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Epigenetic drug combination overcomes osteoblast-induced chemoprotection in pediatric acute lymphoid leukemia

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Abstract

Although there has been much progress in the treatment of acute lymphoblastic leukemia (ALL), decreased sensitivity to chemotherapy remains a significant issue. Recent studies have shown how interactions with the bone marrow microenvironment can protect ALL cells from chemotherapy and allow for the persistence of the disease. Epigenetic drugs have been used for the treatment of ALL, but there are no reports on whether these drugs can overcome bone marrow-induced chemoprotection. Our study investigates the ability of the DNA methyltransferase inhibitor azacitidine and the histone deacetylase inhibitor panobinostat to overcome chemoprotective effects mediated by osteoblasts. We show that the combination of azacitidine and panobinostat has a synergistic killing effect and that this combination is more effective than cytarabine in inducing ALL cell death in co-culture with osteoblasts. We also show that this combination can be used to sensitize ALL cells to chemotherapeutics in the presence of osteoblasts. Finally, we demonstrate that these effects can be replicated *ex vivo* in a number of mouse passaged xenograft lines from both B-ALL and T-ALL patients with varying cytogenetics. Thus, our data provides evidence that azacitidine and panobinostat can successfully overcome osteoblast-induced chemoprotection *in vitro* and *ex vivo* in both B-ALL and T-ALL cells.

Graphical abstract

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Keywords

azacitidine; panobinostat; acute lymphoid leukemia; epigenetic; osteoblasts; chemoprotection

Introduction

Acute lymphoblastic leukemia (ALL) is the most commonly diagnosed pediatric malignancy. While 90% of patients will undergo remission during treatment, around 20% of those patients will have a recurrence of the disease, which is often much more aggressive and difficult to treat [1, 2]. Moreover, T-ALL patients have a worse prognosis. [3]. The higher rate of relapse and treatment challenges in ALL can be partially attributed to the role of the bone marrow microenvironment in leukemogenesis and leukemia progression [4-6]. The bone marrow allows leukemic cells to interact with different cell types such as: osteoblasts, stromal cells, endothelial cells, and mesenchymal stem cells, as well as extracellular matrix (ECM) proteins like fibronectin and osteopontin. These interactions can activate intracellular signaling pathways that protect leukemic cells from chemotherapy [4, 7].

In order to decrease the high relapse rate in ALL and improve patient outcomes, treatment options that either diminish or circumvent the effects of bone marrow-induced chemoprotection must be explored. Our lab has previously identified that disrupting the interaction between p11 protein (S100A10) and annexin II was able to chemosensitize ALL cells in co-culture with osteoblasts [8]. Natalizumab, which specifically targets integrin alpha4 and disrupts stromal adhesion of ALL cells, is currently being tested in clinical trials to block integrin alpha4 chemoprotective activity [9].

Aberrant epigenetic modulation is a crucial mediator of leukemogenesis [10]. Epigenetic alterations may also play a role in the development of chemotherapy resistance in ALL [11]. These epigenetic alterations offer new therapeutic targets as they are easily amenable to change unlike genetic mutations. While there has been some success in the clinical use of epigenetic drugs, their role in overcoming microenvironment-induced chemoprotection in ALL has not been characterized.

In this study, we investigate the ability of the epigenetic drugs azacitidine (DNA methyltransferase inhibitor) and panobinostat (histone deacetylase inhibitor) to kill ALL cells and overcome osteoblast-induced chemoprotection in a variety of ALL samples. We identify that azacitidine and panobinostat show synergy in inducing ALL cell death *in vitro* and *ex vivo* and that the combination is more successful in overcoming osteoblast-induced protection than other DNA-damaging chemotherapeutics. Additionally, we show that azacitidine and panobinostat can sensitize ALL cells to chemotherapy treatments and these effects are replicated *ex vivo* in a number of patient samples with varying cytogenetics.

Materials and Methods

Cell lines, patient samples, and reagents

REH (CRL-8286), CCRF-CEM (CCL-119), and Saos-2 (HTB-85) cells were obtained from American Type Culture Collection (ATCC), Manassas, VA. Nalm6 cells were purchased from DSMZ-German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany. Leukemic cell lines were cultured in RPMI-1640 culture medium supplemented with 10% fetal bovine serum (FBS), 2 mM/L L-glutamine, 25 U/mL penicillin, and 25 µg/mL streptomycin. Saos-2 cells were cultured in DMEM/F12 (1:1) with supplements described above.

