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Trypanotoxic activity of thiosemicarbazone iron chelators

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A B S T R A C T

Only a few drugs are available for treating sleeping sickness and nagana disease; parasitic infections caused by protozoans of the genus *Trypanosoma* in sub-Saharan Africa. There is an urgent need for the development of new medicines for chemotherapy of these devastating diseases. In this study, three newly designed thiosemicarbazone iron chelators, TSC24, Dp44mT and 3-AP, were tested for *in vitro* activity against bloodstream forms of *T. brucei* and human leukaemia HL-60 cells. In addition to their iron chelating properties, TSC24 and Dp44mT inhibit topoisomerase IIα while 3-AP inactivates ribonucleotide reductase. All three compounds exhibited anti-trypanosomal activity, with minimum inhibitory concentration (MIC) values ranging between 1 and 100 μM and 50% growth inhibition (GI_{50}) values of around 250 nM. Although the compounds did not kill HL-60 cells (MIC values >100 μM), TSC24 and Dp44mT displayed considerable cytotoxicity based on their GI_{50} values. Iron supplementation partly reversed the trypanotoxic and cytotoxic activity of TSC24 and Dp44mT but not of 3-AP. This finding suggests possible synergy between the iron chelating and topoisomerase IIα inhibiting activity of the compounds. However, further investigation using separate agents, the iron chelator deferoxamine and the topoisomerase II inhibitor epirubicin, did not support any synergy for the interaction of iron chelation and topoisomerase II inhibition. Furthermore, TSC24 was shown to induce DNA degradation in bloodstream forms of *T. brucei* indicating that the mechanism of trypanotoxic activity of the compound is topoisomerase II independent. In conclusion, the data support further investigation of thiosemicarbazone iron chelators with dual activity as lead compounds for anti-trypanosomal drug development.

Keywords:
Trypanosoma brucei
Sleeping sickness
Topoisomerase

Thiosemicarbazone iron chelators

1. Introduction

African trypanosomes are the etiological agents of sleeping sickness in humans and nagana disease in cattle (Steverding, 2008). The parasites are transmitted by the bite of infected tsetse flies (*Glossina* spp.) and live and multiply in the blood and tissue fluids of their mammalian host. Trypanosomiasis affects both humans and animals mainly in rural sub-Saharan Africa where the disease imposes significant burden on public health and economic development. Without treatment, both sleeping sickness and nagana disease are fatal. Sadly, few drugs are available for chemotherapy of African trypanosomiasis (Holmes et al. 2004; Steverding, 2010). In addition, most drugs are outdated and difficult to administer. Moreover, drug resistance in African trypanosomes is an increasing problem in the therapy of both sleeping sickness and nagana disease (Matovu et al., 2001; Delespaux and de Koning, 2007). Thus, new strategies are needed if novel chemotherapies are to be developed.

One strategy to improve the activity of drugs is the conjugation of two bioactive moieties. For instance, the conjugate of the iron chelator deferiprone and a chloroquine fragment (7-chloro-4-aminoquinoline) has been shown to display higher trypanotoxic activity than both parent compounds alone (Gehrke et al., 2013). Other examples of compounds with dual activity are thiosemicarbazones. For instance, the compounds Dp44mT and TSC24 (Fig. 1) possess both iron chelating and topoisomerase IIα inhibiting activity (Rao et al., 2009; Huang et al., 2010) while the compound 3-AP (Fig. 1) exhibits iron chelating and ribonucleotide reductase inhibiting activity (Finch et al., 1999; Aye et al., 2012). As topoisomerases and ribonucleotide reductase are essential enzymes involved in the metabolism and replication of DNA (Corbett and Berger, 2004; Nordlund and Reichard, 2006), and as iron chelation has been shown to limit the proliferation of bloodstream form trypanosomes (Breidbach et al., 2002; Merschjohann and Steverding, 2006), inhibition of these enzymes in combination with iron depletion may be an interesting option for the development of novel anti-trypanosomal chemotherapies. For this reason, we studied the *in vitro* trypanotoxic activity of the thiosemicarbazones TSC24, Dp44mT and 3-AP using bloodstream forms of *Trypanosoma brucei*. In addition, we investigated whether the combination of iron chelation and topoisomerase inhibition shows synergy.

