Targeting of CD34⁺CD38⁻ cells using Gemtuzumab ozogamicin (Mylotarg) in combination with tipifarnib (Zarnestra) in acute Myeloid Leukaemia

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Abstract

Background: The CD34⁺CD38⁻ subset of AML cells is enriched for resistance to current chemotherapeutic agents and considered to contribute to disease progression and relapse in Acute Myeloid Leukaemia (AML) patients following initial treatment.

Methods: Chemosensitivity in phenotypically defined subsets from 34 primary AML samples was measured by flow cytometry following 48 hr in vitro treatment with gemtuzumab ozogamicin (GO, Mylotarg) and the farnesyltransferase inhibitor tipifarnib/zarnestra. The DNA damage response was measured using flow cytometry, immunofluorescence and immunohistochemistry.

Results: Using a previously validated in vitro minimal residual disease model, we now show that the combination of GO (10 ng/ml) and tipifarnib (5 μM) targets the CD34⁺CD38⁻ subset resulting in 65% median cell loss compared to 28% and 13% CD34⁺CD38⁻ cell loss in GO-treated and tipifarnib-treated cells, respectively. Using phosphokinome profiling and immunofluorescence in the TF-1a cell line, we demonstrate that the drug combination is characterised by the activation of a DNA damage response (induction of γH2AX and thr68 phosphorylation of chk2). Higher induction of γH2AX was found in CD34⁺CD38⁻ than in CD34⁺CD38⁺ patient cells. In a model system, we show that dormancy impairs damage resolution, allowing accumulation of γH2AX foci.

Conclusions: The chemosensitivity of the CD34⁺CD38⁻ subset, combined with enhanced damage indicators, suggest that this subset is primed to favour programmed cell death as opposed to repairing damage. This interaction between tipifarnib and GO suggests a potential role in the treatment of AML.

Keywords: Tipifarnib/Zarnestra, Gemtuzumab ozogamicin/ Mylotarg, AML CD34⁺CD38⁻ cells, DNA damage response

Background

Acute myeloid leukaemia is a disease in which patients tend to respond well to remission induction chemotherapy, but relapse is common because current therapy cannot totally eradicate the leukaemic cells. Cells which survive chemotherapy may have a distinctive biology (e.g. active survival pathways, quiescent cell cycle status) compared to the bulk of cells in the clone, and/or may be protected by the bone marrow niche microenvironment in which they reside. For this reason the identification of a post-remission chemotherapy that can specifically target these cells is crucial. The CD34⁺CD38⁻ cell subset was originally thought to contain all the leukaemia initiating cells [1]. Whilst leukaemia initiating cells with a more mature phenotype have now also been found [2,3], the subset remains of particular interest since it is enriched for quiescent, chemoresistant cells which are associated with the likelihood of relapse [4-6].

Gemtuzumab ozogamicin/GO (Mylotarg) is a chemotherapeutic agent that consists of a humanised anti-CD33 antibody (Hp67.6) conjugated to N-acetyl-calicheamicin 1,
2-dimethyl hydrazine dichloride, a potent enediyne antitu-
mour antibiotic. In the MRC AML 15 trial, AML patients 
<60 years with favourable risk disease who were treated with 
GO in combination with induction chemotherapy showed a 
significant survival benefit, and a trend was also documented 
for patients with intermediate risk [7]. This benefit to good 
risk patients was found in a similar study conducted by the 
Southwest Oncology Group (SWOG) [8]. Older patients, in-
cluding those with intermediate risk cytogenetics, also ben-
fit from the addition of GO to remission induction 
chemotherapy [9,10]. Using an in vitro short-term culture 
system consisting of a defined “niche-like” microenvironment 
we previously showed that GO treatment can target CD34 
+CD38- cells [11]. We therefore investigated whether CD34 
+CD38- cell sensitivity to GO could be enhanced by another 
anti-leukaemic chemotherapeutic agent for which clinical ef-
cacy has already been established. Several agents were 
examined in a preliminary study, of which tipifarnib 
appeared to be the most promising. Tipifarnib is an orally 
examined in a preliminary study, of which tipifarnib 
was found to cause better CR duration and higher CR rates in AML 
patients with chromosome 5/7 abnormalities [19]. We therefore investigated whether CD34 
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patients with chromosome 5/7 abnormalities [19].

In this report we establish the efficacy of combining 
tipifarnib with GO in vitro, particularly in CD34+CD38- 
AML cells, and investigate the mechanisms involved.

Methods

Cell samples

Blood or bone marrow samples were obtained with writ-
ten informed consent from AML patients and healthy 
stem cell donors. Use of these samples was approved by 
the Nottingham 1 Ethics Committee and the Nottingham 
University Hospitals NHS Trust. Mononuclear cells were 
isolated using a standard density gradient centrifugation 
method with Histopaque.

