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European Association of Urology

Platinum Priority – Bladder Cancer

Editorial by Samantha Conroy, Alana H.T. Nguyen on pp. 271–272 of this issue

Molecular and Pharmacological Bladder Cancer Therapy Screening: Discovery of Clofarabine as a Highly Active Compound

Iris E. Ertl[a], Ursula Lemberger[a], Dafina Ilijazi[a], Melanie R. Hassler[a], Andreas Bruchbacher[a], Robert Brettner[a], Hannah Kronabitter[a], Michael Gutmann[b], Petra Vician[b], Gerhard Zeitler[b], Anna Koren[d], Charles-Hugues Lardeau[d], Thomas Mohr[b], Andrea Haitel[e], Eva Compérat[e], André Oszwald[e], Gabriel Wasinger[e], Thomas Clozel[f,g], Olivier Elemento[c,g,h], Stefan Kubicek[d], Walter Berger[b,], Shahrokh F. Shariat[a,i,j,k,l,m]*

[a] *Department of Urology, Comprehensive Cancer Center, Medical University of Vienna, Vienna, Austria;* [b] *Department of Medicine I, Institute of Cancer Research and Comprehensive Cancer Center, Medical University Vienna, Vienna, Austria;* [c] *Institute for Computational Biomedicine, Weill Cornell Medicine, New York, NY, USA;* [d] *CeMM Research Center for Molecular Medicine, Vienna, Austria;* [e] *Department of Pathology, Medical University of Vienna, Vienna, Austria;* [f] *OWKIN, New York City, NY, USA;* [g] *Englander Institute for Precision Medicine, Weill Cornell Medicine-New York Presbyterian Hospital, New York, NY, USA;* [h] *Meyer Cancer Center, Weill Cornell Medicine, New York, NY, USA;* [i] *Department of Urology, Weill Cornell Medical College, New York, NY, USA;* [j] *Department of Urology, University of Texas Southwestern, Dallas, TX, USA;* [k] *Department of Urology, Second Faculty of Medicine, Charles University, Prague, Czech Republic;* [l] *Institute for Urology and Reproductive Health, I.M. Sechenov First Moscow State Medical University, Moscow, Russia;* [m] *Hourani Center for Applied Scientific Research, Al-Ahliyya Amman University, Amman, Jordan*

Article info

Article history:
Accepted March 10, 2022

Associate Editor:
James Catto

Keywords:
Bladder cancer
Drug repurposing
Drug screen
Sarcomatoid carcinoma
Urothelial carcinoma
Clofarabine

Abstract

Background: The heterogeneity of bladder cancers (BCs) is a major challenge for the development of novel therapies. However, given the high rates of recurrence and/or treatment failure, the identification of effective therapeutic strategies is an urgent clinical need.
Objective: We aimed to establish a model system for drug identification/repurposing in order to identify novel therapies for the treatment of BC.
Design, setting, and participants: A collection of commercially available BC cell lines (n = 32) was comprehensively characterized. A panel of 23 cell lines, representing a broad spectrum of BC, was selected to perform a high-throughput drug screen.
Outcome measurements and statistical analysis: Positive hits were defined as compounds giving >50% inhibition in at least one BC cell line.
Results and limitations: Amongst >1700 tested chemical compounds, a total of 471 substances exhibited antineoplastic effects. Clofarabine, an antimetabolite drug used as third-line treatment for childhood acute lymphoblastic leukaemia, was amongst the limited number of drugs with inhibitory effects on cell lines of all intrinsic subtypes. We, thus, reassessed the substance and confirmed its inhibitory effects on commercially available cell lines and patient-derived cell cultures representing various disease stages,

* Corresponding author. Department of Medicine I, Institute of Cancer Research and Comprehensive Cancer Center, Medical University Vienna, Vienna, Austria. Tel. +43-1-40160-57555; Fax: +43-1-40160-957555.
E-mail address: walter.berger@meduniwien.ac.at (W. Berger).

https://doi.org/10.1016/j.eururo.2022.03.009

intrinsic subtypes, and histologic variants. To verify these effects *in vivo*, a patient-derived cell xenograft model for urothelial carcinoma (UC) was used. Well-tolerated doses of clofarabine induced complete remission in all treated animals (n = 12) suffering from both early- and late-stage disease. We further took advantage of another patient-derived cell xenograft model originating from the rare disease entity sarcomatoid carcinoma (SaC). Similarly to UC xenograft mice, clofarabine induced subcomplete to complete tumour remissions in all treated animals (n = 8).

Conclusions: The potent effects of clofarabine *in vitro* and *in vivo* suggest that our findings may be of high clinical relevance. Clinical trials are needed to assess the value of clofarabine in improving BC patient care.

Patient summary: We used commercially available cell lines for the identification of novel drugs for the treatment of bladder cancer. We confirmed the effects of one of these drugs, clofarabine, in patient-derived cell lines and two different mouse models, thereby demonstrating a potential clinical relevance of this substance in bladder cancer treatment.