2. Materials and methods

2.1. Reagents

Deferoxamine mesylate, di-2-pyridylketone-4,4,-dimethyl-3-thiosemicarbazone (Dp44mT), 3-aminopyridine-2-carboxaldehyde thiosemicarbazone (3-AP) and ammonium ferric citrate were purchased from Sigma-Aldrich (Gillingham, U.K.). (E)-N,N-dimethyl-2-(quinolin-2-ylmethylene)hydrazinecarbothioamide (TSC24) was from Merck Chemicals Ltd. (Nottingham, U.K.). Epirubicin hydrochloride was obtained from Cambridge Bioscience Ltd. (Cambridge, U.K.).

2.2. Cell cultures

Bloodstream forms of *T. brucei* clone 427-221a (Hirumi et al., 1980) and human myeloid leukaemia HL-60 cells (Collins et al., 1977) were grown in Baltz medium (Baltz et al., 1985) and RPMI medium (Moore et al., 1967), respectively. Both media were supplemented with 16.7% (v/v) heat-inactivated foetal calf serum. All cultures were maintained in a humidified atmosphere containing 5% CO_2 at 37°C.

2.3. Toxicity assays

Trypanosomes and HL-60 cells were seeded in 24-well plates in a final volume of 1 ml culture medium containing various concentrations of thiosemicarbazones dissolved in 100% DMSO. Controls contained DMSO alone. In all experiments, the final DMSO concentration was 1%. The seeding densities were 10^4/ml trypanosomes and 10^5/ml HL-60 cells. For toxicity assays including iron supplementation, 10 µl of medium was replaced with 10 µl of a 1.93 mg/ml ammonium ferric citrate solution to give a final iron(III) concentration of 50 µM. After 48 h of incubation, living cells were counted with a Neubauer haemocytometer. The 50% growth inhibition (GI_{50}) values, i.e. the concentration of compounds necessary to reduce the growth rate of cells by 50% to that of controls, was determined by linear interpolation according to the method described in (Huber and Koella, 1993). The minimum inhibitory concentration (MIC) values, i.e. the concentration of the compounds at which all cells were killed, was determined microscopically.

2.4. Flow cytometric analysis

Flow cytometric analysis was performed as described previously (Phillips et al., 2013). Bloodstream form trypanosomes (1-5 × 10^6/ml) were incubated with 500 nM TSC24, 50 µM ammonium ferric citrate, 5 µM TSC plus 50 µM ammonium ferric citrate or 1% DMSO for 24 h. After harvesting by centrifugation at 850×*g* and washing twice with PBS/1% glucose, cells were fixed in 100 µl ice-cold methanol for 5 min and then diluted with 1 ml PBS. After centrifugation, the cell pellets were re-suspended in PBS and stained with propidium iodide (final concentration 50 µg/ml). Cells were analysed on a BD Accuri C6 Flow Cytometer. Debris was excluded from analysis through gating on forward scatter and side scatter properties. Singlets were identified and doublets excluded through gating on FL-2 (585/40 nm) area versus height. A minimum of 10,000 cells were collected for analysis. Data was analysed using FlowJo version 10.

2.5. Isobolographic analysis

The interaction of the iron chelator deferoxamine and the topoisomerase II inhibitor epirubicin was evaluated using the isobolographic method as described previously (Steverding and Wang, 2009). First, the GI_{50} value for each drug was determined. Based on the GI_{50} values, bloodstream form trypanosomes were incubated with twofold serially diluted 1:1 ratios of drug combination. For controls, trypanosomes were cultured with twofold serially diluted concentrations of each drug alone. After 48 h incubation, live cells were counted and the GI_{50} value for each drug in the absence and in the presence of the other co-administered drug was determined. The combination index (CI) for the drug combination was calculated using the equation

$$CI = \frac{GI_{50(DFO,com)}}{GI_{50(DFO,sin)}} + \frac{GI_{50(EPI,com)}}{GI_{50(EPI,sin)}}$$

where $GI_{50(DFO,com)}$ and $GI_{50(EPI,com)}$ are the concentrations of deferoxamine and epirubicin used in the combination to achieve 50% growth inhibition and $GI_{50(DFO,sin)}$ and $GI_{50(EPI,sin)}$ are the concentrations of deferoxamine and epirubicin alone to achieve the same effect. A CI value of <1, $=1$, and >1 indicates synergism, additive effect, and antagonism, respectively.[6]