Materials

Recombinant cytokines were obtained from R&D Sys-
tems (Abingdon, UK). Phentyping antibodies were from 
Becton Dickinson (Cowley, UK). GO was kindly pro-
vided by Wyeth Pharmaceuticals (PA, USA) and tipifarn-
ib by Johnson and Johnson Pharmaceutical Research 
and Development (NJ, US). Unless otherwise stated, all 
other reagents were purchased from Sigma (Poole, UK). 
Stock solutions were prepared as follows: GO (1 mg/mL), 
daunorubicin (1 mM), vinblastine (4.5 mg/ml) and 
verapamil (50 mg/ml) were reconstituted in water; tipifar-
nib in 1 V HCl: 2 V Methanol (25 mM), cyclosporin A in 
100% ethanol (25 mg/mL).

Cell culture

Cell lines

The KG-1a and U937 cell lines were purchased from the European Collection of Animal Cell Cultures and 
the TF-1a cell line from the American Tissue Culture 
Collection (ATCC). U937 and TF-1a cells were maint-
tained in RPMI 1640 with 10% foetal calf serum 
(FCS; First Link), 2 mmol/L L-glutamine, 100 U/mL 
penicillin, and 10 μg/mL streptomycin (R10). KG-1a 
were maintained as above but with 20% FCS. All cul-
tures were kept at 37°C in 5% CO2 and all experi-
ments were done with cell lines in log phase. 
Continued testing to authenticate these cell lines was 
done using a panel of monoclonal antibodies toward 
the final passage of each batch thawed.

Primary AML cells

Fresh or cryopreserved AML cells were cultured at 10⁶/ml 
for 48 hours, in triplicate in serum-free medium consisting of 
Iscove’s modified Dulbecco medium supplemented with 
200 μg/ml transferrin, 10 μg/ml insulin, 1% L-glutamine, 
2% bovine serum albumin and 10⁻⁴ M mercaptoethanol. 
Fibronectin-coated wells and serum free medium (SFM) 
were used as previously described [20]. For maintenance of 
CD34+CD38- cell phenotype in 48 hours culture we treated 
AML samples in serum-free medium with immobilized 
fibronectin along with a combination of cytokines consist-
ing of IL-3 (20 ng/ml), SDF-1 (100 ng/ml), SCF (50 ng/ml) 
and TPO (50 ng/ml).

Phosphorylated protein detection

For the detection of the relative phosphorylation levels of 
46 intracellular kinases we used the Human Phospho-
Kinase Antibody Array (Catalogue # ARY003, R&D Sys-
tems, Abingdon, UK) according to manufacturers’ instruc-
tions. Proteins were visualized using chemiluminescence 
(Hyperfilm ECL; Amersham), scanned using a Syngene 
densitometer, and analyzed using the GeneSnap software 
(Syngene).

Flow cytometry

Chemosensitivity assays and immunophenotyping

Phenotyping was carried out using antibodies to CD34, 
CD38, CD123 and CD33. A preliminary comparison of 
test/control relative fluorescence intensity (RFI) values 
in leukaemic samples versus those of CD34+CD38- cells 
from healthy donor samples was carried out to establish 
cut-off points to verify the leukaemic nature of samples 
asessed (for CD33, n = 11, mean RFI + 2 standard devi-
ation cut-off point = 8.67; for CD123, n = 12, mean RFI +
2 standard deviation cut-off point = 17), Flow cytometric enumeration of CD34+CD38- and bulk AML cells were measured in leukaemic samples as previously described [20]. Briefly, two flow cytometric analyses were used in parallel for the analysis of CD34+CD38- cell survival. One assay allowed reproducible measurement of the concentration of viable cells at the end of the experiment using 10 μg/ml 7-amino-actinomycin D (7-AAD) and fixed CD45-stained normal mononuclear cells as internal standard [21]. The second assay allowed the determination of the percentage of CD123+CD34+CD38- cells within the viable population using CD34 FITC and CD38 APC and CD123 PE (or isotype controls) with 7-AAD. From these 2 analyses we calculated the concentration of leukaemic CD34+CD38- cells from the cell count and the proportion of viable cells which are CD34+CD38+CD123-.

**Determination of RNA status**

The method of Toba 1995 was used, using 7-AAD to label DNA and pyronin Y to label RNA [22]. RNA was also measured on unselected cells by spectrophotometry (Nanodrop 2000, ThermoScientific, UK distributor Fisher Scientific, Loughborough, UK).