1. Introduction

Bladder cancer (BC) is the tenth most common cancer worldwide, with an estimated 549.000 new cases and 200.000 deaths annually [1]. BC has profound personal and socioeconomic impact, representing one of the most expensive malignancies per person [2]. There is a high, unmet need for effective treatments that reduce the risk of disease recurrence/progression/mortality, while maintaining or even improving Health-Related Quality of Life (HRQoL). Enriched understanding of the molecular underpinning of BC holds the promise of unravelling novel effective therapeutic strategies. For example, recent studies showed that BC can be classified into various intrinsic subtypes with variable sensitivities to currently available therapies [3]. These discoveries also revealed the heterogeneous and complex nature of BC, leading to frequent and early resistances and high rates of treatment failure [4].

In this study, we aimed to establish a system for the identification of novel and repurposed substances for the treatment of various subtypes of BC. To this end, we selected a panel of commercially available cell lines (n = 23), representing a wide spectrum of BC, on which we performed a high-throughput drug screen, leading to the identification of >470 substances with cytotoxic/cytostatic effects. We then selected the purine analogue clofarabine to be studied in more depth, as it exhibited inhibitory effects on cell lines of all intrinsic subtypes and due to its clinical availability as an orphan drug for childhood acute lymphoblastic leukaemia. Moreover, clofarabine belongs, like many other identified active substances, to the class of antimetabolites and was originally developed for haematologic cancers. Beyond that, it was previously shown that the drug is efficiently taken up by bladder tissue [5].

We confirmed concentration-dependent effects of clofarabine in both commercially available cell lines and patient-derived cultures (PDCs). Furthermore, we observed that it caused massive tumour shrinkage/complete remission in mouse xenograft models generated by implantation of PDCs representing the urothelial and sarcomatoid carcinoma variants. These findings validate our approach and implicate a potential clinical value to repurpose this antileukaemic drug for BC treatment.

2. Materials and methods

Commercially available cell lines either were bought at ATCC (Manassas, VA, USA) or DMSZ (Braunschweig, Germany), or were a kind gift from the Shariat Laboratory at the Cornell Medical School (New York, NY, USA). These cell lines were authenticated (Supplementary Table 1). Patient-derived cell lines were established from specimens obtained by surgical interventions performed at the Vienna General Hospital as described previously [6]. The epithelial origin of PDCs was verified by immunostaining (Supplementary Fig. 1). Expression profiles of commercially available cell lines were established by RNA sequencing (RNA-seq) performed on an Illumina HiSeq2000 sequencer (Illumina, San Diego, CA, USA). Mutations in protein-coding genes were identified by RNA-seq as described previously [7], and verified by Sanger sequencing. For the drug screen, the sensitivity of BC cell lines (n = 23) to 1707 substances included in six different drug libraries was tested. Cell viability was measured after 72 h; positive hits were defined as compounds giving >50% inhibition. To determine half-maximal inhibitory clofarabine concentrations (IC_{50}), cell lines were treated with increasing concentrations (1 nM–10 μM) and cell viability was measured after 48 h. Urothelial carcinoma (UC) and sarcomatoid carcinoma (SaC) xenograft models were established by subcutaneous injection of the PDCs VUC38 or VUC48 in the right flank of 8–12-wk-old male CB17/SCID mice. Clofarabine (50 mg/kg) was administered by oral gavage once daily for 5–10 d.

For a more detailed description of material and methods, see the Supplementary material.

3. Results

3.1. Subtype classification of commercially available BC cell lines

Thirty-two commercially available BC cell lines derived from patients with non–muscle-invasive (NMIBC) or muscle-invasive (MIBC) bladder cancer of various stages were used for the study. These cell lines included well-characterised (eg, 5637 and J82) and less well-characterised (eg, 97-1 and 94-10) BC models (Table 1) [8–11].