3. Results

The trypanotoxic activity of the thiosemicarbazones TSC24, Dp44mT and 3-AP was determined with bloodstream forms of the *T. brucei* strain 427-221a while the general cytotoxicity of the compounds was evaluated with human myeloid leukaemia HL-60 cells. All three thiosemicarbazones showed a dose-dependent effect on the inhibition of the growth of trypanosomes in cell culture with similar GI_{50} values ranging between 0.226 and 0.287 μM (Table 1). Statistical analysis revealed no significant difference between the GI_{50} values of the three compounds (ANOVA, p =

0.574). Both TSC24 and Dp44mT displayed a promising MIC value of 1 μM while 3-AP a less favourable value of 100 μM (Table 1) demonstrating that all three compounds are trypanocidal. By comparison, clinically used anti-sleeping sickness drugs display much higher anti-trypanosomal activities. For example, pentamidine, melarsoprol and sur~~m~~amin exhibit GI_{50} values of 0.001 μM, 0.016 μM and 0.032 μM, and MIC values of 0.006 μM, 0.1 μM and 1 μM, respectively (Merschjohann et al., 2001; Caffrey et al., 2007; Steverding et al., 2014). The thiosemicarbazones also inhibited the proliferation of HL-60 cells but with GI_{50} values varying between 0.005 and 0.673 μM (Table 1). Dp44mT and TSC24 proved to be more effective in inhibiting the growth of HL-60 cells than that of trypanosomes. However, all three compounds had a MIC value of >100 μM indicating that they were cytostatic rather than cytocidal. Anti-sleeping sickness drugs, on the other hand, are much less toxic to HL-60 cells. For example, the GI_{50} values of pentamidine, melarsoprol and suramin are 33 μM, 4 μM and >100 μM, respectively, while their MIC values are ≥100 μM (Merschjohann et al., 2001; Caffrey et al., 2007; Steverding et al., 2014). As a result, the GI_{50} and MIC ratios of cytotoxic to trypanotoxic activities (selectivity indices) for the thiosemicarbazones were much less favourable than those of anti-sleeping sickness drugs. TSC24 and Dp44mT had a GI_{50} ratio of <1 while their corresponding MIC ratio was, at >100, more promising (Table 2). The GI_{50} and MIC ratios for 3-AP were 2.85 and >1 indicating poor selectivity of this drug. In contrast, the GI_{50} and MIC ratios of anti-sleeping sickness drugs are much higher (pentamindine: 9,800 and 13,000; melarsoprol: 267 and >1,000; suramin: >100 and >1,000) (Merschjohann et al., 2001; Caffrey et al., 2007; Steverding et al., 2014).

Supplementation of iron partially reversed the trypanotoxic activity of TSC24 and Dp44mT causing a 13- and 100-fold increase of their GI_{50} and MIC values, respectively (Table 1). This finding supports the notion that both thiosemicarbazones could chelate iron in cells, which may have contributed to the trypanotoxic activity of the compounds. In contrast, addition of iron did not impair the anti-trypanosomal activity of 3-AP (Table 1). Iron supplementation also reduced the cytotoxicity of the compounds (Table 1). However, the GI_{50} values for TSC24 and Dp44mT for HL-60 cells increased only 5- and 7-fold, respectively, which was lower than those observed for the compounds for trypanosomes. As the addition of iron shifted the trypanotoxic and the cytotoxic activity of the compounds in the same direction, no change in the MIC and GI_{50} ratios were observed apart from a 100-fold drop in the MIC ratios for TSC24 and Dp44mT (Table 2).

As TSC24 and Dp44mT are inhibitors of topoisomerase IIα and displayed almost equal trypanotoxic activities indicating that their mechanism of anti-trypanosomal action is identical, TSC24 was chosen to investigate the effect of this thiosemicarbazone on the cell cycle progression in *T. brucei*. Bloodstream form trypanosomes were incubated for 24 h in the absence or presence of iron with TSC24 at concentrations sufficient to inhibit the growth of the cells without killing them. The iron supplementation control showed little change in the cell cycle distribution compared to the DMSO control (Fig. 2A). TSC24 treatment increased the population of cells with sub-G1 and post-G1 DNA content (Fig. 2B). This action of TSC24 is in contrast to the effect of the compound on the cell cycle progression in mammalian cells where the thiosemicarbazone has been reported to induce a G1-S arrest (Huang et al., 2010). However, our finding is reminiscent of the action of idarubicin, a classical topoisomerase II inhibitor, on *T. rangeli* where the drug has also been demonstrated to lead to DNA degradation (Jobe et al., 2012). When bloodstream forms of *T. brucei* were incubated with TSC24 in the presence of iron, an increase in cells in the G1 phase was observed (Fig. 1C). This result resembles the action of Dp44mT and TSC24 found for mammalian cells where the compounds induce a G1-S cell cycle arrest (Rao et al., 2009; Huang et al., 2010).