**Measurement of P-glycoprotein protein and function**

P-glycoprotein (Pgp) protein and function was measured using flow cytometric methodology as previously described [23]. Each assay involved labelling with the fluorescent probe or antibody of interest and with an antibody against CD45 to allow leukemic (CD45 low/side scatter low) cells to be gated. The peridinin chlorophyll protein conjugate to CD45 was chosen to avoid spectral overlap with FITC labels. Briefly, Pgp substrate efflux modulation by tipifarnib, Cyclosporin A, vinblastine and verapamil was determined in a modulation assay using rhodamine 123 based on the report by Broxterman et al. [24]. For protein measurement, MRK16 anti-Pgp (Kamiya Biomedical) antibody (30 minutes, room temperature) was used followed by 20% normal rabbit serum to block (30 minutes, 4°C) and FITC-conjugated goat anti-mouse secondary antibody (30 minutes, 4°C; Dako). U937 (Pgp negative) and KG1a (Pgp positive) were used as controls.

**Measurement of γH2AX and phospho-Chk2(Thr68)**

Cells were fixed and permeabilised using the Leucoperm kit (AbdSerotec) with an additional 10 minute incubation in ice-cold methanol after the first fixation step. For γH2AX, 2 μg/mL mouse monoclonal antibody (Upstate) or mouse isotype control (DAKO) was added (2 hours, RT) and goat anti-mouse IgG FITC F(ab')2 secondary antibody (DAKO) was used as second layer. For phosphorylated Chk2 (Thr68), a rabbit polyclonal antibody (1:25 dilution Cell Signalling Technology) was added or not for 1 hour at RT and goat anti-rabbit FITC was used as second layer. Data were analyzed on a FACS CANTO using the DIVA software with the phosphorylation status expressed as a ratio between test and negative control antibodies (relative fluorescence ratio; RFI). For primary cell work, cryopreserved cells were used (65–99% blasts). These cells were counterstained with CD34 PerCP and CD38 APC. The PE channel was not used in order to maximise accuracy of FITC specific fluorescence without recourse to fine-tuning compensation for individual samples. Isotype control antibodies were used to set gates for CD34 and CD38 fluorescence.

**Immunohistochemistry and immunofluorescence**

For immunohistochemistry, cells were fixed on glass slides, labeled with γH2AX and counted using the H score, a semi-quantitative measurement of damage foci per 100 cells, as previously described [25]. For immunofluorescence, cells were labeled as for flow cytometry and were mounted on glass slides in DAPI-containing mounting medium.

**Cytogenetics, FLT3 and NPM1 status**

FLT3/ITDs were analysed by previously described methods [26]. NPM1 mutation status was identified as described by Noguer et al. [27]. Stratification into favourable, intermediate, and adverse cytogenetics groups was made according to guidelines established in Medical Research Council studies [28].

**Data output and statistical analysis**

Statistical analysis was carried out using the Statistical Package for Social Sciences, version 16 (SPSS). Tests used were based on the assumption that cell line data was parametrically distributed and patient cell data non-parametrically distributed. P values of ≤ 0.05 were considered to represent significance. The supra-additive nature of the combination of tipifarnib and GO was made by comparing the toxicity of the combination with the sum of the toxicities of the two agents individually by Wilcoxon signed rank tests.

**Results**

**Assessment of the leukaemic nature of CD34+CD38- cells**

The frequency of CD34+CD38- cells in normal bone marrow has been calculated as 0.02 ± 0.01% mononuclear cells [29]. In 27 of our original cohort of 38 samples, the proportion of CD34+CD38- cells was >2%, i.e. at least 100X the normal value and therefore judged to be overwhelmingly leukaemic. Of the remaining 11 samples, 7 over-expressed both CD123 and CD33 on their CD34+CD38- cells, 1 over-expressed CD123 but not CD33 and 1 over-expressed CD33 but not CD123. In 2
samples a defining leukaemic phenotype was not found and these samples were excluded from further analysis. In addition, 2 samples had insufficient viable CD34+CD38- cells for analysis after 48 hour culture, such that the final cohort analysed comprised 34 samples.

