Cell membrane permeability and defective G2/M block as factors potentially contributing to increased cell chemosensitivity. SeAx cell line as an example

Edyta Biskup *, Omid Niazi, Vibeke Pless
Department of Dermatology, Bispebjerg Hospital, Bispebjerg Bakke 23, 2400, Copenhagen, Denmark

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ABSTRACT

Background: Immortalized mammalian cell lines are a valuable research tool, though they represent a highly simplified model. Due to accumulated mutations they may not reflect characteristics of the disease or even the tissue they derive from.

Objective: We aim to pinpoint factors distinguishing SeAx cells from two other cutaneous T-cell lymphoma (CTCL) cell lines, namely Hut78 and MyLa2000. Of note, these factors may influence cell sensitivity in an unspecific way and therefore should be taken under consideration.

Methods: We evaluated transcriptional levels of drug transporters across cell lines, cell membrane permeability, functionality of pathways related to DNA damage response and activation of G2/M block.

Results: Analysis of the transcriptional levels of genes coding drug efflux pumps indicated that they are not consistently down-regulated in SeAx. However, we noted that SeAx cell membrane is markedly more permeable than Hut78 and MyLa2000, which may contribute to increased chemosensitivity in an unspecific way.

Moreover, though DNA damage response seemed to be at least partly functional in SeAx cells, they fail to activate G2/M block in response to psoralen + UVA treatment. Any DNA damage should be repaired before cells enter mitosis, in order to uphold genome integrity. Thus, a defective cell cycle block may contribute to cell sensitivity.

Conclusions: We believe that factors such as increased membrane permeability or defective cell cycle block should be accounted for when comparing sensitivity of cell line panels to chemotherapeutics of interest. It is worth to exclude a simple, indiscriminative mechanisms of cell resistance or sensitivity before attempting comparisons.

Cell lines that are indiscriminately sensitive to a broad range of chemicals may contribute to overestimating the cytotoxic potential of tested compounds if used in cytotoxicity studies.

1. Introduction

Mammalian cell lines are widely used in molecular and cell biology, especially in cancer studies, even though they represent a highly simplified preclinical model [1]. Cancer cells tend to accumulate mutations both in the course of the disease and in prolonged culture, and may not always be representative for the condition they derive from. These alterations often render cancer cells more sensitive or more resistant to treatment, either specifically to certain therapeutics or in a more general way.

In simple terms, such mechanisms can be divided into three categories: 1) mutations influencing cell resistance to specific chemotherapeutics, 2) semi-discriminative alterations, changing resistance to a group of functionally similar drugs or 3) indiscriminative alterations contributing to chemo-resistance or chemo-sensitivity to broad range of compounds.

The first category is vital for designing targeted therapies, and encompasses (over)expression of potential drug targets as well as mutations and genomic rearrangements, resulting in formation of new drug targets. Hence, presence of estrogen receptor renders breast cancer cells sensitive to tamoxifen, while a BCR-ABL fusion kinase, resulting from a chromosomal translocation in chronic myeloid leukemia, serves as a target for imatinib [2,4]. Conversely, point mutations in BCR-ABL kinase would directly change drug-target interactions, making cells resistant to imatinib treatment [2].

Alterations in the DNA damage response (DDR) fall into the second
were seeded at 10^4 and culture conditions have been described elsewhere [10]. (Sezary syndrome) and MyLa2000 (mycosis fungoides). Cell line sources:

2.2. Cell lines and experimental conditions

Francisco, CA).

- cisplatin (Accord Healthcare Ltd., North Harrow, UK), etoposide, KU55933, KU60019, Olaparib (all from Selleckchem, Houston, TX),
- VE-821, VE-822, AZD6738, VE-822, AZD6738, KU55933, KU60019, Olaparib (all from Selleckchem, Houston, TX),
- cisplatin which was dissolved in sterile distilled water. DDR inhibitors, if needed, were added 30 min prior to genotoxic agents.

Among mechanisms changing cell sensitivity and resistance to a wide spectrum of chemotherapeutics are those influencing cellular drug concentration. This may be achieved via altered expression of drug efflux pumps (for example ATP-binding cassette transporters; ABC transporters) [7] as well as altered composition of cell membrane, which influences its fluidity and hence permeability [8]. Eventually, defects in the apoptotic pathways, which favour survival, would make neoplastic cells more resistant. For instance, aberrant expression of Bel-2 family members and the NFXB signaling pathway helps to evade apoptosis [7].