To investigate whether the trypanotoxic action of TSC24 and Dp44mT was the result from a synergistic effect of their iron chelating and topoisomerase II inhibiting activity, a combination assay was carried out. Although the iron chelating properties of TSC24 and Dp44mT is known to be due to their thiosemicarbazone scaffold, the part of the molecules responsible for their topoisomerase inhibiting properties is not known. Therefore, a combination assay was designed using two separate agents, the iron chelator deferoxamine and the topoisomerase II inhibitor epirubicin. The combination of deferoxamine with epirubicin showed an antagonistic effect with a CI of 1.49±0.25 (Fig. 3). Whereas the GI_{50} of deferoxamine dropped from 10.8±2.1 μM to 4.5±0.6 μM, the GI_{50} of epirubicin remained unchanged (108±17 nM vs 113±14 nM). This result suggests that iron chelation and topoisomerase inhibition probably do not show trypanocidal synergy.

3. Discussion

As bloodstream forms of *T. brucei* contain only four iron-dependent enzymes (aconitase, alternative oxidase, ribonucleotide reductase and superoxide dismutase) and do not express any iron storage proteins, they are more prone to iron-depletion than mammalian cells (Breidbach et al. 2002). Thus, iron chelation could be an interesting approach for the development of new trypanocidal drugs. In this study, we investigated the trypanotoxic activity of newly designed thiosemicarbazones that in addition to their iron chelating properties display inhibitory activities against different enzymes. Aiming simultaneously at two biological targets with one drug may achieve greater therapeutic efficacy due to synergistic effects.

All three thiosemicarbazones studied showed similar trypanotoxic activities. The addition of iron reduced the anti-trypanosomal action of TSC24 and Dp44mT but not that of 3-AP. This may be explained by the different inhibitory mechanism of the compounds. Whereas the anti-proliferate effect of Dp44mT and TSC24 have been attributed to both iron chelation and inhibition of topoisomerase IIα (Rao et al., 2009; Huang et al., 2010), that of 3-AP is due to the destruction of the tyrosyl radical of the β2 subunit of ribonucleotide reductase through the active reductant [Fe(II)-(3-AP)] (Aye et al., 2012). As the activity of 3-AP requires binding of iron, supplementation of the metal would not be expected to significantly affect the toxic action of the compound. An alternative mode of action was reported for Dp44mT involving redox cycle of the iron-Dp44mT complex to generate reactive oxygen species (ROS) (Yuan et al., 2004). Similar to 3-AP, iron supplementation should not affect this activity of Dp44mT as the production of ROS requires the metal. However, as the addition of iron reduces the anti-trypanosomal effect of Dp44mT, this additional mode of action involving the production of ROS does~~seems~~ not seem to be responsible for the trypanotoxic activity of the compound.

Comment [DS1]: 'Seems not' is perfectly correct but is more archaic and less used today. So my correct is only a suggestion.

The cytotoxic activity of Dp44mT and TSC24 has been associated with the ability of the compounds to induce cell cycle arrest at the G1-S checkpoint (Rao et al., 2009; Huang et al., 2010) which is consistent with previous reports of most iron chelators (Brodie et al., 1993; Yu et al., 2007). Our results indicate that the mechanism of action of the two thiosemicarbazones on the cell cycle in bloodstream forms of *T. brucei* is different from that in cancer cells. In the absence of iron, the compounds caused a reduction in the DNA content in many cells. This finding is indicative for degradation of DNA suggesting a topoisomerase II independent mechanism of trypanotoxic action for the compounds similar to that of idarubicin described previously for *T. rangeli* (Jobe et al., 2012). In the presence of iron the thiosemicarbazones caused an increase of bloodstream form trypanosomes in the G1 phase which suggests that some of the trypanosomes had undergone cell cycle arrest at the G1-S boundary. It appears that in the absence of iron Dp44mT and TCS24 display different actions towards trypanosomes than to cancer cells. However, it should be mentioned that bloodstream forms of *T. brucei* have a much lower iron content than mammalian cells (Schell et al., 1991). Therefore, it is possible that the thiosemicarbazones within cancer cells quickly bind iron and execute their activity only as an iron complex while in bloodstream form trypanosomes they operate mainly as iron-free compounds. This suggestion is supported by the fact that iron supplementation has a much greater abrogating effect on the trypanotoxic activity of Dp44mT and TSC24 (13-fold reduction) than on their cytotoxic activity (~6-fold reduction). That iron supplementation has only a minor effect on the cytotoxic activity of TSC24 has been previously demonstrated (Huang et al., 2010).