Chemosensitivity to tipifarnib/GO combination in primary AML cells
We used an in vitro model comprising immobilised fibronectin, serum-free medium and a mixture of the cytokines IL-3, SCF, TPO and SDF-1 to support the survival of CD34+CD38- cells in culture without loss of phenotype [11]. The response of primary AML blasts to GO at 10 ng/ml has previously been reported [11], and this was maintained in the current study. With insufficient CD34+CD38- cells in most samples to study more than one concentration of each drug, we carried out a preliminary study to establish a concentration of tipifarnib (5 μM) that would induce a low level (10-30%) cell kill as a single agent (data not shown). When the cohort was expanded to 34 patient samples, tipifarnib (5 μM) treatment (48 hours) was found to induce a median 20% bulk cell kill versus 13% in CD34+CD38- cells. As previously found [11] GO (10 ng/ml) treatment (48 hours) alone caused a greater decrease in viable cells in the CD34+CD38- subset than in bulk cells (14% in bulk cells versus 28% in CD34+CD38- cells, P = 0.003). The combination of 5 μM tipifarnib and 10 ng/ml GO resulted in a median bulk cell kill of 51% and median CD34+CD38- cell kill of 65% (Tables 1 and 2 and Figure 1A). Excluding the 1 bulk cell sample and 5 CD34+CD38- samples in which the sum of the individual toxicities of tipifarnib and GO was ≥100%, we determined that the combination was supra-additive in bulk cells (n = 33, P = 0.009). There was a non-significant trend towards a supra-additive effect in CD34+CD38- cells (n = 29, P = 0.066, Figure 1B). Cytogenetics were available for 23 samples (Tables 1 and 2). By MRC criteria [28] most samples were of intermediate prognostic risk (n = 15). Only 5 samples belonged to the poor risk group and three to the good risk group, rendering any subgroup analysis on these two latter groups inappropriate. Sensitivity to the drug combination correlated strongly with sensitivity to the drugs used individually (rho = 0.7, P < 0.001 for tipifarnib and rho = 0.43, P = 0.01 for GO in bulk cells, rho = 0.61, P < 0.001 for tipifarnib and rho 0.64, P < 0.001 for GO in CD34+CD38- cells).

The tipifarnib/GO combination induces a DNA damage response pathway
In order to probe for factors which might support supra-additive killing of AML cells by the combination of GO and tipifarnib, a preliminary study was carried out in which the expression of 46 known human phospho-

Impaired resolution of damage foci in dormancy-enriched leukaemia cells
CD34+CD38- leukaemia cells are largely quiescent [4,30] and reported to be resistant to chemotherapeutic drugs [4,5]. However, we have shown sensitivity to tipifarnib + GO in this subset, together with enhanced γH2AX expression. γH2AX induction is associated with double strand breaks and initiates the homologous recombination repair pathway [31,32] which is only functional in proliferating cells. To confirm that dormant CD34+CD38- cells are sensitive to drugs that induce a double strand break response, we compared the DNA damage response in proliferating and non-proliferating CD34+CD38- leukaemia cells by inducing damage in CD34+CD38- KG-1a AML cells which had been enriched for dormancy by inhibition of the mTOR pathway. In contrast to cells enriched for dormancy by serum withdrawal, the mTOR inactivation method produced cells that remained 100% viable over several days (data not shown). Low RNA content is a
hallmark of quiescent leukaemic stem/progenitor cells [33], and rapamicin-treated KG-1a cells displayed a major loss of RNA, measured as 3.5fold increase in Pyronin Ylow cells, from 13.6 to 48.6% cells, and a decrease in average RNA per cell (measured by spectrophotometry) of 54% (Figure 4a).

We treated the proliferating parent and dormancy-enriched KG-1a cells with daunorubicin. The reason to use daunorubicin rather than GO in this experiment is that daunorubicin induces DNA damage rapidly [26] and provides a clear-cut model for monitoring damage induction and resolution before the onset of confounding apoptosis. So, whereas cell lines had been exposed to GO for 24 hours before analysis of \( \gamma H2A.X \) expression, the KG-1a cells were exposed to daunorubicin for just 2 hours. Immunocytochemistry was used in order to measure the DNA damage response (DDR) after 2 hours treatment with daunorubicin with or without an additional 2 hours of incubation following drug withdrawal. The \( \gamma H2AX \) antibody was used as a marker of the DDR: H-scores were recorded to demonstrate the extent