Still, cell lines remain a valuable research tool and therefore it is essential to thoroughly characterize and describe them in order to acquire credible data. SeAx is one of few (next to Hut78/Hut9 and Sz4/SZ4) established cell lines, derived from Sezary syndrome (Sez), a leukemic variant of cutaneous T-cell lymphoma (CTCL) [9]. In the course of studies performed by our group we observed a pattern suggesting that SeAx is generally more sensitive towards a range of structurally and functionally unrelated compounds than two other cell lines routinely used in our studies, namely Hut78 (Sezary syndrome) and MyLa2000 (mucositis fungoides). Subsequently, we were able to pinpoint at least two factors (altered membrane permeability and deficient G2/M block) distinguishing SeAx from the other two cell lines, which may potentially contribute to its increased sensitivity. These factors are by no means unique to SeAx and they should be taken under consideration for example when presenting drug sensitivity panels using various cell lines. They may contribute to cell sensitivity, irrespective of the actual mechanism of a drug in question.

2. Materials and methods

2.1. Reagents

The following reagents were used: VE-821, VE-822, AZD6738, cisplatin (Accord Healthcare Ltd., North Harrow, UK), etoposide, doxorubicin, gemcitabine, cyclosporin A, elacridar, NSC130813 (all from Sigma-Aldrich/Merck St Louis, MO), staurosporin (Cell Signaling, Beverly, MA), 8-MOP (Fluka, St. Louis, MO) and MK571 (BioVision San Francisco, CA).

2.2. Cell lines and experimental conditions

Three CTCL cell lines were used as preclinical models, Hut78, SeAx (Sezary syndrome) and MyLa2000 (mucositis fungoides). Cell line sources and culture conditions have been described elsewhere [10]. In the experiments involving treatment with chemotherapeutics cells were seeded at 10^5/2 ml in 12 well plates and treated with cytotoxic agents dissolved in 99.8% DMSO (Sigma-Aldrich) with the exception of cisplatin which was dissolved in sterile distilled water. DDR inhibitors, if used, were added 30 min prior to genotoxic agents.

2.3. Viability assessment

Viability assessment was performed by propidium iodide exclusion assay 24 h, 48 h and 72 h after treatment in case of all cytotoxic agents apart from doxorubicin when, due to its red fluorescence, SYTOX Green was used. Briefly, cells were stained with 4 μg/ml propidium iodide (PI; Sigma-Aldrich, St. Louis, MO) or 50 ng/ml SYTOX Green (ThermoFisher Scientific, Chicago, IL) for 15 min and analyzed using a Cell Lab Quanta SC MPL flow cytometer (Beckman Coulter, Fullerton, CA). The proportion of PI-negative/SYTOX Green-negative (viable) cells was normalized to the vehicle-treated control.

Apoptosis was determined by flow cytometry using the FITC-annexin V/propidium iodide protocol according to manufacturer instructions (Beckman Coulter).

2.4. G2/M block

G2/M block was as induced by treatment with 8-MOP + UVA (PUVA). Cells were seeded at 10^5/ml in 6-well plate and treated with 1–2 μM 8-MOP for 2 h before irradiation with UVA at 0.4–2.4 mJ/cm^2.

2.5. Cell cycle distribution

Analysis of cell cycle distribution was performed 20 h after 8-MOP + UVA treatment. Cells were washed in PBS, fixed in ice-cold 70% ethanol for at least 2 h, re-hydrated with PBS and stained for 30 min with 7AAD. Flow cytometry was used to determine the cell cycle distribution. Kaluza software (Beckman Coulter) was used for data analysis and graphic presentation for all experiments involving flow cytometry.

2.6. DDR repair pathways functionality

In order to confirm if a DDR repair pathway is functional we combined genotoxic drugs with inhibitors of proteins crucial for a given pathway. In case of a synergy, the pathway was regarded as functional. Bliss independence model was used to distinguish synergistic from additive effect [11]. The observed inhibitory effect (Y_{AB,O}) of a genotoxic agent (A) combined with a DDR inhibitor (B) was compared with a predicted inhibitory effect for two agents working independently (Y_{AB,p}), calculated with the following formula: Y_{AB,p} = Y_A + Y_B - Y_A*Y_B. In this model Y_{AB,O} > Y_{AB,p} means synergy, while Y_{AB,O} = Y_{AB,p} means additive effect [11].