Primary B-ALL and T-ALL cells isolated from bone marrow aspirates or peripheral blood of patients treated at Nemours/Alfred I. duPont Hospital for Children are banked by the Nemours BioBank. Samples were collected under a Nemours Delaware Institutional Review Board (IRB) protocol approved by the Nemours Office of Human Subjects Protection. Patient samples were passaged in mice following the guidelines of the Nemours Institutional Animal Care and Use Committee (IACUC) as described previously [12]. Mouse passaged primary ALL cells were used for *ex vivo* studies.

Azacitidine (S1782), panobinostat (S1030), cytarabine (S1648), and daunorubicin (S3035) were obtained from Selleckchem. Powder was dissolved in DMSO to appropriate concentrations.

Determination of IC50 concentrations

Leukemic cells (30,000) were plated in 96-well plates and treated with varying concentrations of azacitidine and panobinostat. Drugs were diluted in RPMI-1640 media to the highest concentration used then serially diluted to lowest concentration and added to the corresponding wells. Viability was determined using a NovoCyte flow cytometer (ACEA Biosciences, Inc.) using a forward scatter by side scatter plot to draw independent gates demarking live and dead populations. These populations were confirmed with propidium iodide staining. GraphPad Prism was used to determine IC50 with a log(agonist) vs. response -- variable slope curve analysis using the 95% confidence interval.

Synergy assay

Leukemic cells (30,000) were plated in 96-well plates. IC50 concentrations of azacitidine and panobinostat were diluted in RPMI-1640 both singularly and in combination and added

to the cells for 48 hours. The percentage of viable cells at the end of treatment was determined using flow cytometry as described above. Drug synergy was estimated by calculating relative risk ratio (RRR). RRR is calculated as the ratio between the actual value and expected value of the percentage of surviving cells following treatment. $RRR = \frac{\text{Percentage of viable cells in sample treated with azacitidinepanobinostat combination}}{[(\text{Percentage of viable cells in azacitidine-treated sample} \times \text{Percentage of viable cells in panobinostat-treated sample})/100]}$, as described previously and shown to correlate with combination index [13, 14].

For patient samples, 1×10^6 cells were plated on 24-well plates in the presence of 20,000 Saos-2 cells and treated with specified concentrations of azacitidine and panobinostat alone and in combination for 48 hours. Synergy was determined as mentioned above.

Determination of effectiveness of coculture treatments

20,000 Saos-2 cells/cm² were plated on 24-well plates and left to adhere overnight. Media was removed and 200,000 ALL cells (1×10^6 cells for patient samples) were plated in RPMI-1640 and the appropriate drugs were added to each well for 48 hours. Cell viability was determined by flow cytometry.

Sensitivity assay

Leukemic cells (200,000) plated in a 24-well plate were either pre-treated with azacitidine-panobinostat or left untreated for 48 hours. Cells were then transferred into corresponding wells on a separate 24-well plate containing 20,000 Saos-2 cells that had been plated the day before and left to adhere overnight. Cells were treated with cytarabine or DMSO (0.1%) for additional 48 hours. Cell viability percentage was determined by flow cytometry.

For patient samples, 1×10^6 cells were plated and pre-treated with 1 μ M azacitidine and 1 nM panobinostat for 24 hours and then transferred on to fresh Saos-2 cells and treated with DMSO (0.1%) or indicated concentrations of daunorubicin.

Measuring the effect of soluble factors in chemoprotection

To determine the role of soluble factors in osteoblast-mediated chemoprotection, 20,000 Saos-2 cells were plated on a 12-well plate and left to adhere overnight. Leukemic cells (200,000) were placed in a 0.4 μ m Transwell filter insert (Corning Inc. 3460) suspended over osteoblasts with or without cytarabine for 48 hours. At the end of the incubation, ALL cells were collected from Transwell inserts and cell viability was determined using flow cytometry as described above.

Statistical analysis

T-test: two-sample assuming unequal variance was used to evaluate the variances between two data points captured from 3-4 independent experiments in duplicates. P-value of < 0.05 was considered statistically significant.