Table 1 – Origins of commercially available BC cell lines included in the study

Cell line	Origin	Stage	Grade	Sex	Cell line	Origin	Stage	Grade	Sex
5637	UC [8]	NR	G2 [9,10]	M [9,10]	JMSU1	UC [8,9]	pT4 [9]	G3 [9]	M [9]
253J	UC [8,9]	pT4 [8–10]	G4 [8–10]	F [10]/M [9]	JON	UC [8,10]	NR	NR	M [9]
647V	UC [8]	pT2/3a [9]	G2 [8,9]	M [9]	KU1919	UC [8,9]	pT3b [8,9]	G3 [8,9]	M [9]
94-10	UC [8,9]	pT3 [8,9]	G2/3 [8,9]	M [9]	RT112	UC [8,9]	pTa [9]	G2 [8–10]	F [9]
96-1	UC [8,9]	pT2 [8]/pT3 [9,10]	G2/3 [8–10]	M [9,10]	RT4	UC [8,9]	pT1 [9]/pT2 [8,10]	G1 [8,10]/G1–2 [9]	M [9,10]
97-1	UC [8,9]	pT2 [8]/pT1–2 [9,10]	G1/2 [8–10]	M [9,10]	SCaBER	SCC [8–10]	pT3 [10]	NR	M [9,10]
97-18	UC [8,9]	pT2 [8–10]	G3 [8–10]	NR	SD	UC [8,9]	NR	NR	NR
97-24	UC [8,9]	pT3 [8,9]	G3 [8–10]	NR	SW1710	UC [8,9]	NR	G1 [9]/G3 [8]	F [9,10]
97-7	UC [8,9]	pT1 [8–10]	G2/3 [8–10]	NR	SW780	UC [8,9]	NR	G1 [9,10]	F [9,10]
BC3C	UC [8,9]	pT3 [9]	G4 [8,9]	M [9]	T24	UC [8,9]	pTa [9]	G3 [8–10]	F [9,10]
BFTC905	UC [8,9]	pT4 [9]	G3 [8,9]	F [9]	TCCSUP	UC [8,9]	NR	G4 [8–10]	F [9,10]
CAL29	UC [8,9]	pT2 [8,9]	G4 [8,9]	F [9]	TSU-PR1	UC [a]	NR	NR	NR
DSH1	UC [8,9]	pT1a [8,9]	G2 [8,9]	M [9]	UMUC3	UC [8,9]	pT2-T4 [9]	NR	M [9,10]
HT1197	UC [8,9]	pT2 [8–10]	G4 [8–10]	M [9,10]	UMUC14	UC [9,10]	NR	G4 [9]	M [9]
HT1376	UC [8,9]	pT2 [10]/≥pT2 [9]	G3 [8–10]	F [9,10]	VMCUB1	UC [8,9]	NR	G2 [9,10]	M [9,10]
J82	UC [8,9]	pT3 [8–10]	G3 [8–10]	M [9,10]	VMCUB2	UC [8,9]	pT4 [10]	NR	M [9,10]
SV-HUC1	Uroepithelium immortalised by SV40 [11]								

BC = bladder cancer; G = grade; F= female; M = male; MIBC = muscle-invasive BC; NMIBC = non–muscle-invasive BC; NR = not reported.
Thirty-two BC cell lines established from NMIBC and MIBC of different stages (Ta-T4) and grades (G1-G4) were included in the study. Except for SCaBER, which originates from a squamous cell carcinoma (SCC), all cell lines derive from urothelial carcinoma (UC).
The cell line SV-HUC-1 originates from normal ureter tissue transformed by simian-virus SV40.
[a] TSU-PR1 cells are derivatives of T24 [37].

We performed RNA-seq (Supplementary Table 2) and selected, based on previous publications, expression markers to classify these cell lines into different intrinsic subtypes [3,12,13]. Performing cluster analysis, we identified three different expression types and assigned the cell lines to the luminal (n = 6), basal (n = 18), or unspecific subtype ("nontype"; n = 8; Fig. 1A). This was in accordance with the findings of a previous study that assigned 20 of the cell lines we used to the same intrinsic subtypes [12]. Gene set enrichment analysis (GSEA) of RNA-seq data on BC cell lines classified as luminal or basal revealed an overlap with gene sets known to be differentially expressed between luminal and basal breast cancer subtypes (Supplementary Fig. 2) [14–16]. We also analysed the RNA-seq data on our cell line collection employing the gene set predictor BASE47 and a recently established single sample consensus classifier (Fig. 1B and 1C, Supplementary Fig. 3, and Supplementary Table 3) [17,18]. Both classification systems confirmed our approach (Fig. 1A–C).

3.2. Assessing the genomic landscape of commercially available BC cell lines

Taking advantage of our RNA-seq data, we detected nonsilent point mutations in genes included in the Cancer Gene Census as described previously [7,19]; 192 alterations affecting 79 genes were verified by Sanger sequencing, including 65 previously unreported mutations (Supplementary Table 4) [10,20–24]. Additionally, we extracted publicly available mutational data, thereby obtaining a comprehensive overview of the genomic landscape of our cell line collection (Supplementary Table 4). Evaluating the loci most frequently affected by alterations (three or more cell lines), we identified 48 genes (Fig. 2). KEGG pathway analysis showed enrichment of components involved in 59 signalling pathways, many of which were previously shown to be involved in bladder tumourigenesis and progression (Supplementary Table 5 and Supplementary Fig. 4) [25].

3.3. High-throughput drug screen in a representative panel of BC cell lines

In order to identify novel agents with antineoplastic effects against BC, we performed a high-throughput drug screen. To cover a wide spectrum of BC, we selected cell lines consistently classified as luminal (eg, RT4 and SW780) or basal (eg, KU1919 and VMCUB1) by all classification systems (Fig. 1A–C), as well as cell lines classified as neuroendocrine-like by the single sample consensus classifier (UMUC3 and JMSU1; Fig. 1C). We also took the mutational landscapes of the cell lines into consideration; for instance, we chose luminal cell lines with (RT4, RT112, and SW780) and without activating FGFR3 mutations (JON; Fig. 2). Further, we selected cell lines previously shown to be highly sensitive (eg, 253J and 647V) or resistant (eg, HT1197 and RT4) to cisplatin, and excluded cell lines with particularly low proliferation rates (eg, DSH1) [26].