Although TCS24 has been demonstrated to have both iron chelating and topoisomerase inhibiting activities with both actions believed to contribute to its cytotoxicity against a range of cancer cell lines (Huang et al., 2010), it remains unclear whether both activities contribute also to the trypanotoxic action of the compound. The partial reversal of the anti-trypanosomal activity of TSC24 upon iron addition may indicate that both actions play a role and act synergistically. However, combination experiments carried out with the iron chelator, deferoxamine, and the topoisomerase II inhibitor, epirubicin, showed no synergy between iron chelating and topoisomerase inhibiting actions. As, in this test, two separate agents were used, it is possible that the two compounds interfere with each other's activity reducing their efficacy. Another explanation for a possible difference in the interaction of iron chelating and topoisomerase II inhibiting activity of TSC24 and deferoxamine/epirubicin combination may lie in the different topoisomerase inhibition mechanism of TSC24 and epirubicin. Whereas TSC24 is a catalytic inhibitor inactivating topoisomerase II via binding to the APTase domain and blocking the ATP hydrolysis activity of the enzyme (Huang et al., 2010), epirubicin is a topoisomerase poison that intercalates between DNA base pairs and stabilises the DNA-enzyme complex (Coukell and Faulds, 1997).

In summary, the three thiosemicarbazones investigated in this study all show GI_{50} values below 300 nM for bloodstream forms of *T. brucei*. These values are within the range of GI_{50} values reported previously for other topoisomerase inhibitors for trypanosomes (Deterding et al., 2005). In addition, the MIC value of TSC24 and Dp44mT was similar to that of suramin (1 μM), one of the current drugs used to treat sleeping sickness (Merschjohann et al., 2001; Steverding et al., 2014). However, the selectivity of the thiosemicarbazones was poor. While the MIC values showed that the compounds did not kill human HL-60 cells, the GI_{50} values indicated unsatisfactory cytotoxicity of the agents. Nevertheless, the actual clinical selectivity of the thiosemicarbazones may be much higher. As the thiosemicarbazones have been selected for cytotoxic action against cancer cells, their anti-proliferative effect on HL-

60 cells may, therefore, be an overestimate for a healthy cell response. Whether thiosemicarbazone iron chelators are interesting compounds for further anti-trypanosomal drug development remains to be shown.

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Table 1

MIC and GI_{50} values of the thiosemicarbazones TSC24, Dp44mT and 3-AP for *T. brucei* bloodstream forms and human HL-60 cells.

Compound	*T. brucei*				HL-60			
	MIC (μM)		GI_{50} (μM)		MIC (μM)		GI_{50} (μM)	
	−Fe	+Fe	−Fe	+Fe	−Fe	+Fe	−Fe	+Fe
TSC24	1	100	0.287±0.020	3.642±2.068	>100	>100	0.122±0.058	0.617±0.077
Dp44mT	1	100	0.226±0.082	3.069±0.436	>100	>100	0.005±0.002	0.036±0.025
3-AP	100	100	0.236±0.093	0.322±0.046	>100	>100	0.673±0.054	1.537±0.921

Data are mean values± SD of three experiments.

Table 2

MIC and GI_{50} ratios of cytotoxic to trypanotoxic activities of the thiosemicarbazones TSC24, Dp44mT and 3-AP.

Compound	$MIC_{(HL\text{-}60)}/MIC_{(\textit{T. brucei})}$		$GI_{50(HL\text{-}60)}/GI_{50(\textit{T. brucei})}$	
	−Fe	+Fe	−Fe	+Fe
TSC24	>100	>1	0.43	0.17
Dp44mT	>100	>1	0.02	0.01
3-AP	>1	>1	2.85	4.77

MIC and GI_{50} ratios were calculated from MIC and GI_{50} values shown in Table 1.

