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Research paper

Novel phosphonate analogs of sulforaphane: Synthesis, *in vitro* and *in vivo* anticancer activity

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ABSTRACT

A library of over forty, novel, structurally diverse phosphonate analogs of sulforaphane (P-ITCs) were designed, synthesized and fully characterized. All compounds were evaluated for antiproliferative activity *in vitro* on Lovo and LoVo/DX colon cancer cell lines. All compounds exhibited high antiproliferative activity, comparable or higher to the activity of naturally occurring benzyl isothiocyanate and sulforaphane. Assessment of the mechanisms of action of selected compounds revealed their potential as inducers of G_2/M cell cycle arrest and apoptosis. Further antiproliferative studies for selected compounds with the use of a set of selected cell lines derived from colon, lung, mammary gland and uterus as well as normal murine fibroblasts were performed. *In vivo* studies of the analyzed phosphonate analogs of sulforaphane showed lower activity in comparison with those of benzyl isothiocyanate. Our studies demonstrated that newly synthesized P-ITCs can be used for as a starting point for the synthesis of novel isothiocyanates with higher anticancer activity in the future.

1. Introduction

Cruciferous vegetables are a rich source of glucosinolates which, after enzymatic transformation by myrosinase followed by Lossen rearrangement, release isothiocyanates (ITCs) – organic heterocummulenes containing a highly electrophilic –NCS moiety [1]. These compounds, originally recognized as repellents and insecticides [2] in the 1960s, have proven to be potent inhibitors of chemically-induced carcinogenesis [3]. The discovery of sulforaphane (1-isothiocyanato-4-methylsulfinylbutane, **SFN**) in 1992 by Zhang and co-workers [4] was a turning point for studies into naturally occurring isothiocyanates as potential anticancer agents. Subsequently, their chemopreventive and anticancer activity has been evaluated in numerous studies indicating a complex, multi-targeted mode of action [5].

Because of the electrophilic character of the isothiocyanato group, the glutathione [6] and free cysteine –SH groups present in multiple proteins are recognized as natural primary intracellular targets. At least 30 of proteins [7], including microtubules [8], Kelch-like ECH-associated protein 1 and Nrf2 system [9] and proteasome [10], have been found to be directly modified after isothiocyanate accumulation in the cell cytoplasm. Additionally, they can act as indirect modulators of phase I and II enzymes [11],

Abbreviations: AITC, allyl isothiocyanate; ALT, alanine transaminase; An V, annexin V; ANOVA, analysis of variances; APC, allophycocyanin; AST, aspartate transaminase; b.w., body weight; BITC, benzyl isothiocyanate; BTMS, bromotrimethylsilane; BuITC, butyl isothiocyanate; CDDP, cisplatin; CRE, creatinine; CTRL, control group; CY, cyclophosphamide; DOXO, doxorubicin; Hct, hematocrit; Hgb, hemoglobin; *i.p.*, intraperitoneally; IR, index of resistance; ITC, isothiocyanates; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide; NQO1, NAD(P)H dehydrogenase (quinone 1); MAD, median average deviation; MITO, mitoxantrone; MTD, maximal tolerated dose; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; *ort.*, orthotopically; PI, propidium iodide; P-ITC, phosphonate analog of sulforaphane; PLT, platelet; SFN, sulforaphane; SRB, sulforhodamine B; TGI, tumor growth inhibition; TIW, three times a week; UREL, urea; WBC, white blood cell.

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inhibitors of cell cycle progression and inducers of oxidative stress *via* glutathione depletion [6,12], inhibition of thioredoxin [13] or even by direct causation of mitochondrial leakage [14]. Some of the observed biological properties of ITCs can be assigned to the activity of their metabolites – mercapturic acids (e.g., histone deacetylase inhibition [15]), which also serve as ITC intracellular depots. Mercapturic acids can undergo hydrolysis and released isothiocyanates can easily re-enter cells [16] further affecting cell metabolism, cycle progression, and *redox* status, eventually leading to cell cycle arrest, cell death *via* apoptosis [17,18] or autophagy [19].

The complex mode of action of isothiocyanates might be considered an advantage, e.g. decreased risk of the development of isothiocyanate–resistance. On the other hand, such complexity creates difficulty to understand isothiocyanate structure–activity relationships. Presence (if any) of additional functional groups found in natural isothiocyanates is limited to C=C double bonds, and aryl or hydroxyl groups. The large group of ITCs which can be considered sulforaphane (**SFN**) analogs comprise a central sulfur atom with different oxidation states connected to an isothiocyanato group by an unbranched carbon linker of different length [20]. To enhance structural diversity and the potentially biological activity of ITCs, attempts to synthesize their diverse synthetic analogs have been undertaken [21]. Among others, replacing isothiocyanate sulfur with selenium resulting in isoselenocyanates [22], substitution of the sulforaphane *S*-methyl group with organofluorine substituents [23], insertion of an –NCS moiety in naphthalimide [24] or implementation of a second –NCS group [25] to the ITC core. Most of the synthetic analogs show at least comparable biological activity to naturally occurring compounds, which raises the question of how far the original structure can be modified without losing its antiproliferative potential. The presence of a second functional group (at least a double C=C bond) in ITC appears to be obligatory [26], but the question then arises as to how significantly these groups can influence a compound's overall activity, and ultimately if the second functional group's character can significantly modulate its mode of action and intracellular target selection.