2.7. Membrane permeability assay

Cells were seeded at 10^5/2 ml in 24 well plates and treated with the following amounts of viability dyes: 100 ng/ml μM SYTOX green, 8 μg/ml PI, 150 ng/ml 7- aminoactinomycin 7AAD; Beckman-Coulter or 2.4 mJ/UVA. Concentrations of efflux pumps inhibitors were earlier adjusted following gene expression assays (Applied Biosystems) were used:

- GAPDH (Hs02786624_g1), ATR (Hs00992123_m1), ABCB1 (Gene Expression Assays (Applied Biosystems, Foster City, CA) specific for each gene of interest (GOI). GADPH expression was used as normalization control. Results were expressed as 2^{ΔΔCt} where ΔΔCt = Ct GOI - Ct GAPDH. The following gene expression assays (Applied Biosystems) were used: GAPDH (Hs02786624_g1), ATR (Hs00992123_m1), ABCB1 (ΔΔCt).
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3. Results and discussion

3.1. SeAx is generally more sensitive to a range of compounds than two other CTCL-cell lines

We observed that SeAx is more sensitive to commonly used chemotherapeutics (cisplatin, etoposide, doxorubicin) than Hut78 and MyLa2000. Moreover, SeAx was also more sensitive to Ataxia Telangiectasia and Rad3 related (ATR) kinase inhibitors, especially VE-822 and AZD6738, but, interestingly, not to Ataxia Telangiectasia Mutated (ATM) kinase inhibitors, KU55933 and KU60019 (Table 1). SeAx was also more sensitive to ATR inhibitors than two leukemic cell lines, often used as preclinical models, namely Jurkat and K562, which we implemented as a reference (Suppl. Table 1). Sensitivity to all the above compounds may suggest defects in DNA damage response (DDR), since these are either genotoxic drugs or inhibitors of enzymes related to DNA damage signaling. However, we also noticed increased sensitivity of SeAx cells as compared to Hut78 and MyLa2000, which may contribute to higher cell sensitivity to some of the compounds tested.

3.2. Sensitivity of SeAx cells cannot be ascribed to low expression of drug efflux pumps

Drug resistance/sensitivity may be mediated by the activity of ABC transporters family members. Among those, three are believed to support chemoresistance in vivo: ABCB1 (P-glycoprotein/MDR1), ABCC2 (Breast Cancer Resistant Protein) and ABCC1 (Multidrug Resistant Protein) [3]. Analysis of expression levels of the efflux pumps genes did not provide a consistent pattern and a convincing explanation: ABCB1 expression was low in Hut78 and barely detectable in SeAx and MyLa2000. ABCC1 was abundantly expressed in all cell lines tested, though indeed its levels in SeAx were lowest, while expression of ABCC2 was highest in SeAx (Fig. 1A – C). Altogether, it did not seem plausible that low transcriptional levels of efflux pump genes account for SeAx chemosensitivity. However, at this stage we were not able to opine about the efflux pump functionality (see below).

3.3. Cell membrane is SeAx cells is more permeable than in other CTCL cell lines tested

Another factor, apart from the efflux pumps, influencing the intracellular amount of a drug is the cell membrane lipid content and, consequently, cell membrane permeability/fluidity [15]. In order to check if the membrane of SeAx cells is more permeable we selected compounds which are otherwise used as viability dyes, i.e. are incapable of passing through the intact membranes of living cells (impermeant). Dyes such as SYTOX Green, propidium iodide (PI) or 7-aminoactinomycin-D (7AAD) are typically used to determine cell viability. They enter through compromised membrane and emit fluorescence upon binding to cell DNA, allowing to distinguish between dead and viable cells. We reasoned that if SeAx cell membrane is more permeable, the fluorescence should increase faster over time than in other cell lines. We incubated SeAx, Hut78 and MyLa2000 cells in the presence of fluorescent viability dyes over 6 h measuring the fluorescence every 30 min. Moreover, we used a similar protocol to monitor changes in fluorescence over time of CTCL cells incubated with doxorubicin.