Results

Azacitidine and panobinostat have a synergistic killing effect in combination

The effect of varying concentrations of azacitidine and panobinostat on ALL cell viability was determined. Panobinostat had a more potent killing effect with IC50 concentrations in the nanomolar range (REH: 20.14 nM, Nalm6: 17.10 nM, CCRF-CEM: 13.10 nM) while azacitidine IC50 concentrations were in the micromolar range (REH: 25.38 μ M, Nalm6: 15.70 μ M, CCRF-CEM: 5.68 μ M) (Fig. 1).

To determine if the combination of azacitidine and panobinostat had a synergistic killing effect, ALL cells were treated with IC50 concentrations of each drug singularly and in combination (Fig. 2A, B, C – compare bars 1 and 3 with bar 5). Synergism between azacitidine and panobinostat was calculated using a relative risk ratio (RRR). A RRR value < 1.00 indicates a synergistic effect. All cell lines had relative risk ratios below 1.00 (REH: 0.8711, Nalm6: 0.0494, CCRF-CEM: 0.2572). These values indicate that azacitidine and panobinostat in combination have a synergistic killing effect on ALL cells.

Azacitidine and panobinostat in combination exhibit more efficient killing in coculture with osteoblasts than cytarabine

As previously mentioned, the bone marrow microenvironment plays a pivotal role in the development of chemoprotection in ALL by inducing survival pathways in ALL cells [15]. For example, we showed earlier that osteoblasts can protect ALL cells from cell death induced by treatment with chemotherapeutic drugs dexamethasone and vincristine [8]. In this study, cytarabine treatments (at IC50 concentrations) had significantly reduced efficiency in inducing cell death in REH, Nalm6 and CCRF-CEM cells cocultured with osteoblasts compared to treatments in monoculture (Fig. 2A, B and C, bars 7 and 8). To determine if the epigenetic modifiers - azacitidine and panobinostat were successful in overcoming these effects, we treated ALL cells with IC50 concentrations of both singularly and in combination in monoculture and in the presence of osteoblasts. The combination of azacitidine and panobinostat was equally effective in killing ALL cells in monoculture as it was in coculture (Fig. 2A, B and C, bars 5 and 6). The success of azacitidine and panobinostat alone to overcome the protective effects of osteoblasts varied both by drug and by cell line (Fig. 2A, B and C, bars 1 and 2; 3 and 4). These data suggest that azacitidine-panobinostat combination is more effective than cytarabine or either drug individually. These data also illustrate that unlike conventional chemotherapy; this epigenetic drug combination kills ALL cells in coculture despite the presence of osteoblast-mediated protective microenvironment.

Lower doses of azacitidine-panobinostat can sensitize ALL cells to cytarabine treatments in coculture with osteoblasts

We next wanted to see if ALL cells pre-treated with lower, non-killing doses of azacitidine-panobinostat could sensitize ALL cells to treatment with cytarabine in the presence of osteoblasts. After pre-treatment with azacitidine-panobinostat combination for 48 hours, the ALL cells were co-cultured with osteoblasts and treated with cytarabine for another 48 hours. Pre-treatment with azacitidine-panobinostat had minimal effect on cell viability (Fig.

3, bar 1). Cells pre-treated with azacitidine-panobinostat showed a significant reduction in cell viability in response to cytarabine than those that had no pre-treatment (Fig. 3A, B and C, compare bars 2 and 3). This effect was observed in all three cell lines tested. These data suggest an ability of azacitidine-panobinostat to sensitize ALL cells to chemotherapy in the presence of osteoblasts.

The chemoprotective effects of osteoblasts on ALL cells require direct cell-cell contact

Microenvironment-induced chemoprotection can be mediated through two prominent components: by soluble factors and by engagement of cell adhesion molecules by direct cell-cell contact [7]. To determine if the chemoprotective effects mediated by osteoblasts are through soluble factors or direct contact, ALL cells were cultured in 0.4 μ m Transwell filters hanging above the osteoblast monolayer. This design allows free dispersion of soluble factors into and out of the Transwells without direct cell-cell contact. The viability of ALL cells cultured in Transwells was significantly lower than the cell viability in osteoblast co-culture (Fig. 4A, B and C, compare bars 2 and 3), indicating that the chemoprotective effects observed in supported cultures require direct contact with osteoblasts.