We assessed the sensitivity of these cell lines (n = 23) to 1707 chemical substances included in six drug libraries (anticancer drug library; NIH clinical collection; libraries of natural, epigenetic and toxic compounds; and CLOUD collection); thereby, we identified 471 chemical agents with inhibitory effects (Fig. 3A and Supplementary Table 6). Neglecting pan-toxic substances (response in >95% of cell lines), approved anticancer drugs such as romidepsin, panobinostat, ponatinib, paclitaxel, and idarubicin were amongst the most potent agents (Supplementary Table 6). Several approved drugs for BC, for example, doxorubicin, vinblastine, and methotrexate, all of which are components of MVAC, were found to be effective (Supplementary Table 6). We further identified antineoplastic effects of numerous substances approved for maladies other than cancer, including cholesterol-lowering medications, anthelmintics, antimalarial agents, and psychotropic substances (Supplementary Table 6). Besides approved drugs, the screen identified various natural compounds extracted from

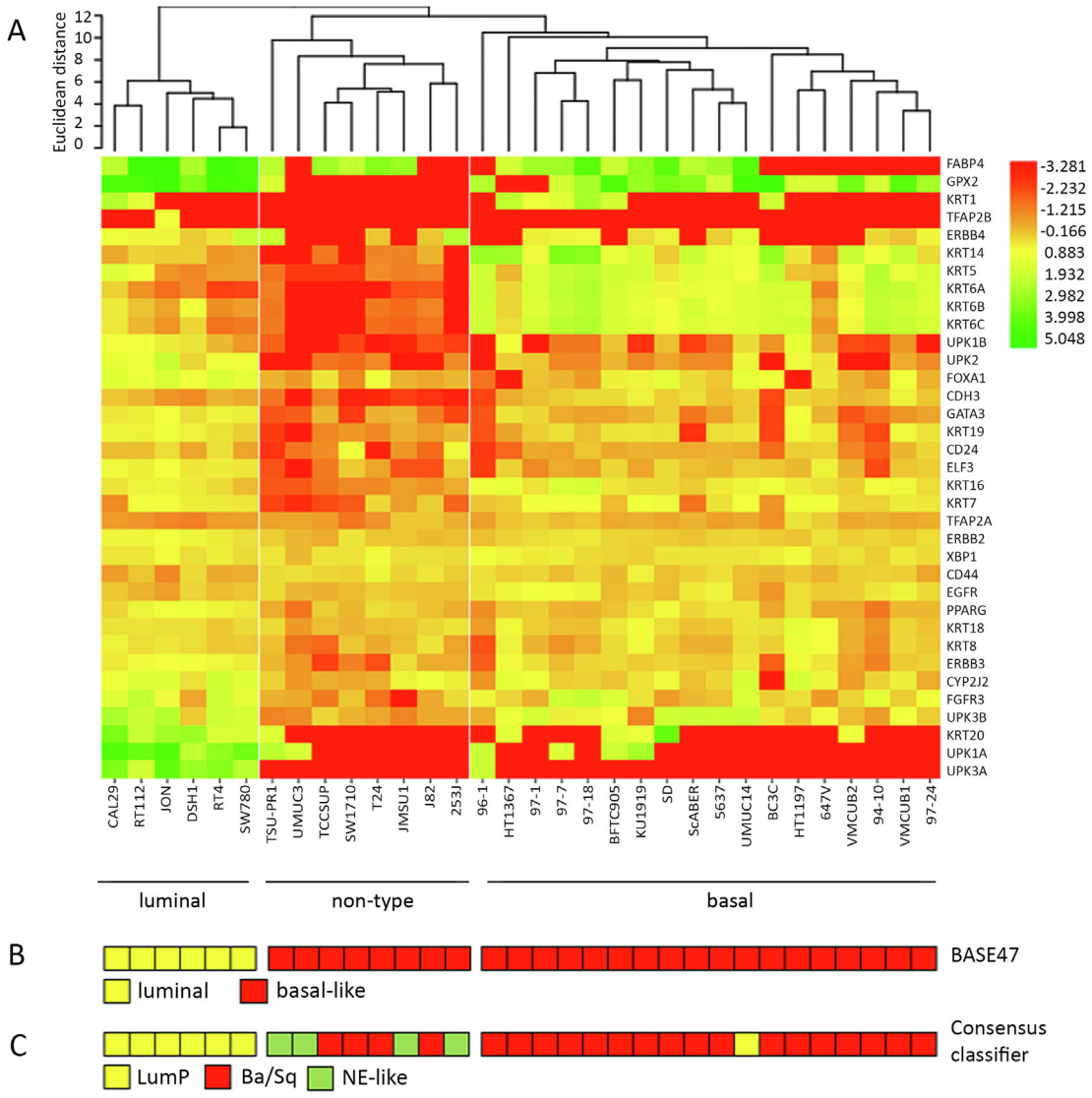

Fig. 1 – Subtype classification of a set of commercially available bladder cancer cell lines. (A) A cluster analysis including 35 expression markers identified three different intrinsic subtypes. One cluster was defined by high expression levels of genes encoding for the uroplakins *UPK1A*, *UPK3A*, and *UPK3B* and the low molecular weight keratin *KRT20*, representing luminal markers. A second cluster was characterised by elevated expression levels of *KRT5* and *KRT6A*, *KRT6B*, and *KRT6C*, which are basal markers for urothelial cells. Eight cell lines exhibited low expression levels of both types of expression markers and were, thus, classified as "nontype". (B) Classification of commercially available cell lines applying the *BASE47* gene set predictor established to distinguish "luminal" and "basal-like" subtypes of high-grade MIBC [17]. A cluster analysis identified two main expression types, of which