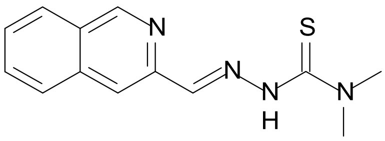
FIGURE LEGENDS

Fig. 1. Structures of the iron-chelating thiosemicarbazones TSC24, Dp44mT and 3-AP. The PubChem Compound Identifier (CID) for each compound is shown in parentheses.

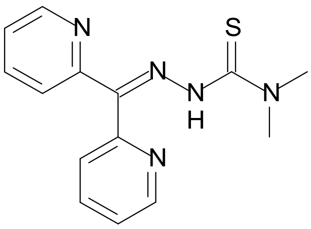
Fig. 2. Cell cycle distribution of *T. brucei* exposed to TSC24. Bloodstream form trypanosomes were treated with 50 μM iron(III) (A), 0.5 μM TSC (B) or 5 μM TSC plus 50 μM iron(III) (C). The dotted trace in each graph is the result of the DMSO control culture. After 24 h incubation, the trypanosomes were stained with propidium iodide and the DNA content analysed by flow cytometry.

Fig. 3. Isobolographic plot for the interaction between the iron chelator deferoxamine and the topoisomerase II inhibitor epirubicin. Bloodstream forms of *T. brucei* were incubated with twofold serial dilutions of the drug combination (1:1) or the drugs alone. After 48 h of incubation, live cells were counted and GI_{50} values determined. The dotted line that connects the GI_{50} points for the single drug treatments (filled squares) is the theoretical additive line. The GI_{50} of the combinations is indicated by the open circle. Each point represents the mean ± SD of three independent experiments.

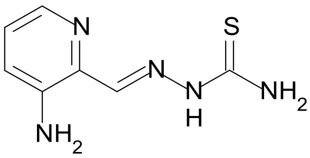
FIG. 1



TSC24 (CID: 46202546)



Dp44mT (CID: 10334137)



3-AP (CID: 9571836)

FIG. 2

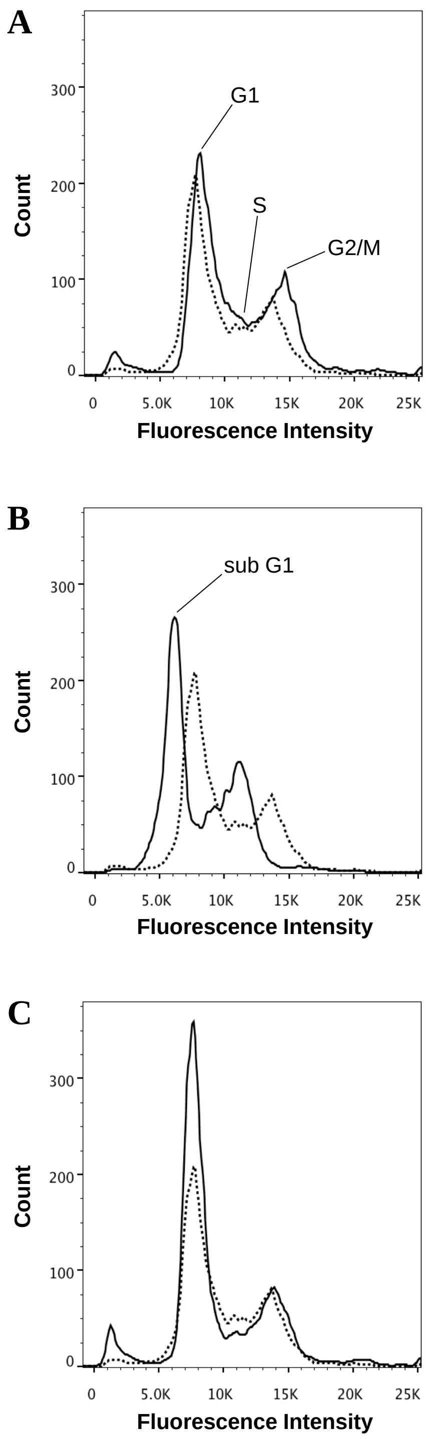

A
G1
S
G2/M
Count
300
200
100
0
0
5.0K
10K
15K
20K
25K
Fluorescence Intensity
B
sub G1
Count
300
200
100
0
0
5.0K
10K
15K
20K
25K
Fluorescence Intensity
C
Count
300
200
100
0
0
5.0K
10K
15K
20K
25K
Fluorescence Intensity

Fig. 3