Responses to epigenetic drug treatment in primary ALL cells *ex vivo*

We used two mouse passaged primary ALL samples *ex vivo*: NTPL-90 (B-ALL) and NTPL-24 (T-ALL), to determine the efficacy of azacitidine-panobinostat combination. Similar to the ALL cell lines, both NTPL-90 and NTPL-24 demonstrated synergistic killing effects of azacitidine and panobinostat in combination with relative risk ratios of 0.71877 and 0.62037 respectively (Fig. 5A and C; compare bars 1-3). NTPL-90 and NTPL-24 cells pre-treated with low concentrations of azacitidinepanobinostat combination for 24 hours in the presence of osteoblasts such that cell death was minimal, significantly reduced viability of cells treated with daunorubicin (Fig. 5B and D, compare bars 2 and 3). These data suggest that the effects of azacitidine and panobinostat on cell viability in co-culture and sensitization to chemotherapy seen in ALL cell lines *in vitro* can be replicated in primary ALL cells *ex vivo*.

***Ex vivo* screening for synergy and sensitivity**

Due to the synergistic killing effects of azacitidine-panobinostat seen in ALL cell lines and in the NTPL-90 and NTPL-24 xenograft lines, we wanted to further investigate these effects in a wider variety of patient samples. To achieve this, we examined the killing effects and synergy of azacitidine and panobinostat in 21 patient samples *ex vivo* with different cytogenetic characteristics [12]. We found that 13 of these samples showed some degree of synergy (RRR < 1.00) (Table 1). These data show that the synergistic killing effects of azacitidine and panobinostat span across many different patient subtypes.

We next wanted to investigate the ability to sensitize ALL cells across different subtypes. We investigated the sensitivity of 10 samples by pre-treating them with azacitidine and panobinostat in co-culture with osteoblasts for 24 hours, and then adding daunorubicin in the presence of osteoblasts for 48 hours. We found that 8 of 10 samples showed increased sensitivity to daunorubicin (viability decreased by > 10%) after being pre-treated with azacitidine and panobinostat (Table 2). These data suggest that the combination of

azacitidine and panobinostat can successfully sensitize ALL cells of various subtypes and cytogenetics.

Discussion

The aim of this study was to determine if the combination of DNA methyltransferase inhibitor azacitidine and histone deacetylase inhibitor panobinostat could be used to increase the effectiveness of ALL treatment in osteoblast supported cultures that mimic the bone marrow microenvironment. The bone marrow plays a major role in chemotherapy resistance in hematologic malignancies including ALL [4, 6, 16]. Our data shows that the epigenetic drugs azacitidine and panobinostat in combination were able to overcome the protective effects of the osteoblasts and sensitize ALL cells to chemotherapeutics.

Leukemogenesis in ALL has been associated with aberrant DNA methylation and histone modifications constituting specific epigenetic signatures [10, 17, 18]. Genetic subtypes of ALL are characterized by definite DNA methylation patterns, which correlate with gene expression profiles [18]. These epigenetic modulations are more apparent at relapse than at diagnosis as they may contribute to the development and persistence of chemotherapy resistance [19]. Therefore, targeted epigenetic drugs offer new opportunity to improve efficacy of treatment. Accordingly, epigenetic drugs like azacitidine and panobinostat have been clinically used. Azacitidine has been studied as a single agent or in combination with chemotherapy in patients with ALL (NCT01861002 and NCT02828358) and so has panobinostat (NCT01321346 and NCT02518750). They have both previously been studied in combination with one another in other hematologic malignancies such as acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS) and showed clinical benefit in high-risk patients [20]. A combination of decitabine (azacitidine analog) and vorinostat (HDAC inhibitor) with chemotherapy also showed clinical benefit in relapsed ALL patients [21].

Intrinsic resistance to chemotherapy is a commonly observed characteristic of relapsed ALL cells, unlike ALL cells at diagnosis. Aberrant epigenetic modulations have been identified as contributors to drug resistance in T-ALL, specifically in response to NOTCH1 inhibitors [22]. A previous report showed decitabine and vorinostat combination chemosensitized B-ALL cells in monocultures, indicating the reversal of relapse-specific gene expression signature by epigenetic reprogramming [23]. This chemoresistance mechanism is distinct from the microenvironment-mediated chemoprotection, which we observed in ALL cells co-cultured with osteoblasts. The role of these epigenetic drugs in chemosensitization in supported cultures has not been studied.

