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Research paper

Applying the designed multiple ligands approach to inhibit dihydrofolate reductase and thioredoxin reductase for anti-proliferative activity

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ABSTRACT

The development of multi-targeting drugs is currently being explored as an attractive alternative to combination therapy, especially for the treatment of complex diseases such as cancer. Dihydrofolate reductase (DHFR) and thioredoxin reductase (TrxR) are enzymes belonging to two unrelated cellular pathways that are known to contribute towards cancer cell growth and survival. In order to evaluate whether simultaneous inhibition of DHFR and TrxR by dihydrotriazines (DHFR-targeting) and chalcones (TrxR-targeting) may be beneficial, breast MCF-7 and colorectal HCT116 carcinoma cells were treated with combinations of selected dihydrotriazines and chalcones at a 1:1 M ratio. Two combinations demonstrated synergy at low-to-moderate concentrations. Based on this result, four merged dihydrotriazine-chalcone compounds were designed and synthesized. Two compounds, **11a** [DHFR IC_{50} = 56.4 μM, TrxR IC_{50} (60 min) = 12.6 μM] and **11b** [DHFR IC_{50} = 2.4 μM, TrxR IC_{50} (60 min) = 10.1 μM], demonstrated *in vitro* inhibition of DHFR and TrxR. The compounds showed growth inhibitory activity against MCF-7 and HCT116 cells, but lacked cytotoxicity. Molecular docking experiments showed **11b** to possess rational binding modes to both the enzymes. In conclusion, this study has not only identified the dihydrotriazine and chalcone scaffolds as good starting points for the development of dual inhibitors of DHFR and TrxR, but also demonstrated the synthetic feasibility of producing a chemical entity that could result in simultaneous inhibition of DHFR and TrxR. Future efforts to improve the antiproliferative profiles of such compounds will look at alternative ways of integrating the two pharmacophoric scaffolds.

1. Introduction

The rational design of producing a single chemical entity that is able to act on multiple targets relevant to a disease is an approach currently gaining popularity. In 2005, the term “designed multiple ligands” was coined by Morphy and Rankovic to describe such compounds [1]. In the field of cancer chemotherapy, designed multiple ligands offer several potential advantages over single target compounds. Multiple pathways of cell survival could be targeted simultaneously, leading to increased therapeutic efficacy [2] and decreased development of cancer drug resistance [3]. Additionally, the use of a single multi-targeted agent instead of administering multiple agents could help to circumvent the gruelling issues associated with complex pharmacokinetic and pharmacodynamic (PK/PD) relationships and potential drug–drug interactions [1].

Dihydrofolate reductase (DHFR) and thioredoxin reductase (TrxR) are two enzymes that are implicated in the growth and survival of cancer cells. As part of the folate pathway, DHFR catalyzes the reduction of dihydrofolic acid to tetrahydrofolic acid, which is essential for the biosynthesis of nucleic acids and amino acids. Methotrexate (MTX) is a DHFR inhibitor that has been used in the clinic as an anticancer agent for more than 60 years. However, its use has become limited by the emergence of acquired resistance to MTX by cancer cells, and this has inspired efforts to develop non-classical DHFR inhibitors that do not contain the glutamate residue found in MTX [4–6]. Being lipophilic, non-classical inhibitors enter cells via passive diffusion, thus circumventing drug resistance due to transport resistance attributed to decreased levels of or mutations in the reduced folate carrier, a putative carrier for active MTX

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transport. Additionally, since non-classical DHFR inhibitors are not polyglutamated, they are not affected by impaired polyglutamylation resulting from reduced folylpolyglutamate synthetase (FPGS) activity. Examples of non-classical DHFR inhibitors are the 4,6-diamino-1,2-dihydro-1,3,5-triazines, first synthesized by E.J. Modest in 1951 [7]. Our laboratory has interest in the design and development of this class of triazine-based compounds as non-classical DHFR inhibitors [8–10].

TrxR and its substrate thioredoxin (Trx) make up the Trx system, which plays an important role in maintaining redox homeostasis and protecting cells against oxidative damage and mutation [11,12]. However, in malignancies, the biological activities of the Trx/TrxR system contribute to tumor growth and progression. Indeed, it has been demonstrated in several human cancers that overexpression of Trx and TrxR are associated with drug resistance and poor patient prognosis [13–16]. The mammalian TrxR enzyme has a highly accessible C-terminal active site containing a penultimate selenocysteine (Sec) residue which is easily attacked by agents that possess an electrophilic center [17–19]. Among such compounds that possess electrophilic character, chalcones containing an α,β-unsaturated carbonyl moiety, also known as a Michael acceptor moiety, have recently been demonstrated to exert anti-proliferative effects against human cancer cells through selective and irreversible inhibition of TrxR [20,21]. The electrophilic β carbon atom within the α,β-unsaturated carbonyl moiety of chalcones is proposed to react irreversibly with the C-terminal Sec residue of the TrxR enzyme via a Michael addition reaction, thereby forming covalent adducts [20].