one consisted of the six cell lines previously classified as luminal. (C) Applying the single sample consensus classifier [18], commercially available cell lines were classified as LumP, Ba/Sq, or NE-like. While all cell lines previously classified as luminal were categorised as LumP, all except one basal cell line were assigned to the Ba/Sq subtype. Cell lines previously not assigned to a specific subtype, were either classified as NE-like or Ba/Sq. Ba/Sq = basal/squamous; LumP = luminal papillary; MIBC = muscle-invasive bladder cancer; NE = neuroendocrine.

medicinal herbs and herbal supplements (Supplementary Table 6). Inhibitory effects of many of these substances (n = 101), including various non-oncology drugs (n = 27), on BC cell lines were also observed in a previous drug repurposing screen (Supplementary Table 7) [27].

Assessing the molecular targets of these 471 substances, we could classify 48% of them into different drug classes, while the mechanism of action of the remaining 52% is unknown or poorly investigated (Supplementary Table 6). The largest fraction of classified active drugs comprised kinase inhibitors (28%), including selective and nonselective inhibitors of receptor tyrosine and nonreceptor tyrosine kinases (Fig. 3B). Epigenetic compounds such as inhibitors of histone deacetylases (HDACs), histone methyltransferases, and histone lysine demethylases accounted for 11%, 4%, and 3% of the identified active drugs (n = 225), respectively (Fig. 3B). Other strongly represented drug classes were antimetabolites (8%), intercalating drugs and/or topoisomerase inhibitors (6%), mitotic inhibitors (5%), as well as modulators of ion channels, neurotransmitter receptors, and hormonal receptors (4%; Fig. 3B).

In agreement with the literature, we found that cell lines classified as luminal, most of which derived from low-grade tumours (eg, RT4 and SW780), had lower general sensitivity than those belonging to the basal cluster (Fig. 3A) [28]. However, the least sensitive cell lines (response to <5% of