Not much is known about the mechanism by which azacitidine and panobinostat can overcome protection in supported cultures and sensitize ALL cells to chemotherapy treatments. Further investigation is required so that this combination could be better utilized in the clinic for pediatric ALL. Cell adhesion molecules like VLA-4 (Integrin $\alpha_4\beta_1$), VLA-5 (Integrin $\alpha_5\beta_1$), LFA-1 (Integrin $\alpha_L\beta_2$), and p11 (S100A10) are known to mediate ALL cell binding to osteoblasts and induce chemoprotective effects [8, 24-28]. Given our data supporting the requirement of direct contact between osteoblasts and ALL cells (Fig. 4), it is possible that the expression levels of cell adhesion molecules may be altered by epigenetic

drugs, rendering the cells sensitive to chemotherapy. Currently, work is in progress in the laboratory to determine if expression levels of cell adhesion molecules are modulated by epigenetic drug combination.

Azacitidine and panobinostat have a synergistic killing effect in ALL cells and are more effective than chemotherapeutic drugs in overcoming chemoprotection induced by the bone marrow elements. This combination can also sensitize ALL cells to chemotherapy treatments in the presence of osteoblasts. This effect was retained in multiple patient samples with varying cytogenetics including B-ALL and the difficult to treat T-ALL samples. These results illustrate the role of azacitidine and panobinostat combination in overcoming microenvironment induced chemoprotection in ALL and showcase the ability for targeted epigenetic therapy to remain broad enough to adapt to inter and intrapatient heterogeneity.

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Highlights

- Azacitidine and panobinostat have a synergistic killing effect on ALL cells
- Direct contact between ALL cells and osteoblasts is required for chemoprotection
- Azacitidine/panobinostat pre-treatment overcomes osteoblast-induced chemoprotection
- Effects can be replicated *ex vivo* across a wide variety of primary patient samples

Fig. 1. Determination of IC₅₀ concentrations of azacitidine and panobinostat

REH, Nalm6, and CCRF-CEM were treated with different concentrations of azacitidine and panobinostat. Cell viabilities were determined using flow cytometry. Azacitidine IC₅₀s were determined to be A) 25.38 μ M B) 15.70 μ M and C) 5.68 μ M, where as panobinostat IC₅₀s were D) 20.14 nM E) 17.10 nM and F) 13.10 nM for REH, Nalm6 and CCRF-CEM respectively. IC₅₀ values were calculated from 3-4 independent experiments performed in duplicates.

Fig. 2. Azacitidine and panobinostat show synergy in combination and overcome the chemoprotection induced by osteoblasts better than cytarabine and the singular treatments Azacitidine, panobinostat, their combination, or cytarabine were used to treat REH, Nalm6, and CCRF-CEM cells either in the absence or presence of osteoblasts. Concentrations used were A) azacitidine: 25 μ M, panobinostat: 20 nM, cytarabine: 600 nM B) azacitidine: 15 μ M, panobinostat: 13 nM, cytarabine: 800 nM C) azacitidine: 5 μ M, panobinostat: 13 nM, cytarabine: 600 nM. Error bars denote SD of the Mean from three independent experiments. * $p < 0.05$ indicates statistical significance.

Fig. 3. Low doses of the combination of azacitidine and panobinostat can sensitize ALL cells to cytarabine treatments in the presence of osteoblasts

ALL cells were pre-treated for 48 hours with non-killing doses of azacitidine-panobinostat. Cells were then transferred into the presence of osteoblasts and treated with cytarabine for 48 hours. Concentrations used were A) 3.75 μ M azacitidine, 3 nM panobinostat, 600 nM cytarabine B) 3.75 μ M azacitidine, 3.25 nM panobinostat, 800 nM cytarabine C) 1.25 μ M azacitidine, 3.25 nM panobinostat, 600 nM cytarabine. Error bars denote SD of the Mean from four independent experiments. * $p < 0.05$ indicates statistical significance.

Fig. 4. The chemoprotective effects of osteoblasts require direct interaction with ALL cells
 ALL cells were treated with cytarabine A) 600 nM B) 800 nM and C) 600 nM in the presence or absence of osteoblasts. ALL cells were placed in Transwell inserts wherever indicated. Error bars denote SD of the Mean from three independent experiments in duplicates. * $p < 0.05$ indicates statistical significance.