In SeAx we observed a marked increase of SYTOX green signal over time, compared to Hut78 and MyLa2000 (Fig. 1D; Suppl. Fig. S1). We saw a similar pattern also for PI, albeit to a lower extent (Fig. 1E), interestingly though not in case of 7AAD (Fig. 1F; Suppl. Fig. S1), which seemed not to penetrate cell membrane. This is in line with our suggestion that the increased penetration of viability dyes results from increased fluidity of SeAx membrane. Since 7AAD has a higher molecular weight (1270.43 g/mol) than SYTOX Green and propidium iodide (600 and 668 g/mol respectively) it may less efficiently cross the membrane. Of note, we also observed that intracellular content of doxorubicin increases faster in SeAx than in Hut78 or MyLa2000 (Fig. 1G; Suppl. Fig. S1).

It should be emphasized that even though the same compounds are used for viability measurement and monitoring cell membrane permeability, these two approaches differ markedly. For viability measurement, a short incubation time (15 min) was used, a time-point when the distinction between viable and dead cell populations can be easily seen. Dead cells in the viability assay present as a separate peak, rather than a shift of a negative (viable) population (see Suppl. Fig. S1).

In order to further verify if the low signal in Hut78 and MyLa2000 is due to more compact cell membrane structure rather than higher efflux pumps activity we also combined the viability dyes/doxorubicin with inhibitors of efflux pumps, namely Elacridar (specific for ABCB1 and ABCC2) and MK571 (specific for ABCC1).

Use of Elacridar, but not MK571, slightly increased membrane penetration of SYTOX Green and doxorubicin. Interestingly, it was observed also in MyLa2000 cells, though the intracellular levels of the viability dyes, even after pump inhibition, were still lower than in steady-state SeAx (Fig. 1D – G). Slight albeit consistent increase of PI membrane penetration in SeAx pre-treated both with Elacridar and MK571 versus no inhibitors further argue that the efflux pumps are functional in SeAx, but their activity is insufficient to compensate for the increased permeability (Fig. 1E).

Collectively, these data speak for increased membrane permeability of SeAx cells as compared to Hut78 and MyLa2000, which may contribute to higher cell sensitivity to some of the compounds tested.
Interestingly, it has been previously demonstrated that membrane lipid composition and, consequently, lipid packaging and membrane fluidity differed between MCF-7 cells resistant and sensitive to doxorubicine [8].

Moreover, membrane architecture has been shown to influence ATPase activity of efflux pumps [16].

3.4. DDR repair pathways operate in SeAx, but G2/M block fails to be activated

Membrane permeability alone does not seem to explain sensitivity of SeAx cells to ATR inhibitor, VE-822. We paid specific attention to the cytotoxicity of VE-822 as it was about 30–50 times more toxic towards SeAx than towards the other CTCL or leukemia cell lines tested (Table 1, Suppl. Table 1), suggesting a highly specific mechanism. Using levels of phosphorylation of Rad17 as a marker of ATR kinase activity we aimed to establish a relation between VE-822 doses and its inhibitory effect. Rad17 rather than Chk1 was used since the former is easier detected in CTCL cell lines and since phosphorylated Rad17 associates with ongoing DNA replication also in unperturbed cells, in the absence of DNA damage [17]. Though VE-822 was by far more toxic to SeAx, it inhibited Rad17 phosphorylation to the same degree in all cell lines (Fig. 2A–C).

Assuming higher permeability of cell membrane in SeAx, we expected that lower doses would be necessary to achieve the same inhibitory effect. This was not the case and therefore we searched for another plausible explanation.

Before setting to do so, however, we addressed the issue of compound specificity. Of note, doses sufficient to completely abrogate Rad17 phosphorylation were still too low to induce cell apoptosis,
suggesting that the toxic effect of VE-822 does not completely overlap with the inhibitory activity. Furthermore, we observed that the ATR down-regulation by a specific siRNA does not recapitulate the effect of chemical inhibitors in terms of cell viability. Even when the SeAx cells were subjected repeatedly to ATR depletion over a nearly three-week period, we observed only up to 20% cell death (Suppl. Fig. S2A–C). The latter may result from a residual kinase activity, still present in cells subjected to ATR knock-down, but nevertheless, the discrepancy between the cytotoxic and inhibitory effect made us wonder if the cytotoxicity of VE-822 in SeAx was not due to an off-target effect.