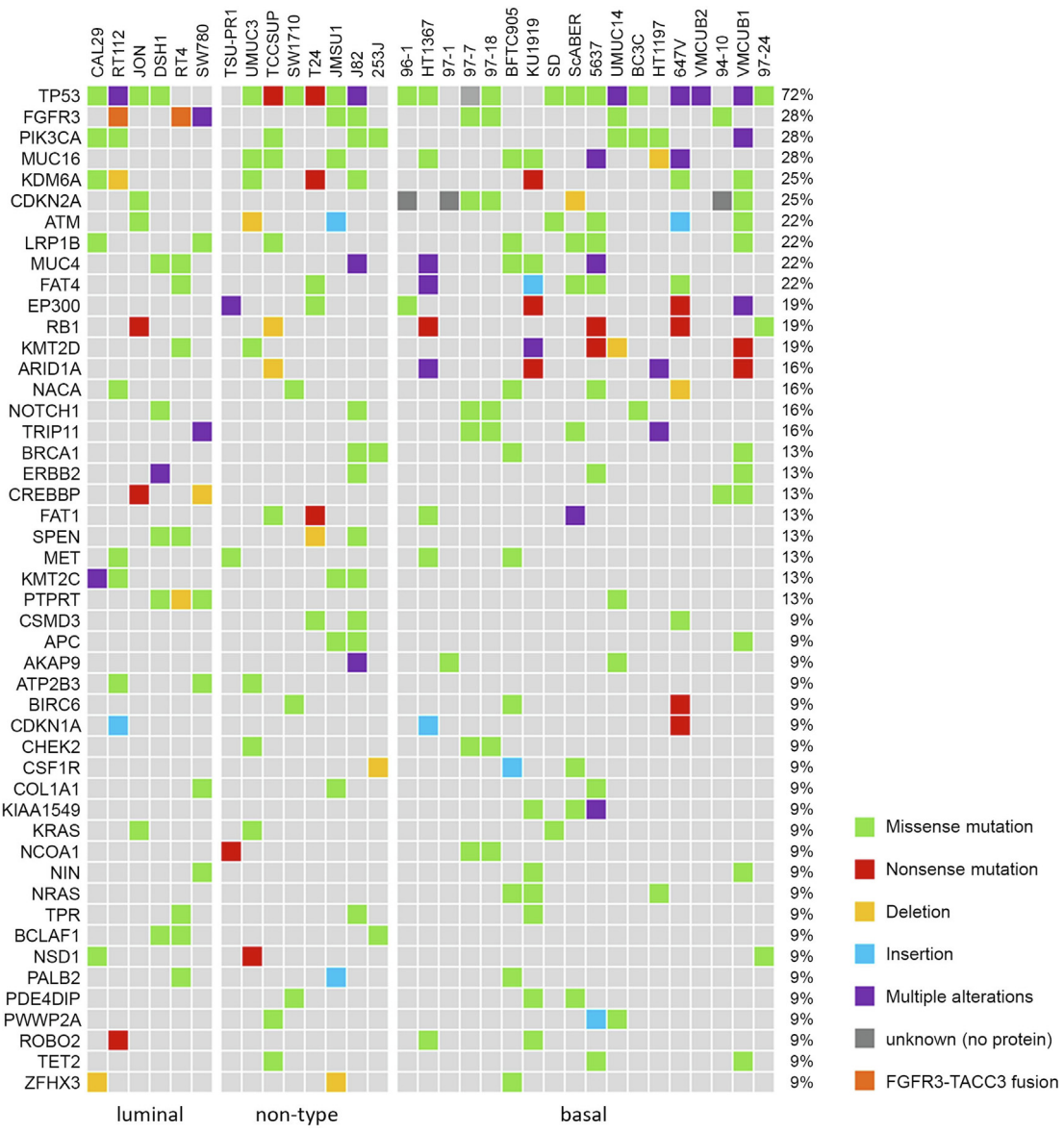

Fig. 2 – Cancer map of most frequent mutations detected in commercially available BC lines. Evaluating the loci most frequently affected by alterations (three or more cell lines), 48 genes were identified, including classical BC-related genes (eg, *TP53*, *PIK3CA*, *FGFR3*, and *RB1*), genes encoding epigenetic regulators of chromatin structure (*ARID1A/BAF250*, *KMD6A*, *EP300*, *CREBBP*, *KMT2C/MLL3*, and *KMT2D/MLL4*), protocadherins (*FAT1* and *FAT4*), cyclin-dependent kinase inhibitors (*CDKN1A* and *CDKN2A*), members of the Ras subfamily of small GTPases (*KRAS* and *NRAS*), and transmembrane mucins (*MUC4 and MUC16*). In congruence with the literature concerning subtype enrichment, mutations of *FGFR3*, *PIK3CA*, and *KMD6A* were amongst the most common alterations in cell lines representing the luminal BC subtype. The luminal cluster also exhibited enrichment of *PTPRT* mutations. Mutations of *EP300* and *ARID1A* were exclusively detected in cell lines belonging to the basal or the unspecific cluster. Common alterations of basal cell lines included co-occurring mutations of *TP53*/*RB1* and *CDKN2A*/*FGFR3*. BC = bladder cancer.

all tested substances) were RT4 and HT1197, of which the latter is classified within the basal subtype (Fig. 3A). These cell lines were previously shown to be highly resistant to cisplatin [26]. HT1197 and RT4 responded almost exclusively to epigenetic drugs, intercalating agents/topoisomerase inhibitors and ion channel inhibitors, of which the latter are known to be involved in the development of multidrug resistance (Supplementary Fig. 5) [29].

3.4. *In vitro validation of one highly active compound identified by drug screening*

Most anticancer drugs exhibiting inhibitory effects on more than one luminal cell line (n = 4) accounted for HDAC inhibitors and intercalating drugs (Supplementary Table 6). Many of these substances are already used in the clinics (eg, epirubicin and doxorubicin) or their antineoplastic effects on BC were reported previously (eg, belinostat and panobinostat; Supplementary Table 6) [30]. Clofarabine, which has not been studied extensively in the context of BC so far, was amongst the agents with inhibitory effects on all intrinsic subtypes (Supplementary Table 6). Retesting the drug in various concentrations, we found decreased viability of both luminal and basal cell lines with IC_{50} values ranging from 0.53 to 4.4 μM (Fig. 4A). However, HT1197 and HT1367 showed only decreases in viability of 21% and 19% in response to the highest tested concentration, respec-