| AML# | Cytogenetics | FLT3 Status | NPM1 Status | CD33 MFI | Pgp status | Tip% cell loss | GO% cell loss | Tip/GO% cell loss | \( \Delta \)tox |
|------|--------------|-------------|-------------|----------|------------|---------------|--------------|------------------|-----------|
| #19  | normal       | WT          | WT          | 1.7      | NEG        | 33            | 70           | 85               | -         |
| #1   | normal       | ITD         | MUT         | 74       | NEG        | 60            | 23           | 82               | -1        |
| #10  | normal       | ITD         | WT          | 3.7      | NEG        | 54            | 3            | 75               | 18        |
| #17  | inv(16)      | ITD         | WT          | 22       | NEG        | 35            | 23           | 75               | 13        |
| #13  | normal       | ITD         | WT          | 19       | POS        | 36            | 0            | 70               | 34        |
| #15  | —            | ITD         | WT          | 3.3      | POS        | 50            | 17           | 69               | 2         |
| #27  | complex      | ITD         | WT          | 38       | NEG        | 0             | 0            | 68               | 68        |
| #33  | del(13)      | ITD         | —           | 198      | NEG        | 38            | 19           | 65               | 8         |
| #14  | normal       | ITD         | MUT         | 8.7      | NEG        | 43            | 4            | 65               | 18        |
| #32  | —            | ITD         | MUT         | 46       | NEG        | 19            | 18           | 65               | 28        |
| #8   | normal       | WT          | MUT         | 83       | NEG        | 20            | 12           | 63               | 31        |
| #12  | inv(16)      | WT          | WT          | 1.5      | NEG        | 56            | 28           | 63               | -21       |
| #26  | —            | ITD         | MUT         | 43       | NEG        | 22            | 31           | 58               | 5         |
| #11  | —            | ITD         | MUT         | 43       | NEG        | 11            | 1            | 55               | 43        |
| #30  | —            | WT          | WT          | 38       | NEG        | 29            | 18           | 54               | 7         |
| #34  | normal       | ITD         | MUT         | 1.3      | NEG        | 4             | 42           | 52               | 6         |
| #20  | —            | WT          | WT          | 30       | POS        | 27            | 23           | 50               | 0         |
| #21  | del(Sq)      | ITD         | MUT         | 59       | NEG        | 40            | 25           | 49               | -16       |
| #5   | normal       | ITD         | MUT         | 75       | NEG        | 0             | 16           | 46               | 30        |
| #25  | dup1, add12, add16 | WT | — | 36 | POS | 18 | 10 | 43 | 15 |
| #9   | normal       | ITD         | MUT         | 8.4      | NEG        | 19            | 19           | 42               | 4         |
| #6   | normal       | ITD         | MUT         | 0.19     | NEG        | 31            | 37           | 41               | -27       |
| #16  | —            | —            | —           | 51       | —          | 21            | 21           | 40               | -2        |
| #4   | —            | ITD         | MUT         | 89       | NEG        | 33            | 0            | 35               | 2         |
| #35  | complex      | WT          | WT          | 18.2     | POS        | 14            | 5            | 21               | 2         |
| #31  | normal       | ITD         | WT          | 11       | POS        | 7             | 0            | 20               | 13        |
| #2   | —            | WT          | WT          | 3.4      | POS        | 2             | 0            | 17               | 15        |
| #7   | plus 11      | WT          | WT          | 23       | NEG        | 0             | 3            | 15               | 12        |
| #29  | normal       | WT          | MUT         | 140      | NEG        | 16            | 0            | 14               | -2        |
| #28  | complex      | —            | —           | 4.9      | POS        | 0             | 2            | 2                | 0         |
| #18  | —5           | WT          | WT          | 2.1      | POS        | 0             | 8            | 0                | -8        |
| #36  | —            | WT          | WT          | 6.3      | —          | 9             | 2            | 0                | -11       |
| #23  | —            | WT          | WT          | 36       | —          | 0             | 1            | 0                | -1        |

34 AML samples used in this study ordered by magnitude of response to tipifarnib + GO (column second from the right).

1% of cells lost after 48 hours treatment (compared to untreated control).

2 \( \Delta \)tox = cell loss with drug combination – (cell loss with tipifarnib + cell loss with GO). No values are displayed when cell loss with tipifarnib + cell loss with GO > 100%. 

Table 1 Characteristics of samples used in this study grouped by responses of cells to Tipifarnib and GO: bulk cells 

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http://www.biomedcentral.com/1471-2407/12/431
As expected, there were more γH2A.X foci in proliferating cells compared with quiescence-enriched cells after daunorubicin treatment (Figure 4b). Strikingly, however, when the drug was removed and cells were allowed two hours to repair, the quiescence-enriched cells were completely unable to repair daunorubicin-induced damage. These data demonstrate that inhibition of proliferation can allow the accumulation of unrepaired damage and therefore indicate a vulnerability in dormant cells.