To verify it, we induced two VE-822 resistant SeAx variants, namely SeAx_0.05R and SeAx_0.08R. As it turned out, both were cross-resistant to all ATR inhibitors tested (irrespective of their structure), but not to ATM inhibitors (Suppl. Figs. S2D and E), arguing against the possible off-target effect. Moreover, all ATR inhibitors prevented Chk1 phosphorylation, following gemcitabine treatment, but failed to block ATM pathway, further supporting the claim that toxicity of VE-822 is indeed due to ATR inhibition (Suppl. Figs. S2F and G). Having confirmed the specificity of VE-822, we asked if the high sensitivity of SeAx cells may result from defects in DNA damage response (DDR).

Defects in DDR pathway have previously been reported to be synthetically lethal with i.a. mutations in XRCC1 (a factor critical for BER pathway [18]), in ATM/p53 [19] or with ERCC1 deficiency (NER pathway [20]). Therefore, presence of these mutations in SeAx would explain their high sensitivity towards ATR blockers. Still, we were not able to identify defective pathways in SeAx.

Subsequently, however, we observed that SeAx cells fail to activate G2/M block in response to combination of psoralen and UVA radiation, a
Fig. 3. SeAx cells have a functional DDR, but a defective G2/M block. A – D. Genotoxic drugs act synergistically with DDR inhibitors, confirming that DNA repair pathways are functional in SeAx. Fields marked in green indicate positive Bliss coefficient values i.e. synergistic effect of a given combination. A – etoposide combined with ATM inhibitors KU55933 or KU60019; B – cisplatin combined with NSC130813, compound blocking interaction between XPF and ERCC1; C – etoposide combined with olaparib; D – cisplatin combined with ATR inhibitors, VE-821 or VE-822. E – SeAx cells fail to activate a G2/M block upon treatment with 8-MOP + UVA. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
therapeutic modality used in CTCL [21], known to induce DNA adducts and interstrand cross-links [22] (Fig. 3E). The latter could indeed contribute to cell chemosensitivity. The physiological role of cell cycle block is to enable cells to complete DNA damage repair before entering mitosis, in order to uphold genome integrity.

3.5. Other factors of potential significance for cell sensitivity

Summarizing, we identified two factors, namely increased cell membrane permeability and defective G2/M block which may contribute to high sensitivity of SeAx cells towards a broad spectrum of chemotherapeutics. Previously, we also reported that both SeAx and Hu78 bear mutated p53 [10]. Since mutations in p53 have been related both to increased and decreased chemosensitivity [23], their p53 status may also play a role. Moreover, we observed that SeAx have a markedly longer cell doubling time as compared to other CTCL cell lines [24]. SeAx was also one of 3 (next to Sez4 and Mac2a) out of 11 CTCL cell lines unable to induce tumor formation in xenograft mouse models [9]). We cannot say how common similar alterations are in SzS patients and therefore how well SeAx represents SzS as a preclinical model. We believe that SeAx should be treated with caution, since they seem to differ from other CTCL-derived and leukemia-derived cell lines in terms of chemosensitivity.

4. Conclusions

We assume that the general sensitivity of SeAx cells is in all likelihood related to a combination of factors rather than a single defect. However, we believe that the features described above should be noted and accounted for in order to produce credible data and avoid drawing false conclusions.

For instance, studies involving comparison of chemosensitivity between non-neoplastic and cancer cell lines or between cell lines positive and negative for certain factors (such as estrogen receptors), i.e. aiming to correlate cell sensitivity to specific mechanism or specific drug-target interaction, may present a potential pitfall. In other words, the fact that a given cell line is markedly more sensitive to a compound or a family of compounds does not automatically prove that the effect is specific. Resistance or sensitivity may still result from unspecified alterations, like membrane fluidity or efflux pump levels and may be erroneously ascribed to a given mutation.

Therefore, we postulate that before drawing conclusions whether a chemical is specific towards cells of given origin (e.g. neoplastic) or that the sensitivity results from certain alterations (e.g. a mutated pathway) a more extensive study should be conducted to confirm that the cell line of choice is not generally sensitive to a broad range chemotherapeutics and rule out indiscriminative, unspecified mechanism. If researchers suspect that a given cell line is indiscriminatively sensitive to a broad range of chemicals, it may be reasonable to exclude such cell line from cytotoxicity studies in order not to overestimate the cytotoxic potential of tested compounds.

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CRedit author contributions

EB: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Project administration; Software; Validation; Visualization; Roles/Writing - original draft.
ON: Investigation, Writing - review & editing.
VP: Resources, Investigation, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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