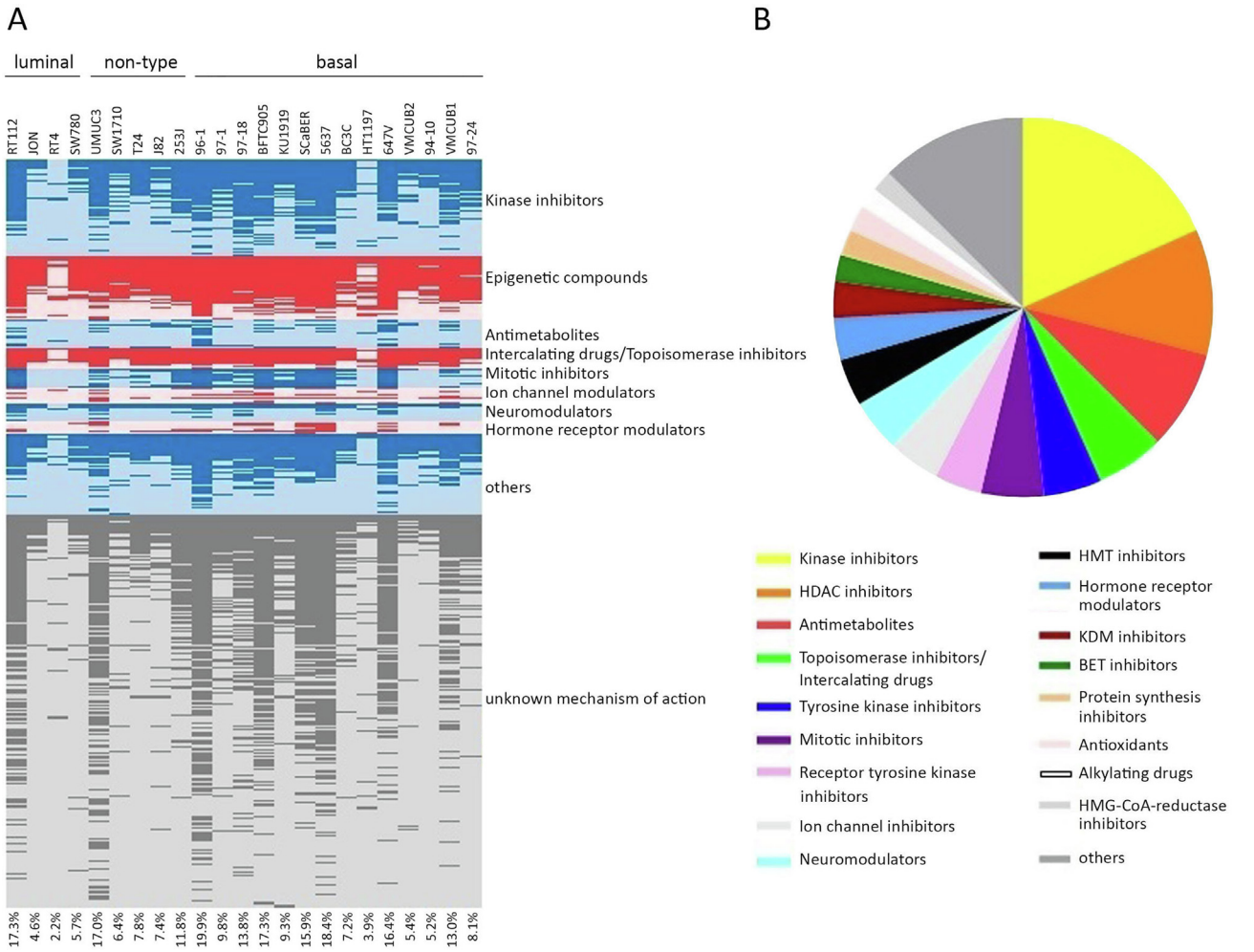

Fig. 3 – Chemical compounds with antineoplastic effects identified by drug screening. (A) A total of 471 chemical compounds exhibited antineoplastic effects on at least one BC line. Dark fields indicate positive hits. The ratio of substances with inhibitory effects (of all tested substances; *n* = 1707) is given for each cell line (bottom). Overall, most luminal cell lines had a lower general sensitivity than those belonging to the basal cluster. The least sensitive cell lines were RT4 and HT1197. (B) The largest fractions of identified substances with a known mechanism of action (*n* = 225) accounted for kinase inhibitors (28%), HDAC inhibitors (11%), and antimetabolite drugs (8%). Moreover, intercalating drugs and/or topoisomerase inhibitors (6%), mitotic inhibitors (5%), modulators of ion channels (4%), and substances targeting neurotransmitter receptors (4%) or hormonal receptors (4%) were strongly represented. BET = bromodomain and extraterminal; HDAC = histone deacetylase; HMT = histone methyltransferase; KDM = histone lysine demethylase.

tively (Fig. 4A). It has previously been shown that the ABC transporter ABCG2 can induce resistance to clofarabine [31]. In congruence, we found that HT1197 and HT1367 had the highest ABCG2 expression of our commercially available cell line collection (Supplementary Fig. 6A).