Investigation of additional factors that may affect relative chemosensitivity to the Tipifarnib/GO combination

Pgp status
Where cells were available, we measured the Pgp status of primary AML samples (31/34 samples, 9 Pgp positive

| AML#  | %CD34⁺CD38 | CD123 MFI | CD33 MFI | Tip% cell loss¹ | GO% cell loss¹ | Tip/GO% cell loss¹ | Δtox² |
|-------|------------|-----------|----------|-----------------|----------------|-------------------|-------|
| #13   | 3          | 92        | —        | 88              | 96             | 100               | -     |
| #19   | 3.4        | 20        | 1.4      | 66              | 91             | 100               | -     |
| #1    | 40         | 51        | 193      | 61              | 40             | 96                | -     |
| #10   | 7.6        | 12        | 0.5      | 80              | 26             | 94                | -     |
| #33   | 0.3        | 46        | 143      | 26              | 67             | 94                | 1     |
| #4    | 1.7        | 35        | 437      | 57              | 57             | 93                | -     |
| #14   | 45         | 319       | 9.7      | 59              | 25             | 93                | 9     |
| #32   | 33         | 62        | 51       | 0               | 45             | 92                | 47    |
| #11   | 2.5        | 35        | 61       | 12              | 53             | 91                | 26    |
| #9    | 0.3        | 34        | 13       | 32              | 57             | 90                | 1     |
| #34   | 2.6        | 70        | 1.5      | 44              | 31             | 86                | 11    |
| #16   | 2.3        | 1.8       | 92       | 0               | 59             | 81                | 22    |
| #17   | 7          | 49        | 16       | 21              | 51             | 81                | 9     |
| #8    | 17         | 53        | 72       | 0               | 0              | 73                | 73    |
| #15   | 78         | 23        | 34       | 47              | 16             | 72                | 9     |
| #5    | 16         | 76        | 74       | 0               | 60             | 70                | 10    |
| #6    | 0.2        | 19        | 57       | 0               | 66             | 66                | 0     |
| #20   | 2.4        | 173       | 31       | 11              | 43             | 64                | 10    |
| #3    | 9          | 8.4       | 12       | 15              | 0              | 63                | 48    |
| #35   | 3.7        | 9.2       | 7.2      | 62              | 1              | 62                | -1    |
| #31   | 0.8        | 55        | 9.6      | 41              | 38             | 52                | -27   |
| #26   | 2.4        | 17        | 9        | 43              | 0              | 43                | 0     |
| #25   | 12         | 42        | 3.2      | 15              | 2              | 38                | 21    |
| #18   | 85         | 20        | 1.9      | 0               | 8              | 33                | 25    |
| #29   | 0.9        | 62        | 31       | 0               | 64             | 27                | -37   |
| #27   | 14         | 20        | 100      | 0               | 0              | 26                | 26    |
| #2    | 54         | 20        | 3.1      | 6               | 5              | 18                | 7     |
| #36   | 53         | 6         | 6.2      | 27              | 9              | 14                | -22   |
| #7    | 23         | 11        | 15       | 5               | 0              | 5                 | -5    |
| #12   | 0.3        | 29        | 7.5      | 0               | 34             | 0                 | -34   |
| #21   | 6.2        | 24        | 53       | 0               | 0              | 0                 | 0     |
| #23   | 0.2        | 42        | 20       | 0               | 2              | 0                 | -2    |
| #28   | 6          | 17        | 3.2      | 0               | 0              | 0                 | 0     |
| #30   | 2.4        | 90        | 34       | 0               | 0              | 0                 | 0     |

³AML samples used in this study ordered by magnitude of response to tipifarnib + GO (column second from the right).
¹% of cells lost after 48 hours treatment (compared to untreated control).
²Δtox = cell loss with drug combination – (cell loss with tipifarnib + cell loss with GO). No values are displayed when cell loss with tipifarnib + cell loss with GO > 100%.

of nuclear damage foci as previously reported [25].
and 22 Pgp negative, Tables 1 and 2). GO resistance in AML blasts is associated with Pgp over-expression [11,34]. In contrast, tipifarnib has been associated with inhibition of Pgp-mediated drug efflux [15,35]. Flow cytometry was used to evaluate the effects of tipifarnib on Pgp-mediated drug efflux using the fluorescent probe rhodamine 123 as a substrate. We compared the Pgp inhibitory activity of tipifarnib with the more commonly used Pgp inhibitors, cyclosporin A, vinblastine and verapamil. Our results indicate that Pgp inhibition by tipifarnib is well within the range of that exhibited by other Pgp inhibitors (Figure 5a), confirming Pgp-reversal activity by tipifarnib. Single tipifarnib and cyclosporin A treatments of three primary AML cells showed a very close correspondence between modulation by tipifarnib and cyclosporin A, (Figure 5b), indicating similar potency between the two agents in inhibiting Pgp activity.

As expected from our previous study [11], Pgp positive cells were relatively insensitive to GO treatment alone compared to Pgp negative cells (P = 0.038 and P = 0.044 for bulk and CD34<sup>+</sup>CD38<sup>−</sup> cells, respectively, Figure 5c). The drug combination also favoured Pgp negative samples (P = 0.038 for bulk cells and P = 0.053 for CD34<sup>+</sup>CD38<sup>−</sup> cells). Our data neither supports nor contradicts the hypothesis that tipifarnib is acting in part as a Pgp inhibitor: in CD34<sup>+</sup>CD38<sup>−</sup> cells median cell kill in the 9 Pgp<sup>+</sup> samples increased from 15% with tipifarnib and 5% with GO to 52% with the combination, but an
increase was only recorded in 6/9 samples and did not reach statistical significance.