Fig 5. Azacitidine and panobinostat's effects can be replicated using *ex vivo* patient samples
 Using samples from one B-ALL patient (NTPL-90) and one T-ALL patient (NTPL-24), the *in vitro* data was replicated in *in vivo*. A) 10 μ M azacitidine and 10 nM panobinostat were used and yielded an RRR value of 0.7188 ± 0.1666 . Daunorubicin concentration was 12.5 nM. C) 10 μ M azacitidine and 7 nM panobinostat had a RRR value of 0.6204 ± 0.0736 . B and D) 1 μ M azacitidine and 1 nM panobinostat were used to sensitize ALL cells for 24 hours in the presence of Saos-2 cells, then were transferred on to fresh Saos-2 cells and treated with 12.5 nM daunorubicin for 48 hours. Both cell lines showed significant differences (* $p < 0.05$) between pre-treated and non-pre-treated cells (B and D Bars 2 and 3).

Table 1
Azacitidine and panobinostat have a synergistic killing effect across many patient samples presenting with different subtype and cytogenetic aberrations

Samples were treated with azacitidine and panobinostat both singularly and in combination. Synergy was determined using a relative risk ratio calculation (RRR) as described in Materials and Methods. A value < 1.00 indicates a synergistic effect. Azacitidine concentrations used were: 3 μ M (NTPL-119), 4 μ M (NTPL-20, 137, and 138), 5 μ M (NTPL-83), 7 μ M (NTPL-59, 84, 87, 92, 103, 109, 150, 155, 164, 300, 313, 454, and 515), and 10 μ M (NTPL-24, 90, and 127). Panobinostat concentrations used were: 3 nM (NTPL-119), 4 nM (NTPL-20, 87, 138, and 164), 5 nM (NTPL-83 and 127), 7 nM (NTPL-24, 59, 84, 92, 103, 109, 137, 150, 155, 300, 313, 454, and 515), and 10 nM (NTPL-90). 13 of 21 samples showed synergy (1 of 3 T-ALL and 12 of 18 B-ALL).

Sample	RRR	Cytogenetics
NTPL-20	0.689	BCR-ABL, P16 deletion
NTPL-24	0.620	T-ALL
NTPL-59	1.446	T-ALL
NTPL-83	0.804	ETV6-RunX1 (TEL/AML1)
NTPL-84	0.608	Hyperdiploid, P16 deletion
NTPL-87	0.804	Other
NTPL-90	0.719	ETV6-RunX1 (TEL/AML1)
NTPL-92	1.039	E2A-PBX
NTPL-103	0.725	Other
NTPL-109	0.823	Other
NTPL-119	1.096	RunX amplification
NTPL-127	0.619	RunX amplification
NTPL-137	1.207	Hyperdiploid
NTPL-138	1.344	Hyperdiploid, P16 deletion
NTPL-150	0.691	Hyperdiploid, P16 deletion
NTPL-155	0.950	P16 deletion
NTPL-164	0.599	P16 deletion
NTPL-300	1.126	Other
NTPL-313	0.944	ETV-RunX1 (TEL/AML1)
NTPL-454	1.430	T-ALL
NTPL-515	1.160	Other

Table 2
Azacitidine and panobinostat can sensitize patient samples with different cytogenetic characteristics

ALL patient samples were either left untreated ('Without Pre-treatment') or pretreated with 1 μ M azacitidine and 1 nM panobinostat for 24 hours ('With Pre-treatment'). At this point cells were transferred on to fresh osteoblasts and treated with 20 nM daunorubicin (12.5 nM for NTPL-24 and 90) for 48 hours. The percentage cell viability was determined from triplicate wells. Reduction in viability owing to pre-treatment is denoted as 'Sensitization percentage'. 5 of 10 samples showed sensitization percentage > 20% and 2 additional samples showed sensitization percentage > 10%.

Sample	Without Pre-treatment	With Pre-treatment	Sensitization percentage
NTPL-24	67.51%	31.44%	36.07%
NTPL-83	70.42%	60.48%	9.94%
NTPL-90	64.05%	23.06%	40.99%
NTPL-109	92.46%	66.40%	26.06%
NTPL-119	76.01%	54.91%	21.10%
NTPL-137	79.29%	65.23%	14.06%
NTPL-138	81.40%	54.84%	26.56%
NTPL-164	71.55%	56.80%	14.75%
NTPL-313	74.79%	69.64%	5.15%
NTPL-454	28.99%	22.08%	6.91%