In the current study, the goal was to harness the combined benefits of simultaneous inhibition of DHFR and TrxR using compounds possessing the respective pharmacophoric scaffolds to bring about enhanced anti-cancer effect. To do this, two approaches were employed and examined. In the first approach, it was hypothesized that the combined use of a 4,6-diamino-1,2-dihydro-1,3,5-triazine and a chalcone would result in enhanced antiproliferative profiles against cancer cells in comparison to the anti-tumor activities of the individual compounds. As such, MCF-7 breast and HCT116 colorectal carcinoma cells were treated with a combination of 4,6-diamino-1,2-dihydro-1,3,5-triazines and chalcones at a 1:1 M ratio. In the second approach, it was hypothesized that the structural combination of the 4,6-diamino-1,2-dihydro-1,3,5-triazine and chalcone scaffolds would produce new molecules that possessed potent antiproliferative profiles correlated to strong DHFR and TrxR inhibitory activities. To test this hypothesis, the 4-6-diamino-1,2-dihydro-1,3,5-triazine and chalcone scaffolds were incorporated synthetically into a single chemical entity; in a preliminary attempt, four merged dihydrotriazine-chalcone compounds were synthesized and evaluated for anti-proliferative activities and possible *in vitro* inhibitory effects on DHFR and TrxR. In summary, findings obtained in this proof-of-concept study have highlighted the feasibility of producing a single chemical entity to bring about simultaneous inhibition of DHFR and TrxR, albeit the chemical approach to merge dihydrotriazine and chalcone pharmacophores to obtain dual-targeted compounds possessing well-correlated enzyme (DHFR and TrxR) inhibitory and anti-proliferative activities would need further optimization.

2. Results and discussion

2.1. Combination treatment

Two 4,6-diamino-1,2-dihydro-1,3,5-triazines, **T1** and **T2**, and three chalcones, **C1**–**C3**, were selected for this study. The structures of the compounds are shown in Fig. 1, and the enzyme inhibitory activities and antiproliferative activities of **T1**-**T2** and **C1**–**C3** are summarized in Tables 1 and 2 respectively. *In vitro* efficacy of enzyme inhibitory activity was expressed as IC_{50} (50% DHFR/TrxR inhibition concentration), while antiproliferative activity was expressed as GI_{50}, which is the concentration of drug at which 50% of growth inhibition was achieved, and LC_{50}, which is the concentration of drug at which 50% of the cells were killed. As shown in Table 1, **T1** and **T2** were moderately potent inhibitors of recombinant human DHFR with IC_{50} values in the low μM range. **T1** and **T2** also inhibited growth of MCF-7 and HCT116 carcinoma cells with GI_{50} values in the low μM range, but were not cytotoxic as evident in the LC_{50} values that were determined to be above 100 μM. This could be related to their DHFR inhibitory activities that caused cells to be unable to produce new DNA, RNA and proteins, thus resulting in growth inhibition but not necessarily cell death. As shown in Table 2, **C1**–**C3** demonstrated moderately potent inhibition of rat liver TrxR upon incubation with the enzyme for 60 min, recording IC_{50} values in the μM range. As evident from the obtained GI_{50} and LC_{50} values in the μM range, the chalcones displayed moderately good anti-proliferative activity and exerted cytotoxicity against MCF-7 and HCT116 cell lines.

In order to evaluate whether the use of 4,6-diamino-1,2-dihydro-1,3,5-triazines and chalcones in combination would result in enhanced anti-proliferative activity, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was carried out on MCF-7 and HCT116 cells treated with the individual compounds and the six possible combinations of a 4,6-diamino-1,2-dihydro-1,3,5-triazine with a chalcone at a 1:1 M ratio. Cell viability after 72 h of incubation with the compounds was then assessed.

The dose–effect curves of the 4,6-diamino-1,2-dihydro-1,3,5-triazines **T1**-**T2** and chalcones **C1**–**C3** were observed to be markedly different from each other. At high doses, the maximum decrease in cell viability effected by **T1**-**T2** was approximately 50%, whereas **C1**–**C3** decreased cell viability by 100%. Additionally, the Hill slope of the dose–effect curves of **T1**-**T2** was ~1, whereas the Hill slope of **C1**–**C3** ranged from ~2 to 4. Synergy analysis using mathematical models such as the Bliss independence model [22], Loewe additivity model [23] and the median effect principle by Chou and Talalay [24] was unsuitable, since an underlying assumption for these models is that the individual drugs will give rise to equal maximal effects. Modified equations based on the Loewe additivity model have been derived by Grabovsky and Tallarida [25] for cases whereby the two individual drugs produce different maximal effects. However, these equations assume that Hill slope = 1 for both drugs. In the absence of an appropriate mathematical model, we therefore adopted the concept of cooperative effect synergy, which is simply defined as an increase in the effect of the drug combination over the agents alone. In a review of synergy methodologies, Geary recommended this simpler definition of synergy because it avoids mathematical difficulties, is easily applicable, meets FDA criteria for evaluation of combination therapies, and is adequate for basic discovery and clinical research [26].

In order to determine whether the drug combinations showed a better effect than their individual components, their dose–effect curves were plotted and compared. Dose-effect curves on MCF-7 and HCT116 cells are shown in Figs. 2 and 3 respectively. In the dihydrotriazine-chalcone combinations **T1** + **C1** and **T1** + **C3**, when used at low-to-moderate concentrations of 1.5–15 μM, both 4,6-diamino-1,2-dihydro-1,3,5-triazine and chalcone components in the combination contributed to a stronger anti-proliferative effect that resulted in a greater reduction in cell viability in comparison to the decrease in cell viability brought about by treatment with the individual components (Figs. 2A, C, 3A, C). Hence, at these concentrations, a small cooperative effect was observed between **T1** and **C1** or **C3**. However, at higher concentrations, the dose–effect