**FLT3, NPM1, CD34+CD38- cell burden, CD123 and CD33 expression**

FLT3 status, nucleophosmin (NPM1) status and CD33 expression did not affect sensitivity to individual drugs or drug combinations (P > 0.05). Strikingly, although GO sensitivity in CD34+CD38- cells was inversely correlated with the percentage of CD34+CD38- in the sample (P = 0.035), this effect was absent in tipifarnib-treated cells (P = 0.82) and the tipifarnib/GO combination (P = 1).

**Discussion**

Despite advances in our understanding of the mechanisms of leukaemogenesis, AML still remains a disease with poor outcome, especially because of disease relapse. This is due to chemoresistant cells surviving the initial exposure to cancer chemotherapy. The characterisation of agents that specifically target relapse-causing cells within their protective niche microenvironment is essential to achieve complete eradication of minimal residual disease cells in AML. We have previously reported that GO targets CD34+CD38- AML subpopulation enriched for stem and progenitor cells [11]. Moreover the recent finding that the addition of GO to standard induction chemotherapy significantly increases disease free survival and reduces relapse risk in two major multi-centre trials [9,10] suggests an *in vivo* effect for GO in targeting cells contributing to minimal residual disease. The other drug in the combination we have studied is tipifarnib, which is clinically available for AML treatment and efficacy of which has been established [19,36]. However, no previous study has attempted to combine these two chemotherapeutic agents. Using 34 primary AML samples, we showed that the combination of GO and Tipifarnib is successful at not only targeting the bulk cells but even more so the CD34+CD38- leukaemia stem and progenitor cell-enriched phenotype is not the only cell subset to initiate leukaemia in transplantation models, this subset is quiescent, chemoresistant and its presence predicts for poor outcome in AML [4-6].
Figure 3 (See legend on next page.)
We have demonstrated a DNA damage response to GO alone and to the tipifarnib + GO combination. The DNA damage response indicator γH2A.X (Figure 3) and chk2-phosphothreonine68 (Figure 2) were elevated in CD34^+CD38^- cells as well as in bulk cells. Leukaemic CD34^+CD38^- cells tend to be dormant [4,30], and despite its canonical role as a checkpoint kinase, chk2 is known to respond to damage in dormant cells [37]. It must be borne in mind that the damage response can favour either repair or apoptosis. Thus, whereas CD34^+CD38^-Lin-cord

Figure 3 γH2AX in treated cells. Cells were treated with 10 ng/ml GO, 5 μM tipifarnib or the combination for the indicated times. FACs data are expressed as γH2AX test–isotype control fluorescence intensity compared to untreated cells. (a,b) Summary charts for TF-1a and U937 cells respectively cells after 48 hours’ treatment (c) Illustration of immunofluorescence and FACs plots showing increases in focal (immunofluorescence) and total (flow cytometry) γH2AX following 24 hours of treatment in U937 cells. (d) Summary charts of flow cytometric values for primary AML: bulk (n = 14); CD34^+CD38^- (n = 9); CD34^+CD38^+ (n = 8). Median values are shown by a solid bar. * represents a P value compared with untreated cells of <0.05 and ** represents P < 0.01. (e) Line graph showing γH2AX induction in CD34^+CD38^- cells compared to CD34^+CD38^+ cells in each of 8 samples.

Figure 4 Differential resolution of γH2AX foci in proliferating and quiescence-enriched KG1a cells. (a) Evaluation of cellular RNA content after treatment with 100nM rapamycin for 48 hours. (i) Flow cytometric dotplots indicating the percentage of cells which are low in RNA (Pyronin Y) as well as low in DNA (7-AAD); (ii) Mean and standard deviation of 5 independent assays; (iii) The total RNA content of lysed cells, measured by spectrophotometry (mean and standard deviation of 3 independent assays). (b) Proliferating and quiescence-enriched KG-1a cells were treated with 3 μM daunorubicin for 2 hours. Cells were then washed twice in ice cold RPMI at 4°C and were re-suspended in fresh culture medium and placed back in the incubator to allow a 2 hour repair period. γH2AX foci were measured by immunohistochemistry using the H score as described. Values correspond to the mean +/- standard deviation for the increase in γH2AX foci compared with untreated controls, n = 3.
blood cells have a delayed double strand break response compared to CD34^CD38^ progenitors [38], chk2 knockdown was found to impair, rather than enhance, apoptosis in stem cells [39]. This is of particular interest because chk2 inhibitors have been developed for the express purpose of sensitising cancer cells to chemotherapy drugs, but in contrast to chk1 inhibitors, these do not have proven efficacy, and in some situations have been found to inhibit rather than enhance apoptotic pathways [40]. The data from Dick and colleagues suggest that apoptosis is favoured by (largely dormant) haemopoietic stem cells with activated chk2. Our data suggest that the same may be true of leukaemic cells, and moreover, by including GO in a combination which induces DNA damage, the CD34^CD38^-cells over-expressed in leukaemic [41,42], but not in normal, adult bone marrow can be targeted.