Based on Posner et al.'s [27] early findings that replacement of a sulfoxide group in SFN with a dimethylphosphinoyl group resulted in a compound of almost equal potency to SFN, and our previous results on antiproliferative activity of α- and β-dialkoxyphosphoryl- [28] and α-diaryloxyphosphoryl- [29] isothiocyanates, we designed and synthesized a library of novel bifunctional sulforaphane analogs – structurally diverse dialkyl and diphenyl ω-(isothiocyanato) alkylphosphonates (P-ITCs) with different steric and electronic effects around the central phosphonato group connected to an –NCS moiety by unbranched carbon linkers of different lengths. Due to the electrophilic character of both isothiocyanate and phosphonate moieties, any such series of compounds should address the issue of whether the second electrophilic center can modulate isothiocyanate's biological properties. To establish a possible correlation between structure and antiproliferative activity, biological properties of synthesized compounds, both *in vitro* and *in vivo*, were determined. Additionally, comparative studies into the mode of actions reflected by selected compounds were performed to assess structure–mechanism of action correlations.

2. Results and discussion

2.1. Chemistry

The number of known ω-(isothiocyanato)alkylphosphonates is limited [30,31]. Only synthesis of diethyl 3-(isothiocyanato)propylphosphonate has been described in patent literature [31]. We applied two strategies in the synthesis of the target P-ITCs **36**–**79** (Schemes 1 and 2). In the first strategy, the appropriate dialkyl or diaryl ω-azidoalkylphosphonates **1**–**35** were converted *via* the Staudinger and aza-Wittig reaction [32,33] with triphenylphosphine/carbon disulfide to the target ITC-Ps **36**–**70** (31–93% yield) after flash chromatography (Scheme 1).

The primary aim of the second strategy as well as one-pot method was to show the possibility of direct conversion of the parent diethyl ω-(isothiocyanto)alkylphosphonates **41**–**45** into selected alkyl or phenyl P-ITCs esters **36**–**40, 51, 56, 70** and **71**–**79** (Scheme 2). Such a strategy enables the use of a few starting diethyl analogs **41**–**45** in the synthesis of the library of isothiocyanatophosphonates. The modification of the protocol established by Molnár et al. [34] for the synthesis of bis(trifluoroethyl) 2-oxoalkylphosphonates was applied in these transformations.

Thus, according to Scheme 2, dealkylation of diethyl ω-(isothiocyanto)alkylphosphonates **41**–**45** by bromotrimethylsilane (BTMS) [35] generated intermediate bis(trimethylsilyl)alkylphosphonates **80**. Next, crude **80** was transformed into the appropriate ω-(isothiocyanto)alkylphosphonic dichlorides **81** as a result of the action of oxalyl chloride [36]. Crude dichlorides **81** were subjected to the reaction with the appropriate alcohol or phenol in the presence of triethylamine to provide moderate yields of the target P-ITCs **36**–**40, 51, 56, 70** and **71**–**79** (12–82%) after flash chromatography.

The starting dialkyl ω-azidoalkylphosphonates **1**–**16, 18**–**25** are accessible in gram quantities *via* microwave-assisted azidation of easily available dialkyl ω-bromoalkylphosphonates [37–40] by sodium azide in water or DMF as solvent, using the procedure established in our laboratory [41]. Bis(trifluoroethyl) and diphenyl ω-azidoalkylphosphonates **26**–**35**, for which synthesis of parent ω-bromoalkylphosphonates is not possible *via* the Michaelis-Arbuzov reaction [42] and diisobutyl 2-azidoethylphosphonate (**17**) were prepared from diethyl ω-azidoalkylphosphonates **6**–**10** in a one-pot synthesis by analogy to the methodology described in Scheme 2. The scope and general character of such a strategy was clearly demonstrated by preparation azides **1**–**5** and **12**–**21** (Scheme 3). The modification of the procedure described for the synthesis of mixed esters of 3-azidopropylphosphonate [43] was applied in the above mentioned transformation.

According to Scheme 3, cleavage of the ester groups in diethyl ω-azidoalkylphosphonates **6**–**10** was accomplished with BTMS [35], followed by the sequential reactions of bis-(trimethylsilyl)esters thus formed with oxalyl chloride and the appropriate alcohol or phenol. The target, novel bis(trifluoroethyl) and diphenyl ω-azidoalkylphosphonates **26**–**35** were isolated after flash chromatography at 50–65% and 55–78% yields, respectively. The other dialkyl azidoesters **1**–**5**, and **13**–**21** were obtained at 38–71% yields. A clear exception was diisopropyl 2-azidoethylphosphonate (**12**), which was formed at 8% yield only. Structures of azidophosphonates **1**–**35** and the target isothiocyanatophosphonates **36**–**79** were confirmed by MS and ^{1}H, ^{31}P and ^{13}C NMR.

2.2. In vitro studies

Over the past few years, results obtained in our laboratories utilizing colorectal adenocarcinoma cell line LoVo as well as its doxorubicin-resistant subline LoVo/DX with a resistant index against doxorubicin in the range 40–60 have proved to be the most informative about the antiproliferative potential of isothiocyanates; thus, all synthesized compounds (**36**–**79**) were first tested for antiproliferative activity against these two cell lines (Table 1). Representative naturally occurring isothiocyanates (**SFN**, benzyl isothiocyanate (**BITC**), butyl isothiocyanate (**BuITC**), allyl isothiocyanate (**AITC**)), as well as doxorubicin (**DOXO**) and cisplatin (**CDDP**) were used as a reference (Table 1). As an indicator of the