. Likewise, in CRC cell lines, p53 is essential for chemotherapy-induced cytotoxicity exhibited by oxaliplatin [32]. In addition, MMR proficient CRC cells are known to survive high doses of cisplatin or oxaliplatin [48] leading to treatment failure and a high incidence of debilitating side effects [29,31]. Sensitivity to these aspirin analogues clearly differs from cell type to cell type due to differences in specific targets by different compounds [9]. Din et al. [9] compared sensitivity to aspirin in CRC cell lines to non-CRC cell lines and found out that the dose of aspirin used was inversely proportional to cell viability in CRC cells while non-CRC cells did not show this relationship. Thus, a compound that will increase sensitivity of SW480 CRC cells to oxaliplatin will lead to reduced dose needed and eventually less serious side effects. As previously reported, aspirin was found to have synergistic effects when combined with cisplatin and oxaliplatin, but not with carboplatin [49]. The combination of cisplatin and PN548 (*meta*-aspirin) [1:400] in SW480 CRC cells (Table 3) had synergistic effect at ED_{90}. In addition, the ED_{50} for cisplatin in

this combination reduced by about 10-fold, which is favourable in cancer therapy [20]. The ED_{50} of cisplatin also reduced by almost 10-fold when in combination with PN549 (*para*-aspirin) [1:250] accompanied by a synergistic effect at ED_{50}, ED_{75} and ED_{90} (Table 3). Cisplatin in combination with PN508 (diaspirin) [1:18] (Table 3) had synergistic effects in SW480 CRC cells with about a 2-fold decrease in ED_{50} for cisplatin. In addition, the synergistic effects of these combinations increased at higher dose, which is advantageous in chemotherapy [20]. Cisplatin in combination with PN590 (*ortho*-thioaspirin) [1:20] had an additive effect in SW480 CRC cells but both of its isomers, PN591 (*meta*-thioaspirin) and PN592 (*para*-thioaspirin) (Table 3) had an antagonistic effect when used in combination with all three platinum compounds. This antagonism between the isomers of thioaspirin and the platinum compounds could be because PN591 and PN592 have lower cytotoxic effects as compared to PN590 against this CRC cell line [50].

In this study, combinations of oxaliplatin with PN548 [1:80] (Table 3) had antagonistic effects at ED_{75} and ED_{90}. Even though there was a synergistic effect at ED_{50} and below, this is unfavourable in cancer therapy because maximum cytotoxicity against the cancer cells is key to effective therapy [20]. Oxaliplatin combination with PN548 [1:80] (Table 3) however resulted in a slight synergistic effect at ED_{50} and ED_{75} increasing to moderate synergism at ED_{90} with a 4-fold decrease in oxaliplatin ED_{50}. The ED_{50} for the platinum compound, oxaliplatin had a 7-fold decrease when combined with PN549 and PN590 with a synergistic effect (Table 3). Although there was a synergistic effect when oxaliplatin was combined with PN508 [1:14] at ED_{50}, the effect regressed to antagonism with an increase in dose (ED_{75} and ED_{90}). With the prominent side effect of oxaliplatin being peripheral neuropathy [27], a reduction in its ED_{50} as a result of the combinations with aspirin [49], PN549 and PN590 may reduce or totally alleviate this side effect because a reduction in drug concentration will result in toxicity reduction and also delay or minimize the induction of drug resistance [20]. Although, there was what seemed to be a synergistic effect between carboplatin with PN508 at ED_{50}, the doses of carboplatin at ED_{50} increased rather than decreased when used in combination with the diaspirin. This increase in ED_{50} of carboplatin when in combination defeats one of the main reasons for combination therapy, which is to achieve a decrease in effective dose in order to reduce or alleviate side effects. Carboplatin, in combination with the thioaspirins, PN590, PN591 and PN592 also had strong synergistic effects (Table 3) against the colorectal cancer cell line. PN549 [1:50] (Table 3) was the only aspirin analogue that showed synergistic effects against SW480 CRC cell line when in combination with carboplatin. There was a 3-fold decrease in carboplatin ED_{50}.

Combinations of aspirin and its analogues with platinum compounds have also been investigated using OE33 oesophageal cancer cell line [49]. However, these combinations should further be investigated in other cancer cell lines.

In the treatment of CRC, oxaliplatin has been used in combination with a variety of cytotoxic agents producing different levels of desired effects [27]. Due to oxaliplatin not having any effect on the drug biotransformation enzyme, P450, its combination with commonly used drugs such as aspirins does not raise any concerns [51].

In conclusion, oxaliplatin was found to exhibit synergistic effects when combined with para-aspirin (PN549), diaspirin (PN508) and ortho-thioaspirin (PN590). Some of these aspirin analogues thus show promising results when combined with platinum compounds for the treatment of cancer and should be further investigated *in vivo*.

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