To specifically examine whether the DNA damage response is enhanced or impaired in dormant CD34^CD38^- cells, we studied mTOR-inhibited KG-1a cells treated with daunorubicin, and found that these incur a smaller double strand break response than proliferating cells during a short pulse of drug, but are almost totally unable to repair the damage, such that, by two hours post-treatment, they have a higher burden of γH2A.X foci than proliferating cells. Hence, our data confirm that a DNA damage response can be induced in dormant CD34^CD38^- leukaemia cells. However, in the case of primary cells treated in vitro with GO and tipifarnib, another potential scenario is predicated on the fact that leukaemic CD34^CD38^- cells, driven by autocrine and paracrine cytokines [33], frequently re-enter the cell cycle. Thus we cannot conclude definitively that the observed damage responses are occurring in truly quiescent cells.

GO alone induced high chk2 phosphorylation in primary cell culture in bulk cells and in the CD34^CD38^- and CD34^CD38^ subsets, consistent with a previous finding [43]. In contrast, tipifarnib did not appear to induce a double strand break response as a single agent. However tipifarnib sensitivity is associated with deficency of the short patch single strand break repair
molecule aprataxin [18,44] and tipifarnib has been reported to induce DNA damage via reactive oxygen species [45]. Of interest here is that both calicheamicin and reactive oxygen species produce 3’phosphoglycolate (3’PG) blocking groups in DNA, which, if not processed efficiently, will result in strand breaks [46]. The combination of a 3’PG-bistrand DNA damage inducer and a reactive oxygen species inducer may result in complex locally damaged sites which in turn may contribute to the large increase in the double strand break response seen with the drug combination. Tipifarnib has many potential molecular targets in AML cells [47] and we acknowledge that any one of these may contribute to the mechanism of its activity and interaction with GO. However, the importance of the DDR in the interaction emerged strongly from our initial phosphokinome profiling.

Sensitivity to GO + tipifarnib in vitro varied from 0% to 100% in our cohort. We reported strong correlations between the toxicity induced by the combination and the toxicities of the individual drugs. The lack of relationship between CD33 expression levels and GO toxicity was unsurprising, given that this has already been explored extensively, with evidence for [48] and against [49,50] the expected association. Jedema and colleagues have previously noted that the failure of excess free CD33 antibody to block GO-mediated toxicity in primary AML blasts is concentration-dependent, and occurs at concentrations greater than 1 ng/ml (we used 10 ng/ml in the current study) [50]. These authors found evidence for antibody uptake by endocytosis, and their work was predicated on the finding in a clinical study that CD33 expression did not clearly correlate with GO response. Walter and colleagues found that CD33 expression had a statistically significant correlation with outcome in 276 AML patients treated with GO monotherapy, but this effect was small and therefore had minimal predictive value [48].

We and others have previously reported a role for Pgp in GO sensitivity [11,34]. In the current study we have shown that a significant association remains when tipifarnib is used together with GO, despite the role of tipifarnib as a Pgp inhibitor. Normal haematopoietic CD34+CD38- cells over-express Pgp, but we have shown previously that leukemic CD34+CD38- subsets express Pgp at the same levels as more mature cells in the sample [11], so we could not expect Pgp over-expression to account for differences in chemosensitivity between bulk cells and CD34+CD38- cells in the same individuals.

Conclusions

In summary, this study is the first to assess combining GO with tipifarnib, both of which separately have shown clinical efficacy in AML. This drug combination targets AML cells in vitro including the CD34+CD38- cells associated with chemo-resistance. The activation of a DDR pathway by GO is amplified by its combination with tipifarnib. Based on our in vitro data we suggest assessing this two drug combination in the clinic as a potential chemotherapy regimen in the treatment of AML.

Competing interest

The authors indicate no potential competing interest.

Authors’ contributions

ML and CS designed and performed research, analyzed data and wrote the paper. MP designed and facilitated the research, analysed data and wrote the paper. NR designed and facilitated the research and edited the paper. KT and NY performed research and analysed data. All authors read and approved the final manuscript.

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