We further tested the effects of clofarabine on PDCs at low passage number, representing various tumour stages/histologic variants, all of which showed a concentration-dependent decrease of cell viability (Table 2 and Fig. 4B). Assessing ABCG2 expression, we found that VUC71 and VUC85, which are least sensitive to clofarabine, had significantly elevated levels compared with the other PDCs (Supplementary Fig. 6B).

To test potential interactions with other drugs, we performed single and double treatments with cisplatin and/or the HDAC inhibitor romidepsin in HT1197. Clofarabine in concentrations of $\geq$1 μM caused a reduction of cell viability, an effect that was significantly increased by combining it with cisplatin, romidepsin, or both drugs (Supplementary Fig. 7A). Studying the interactions between clofarabine and cisplatin or romidepsin in more detail, we found synergistic effects with both agents (Supplementary Fig. 7B).

3.5. *In vivo validation of the antineoplastic effects of clofarabine*

To verify the effects of clofarabine *in vivo*, we generated a subcutaneous mouse xenograft model using patient-derived VUC48 cells, which originate from a conventional UC, and started treatment when all animals (*n* = 16) harboured palpable tumours (Table 2). After receiving seven dosages of clofarabine without showing obvious side effects, all treated mice (*n* = 8) underwent complete remission (Fig. 5A, left). To assess the effects of clofarabine on more advanced disease stages, we allowed VUC48

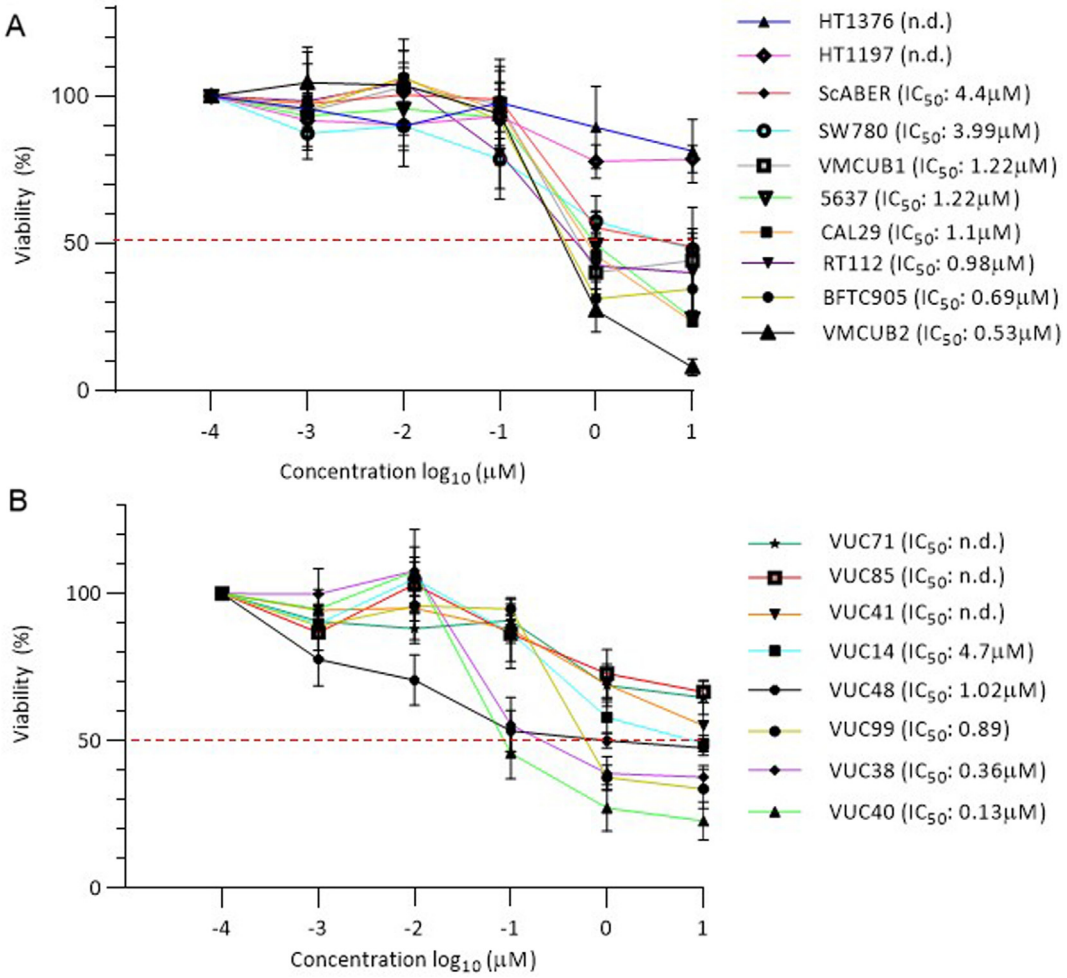

Fig. 4 – Response of commercially available and patient-derived BC cell lines to varying concentrations of clofarabine. (A) Commercially available cell lines representing different intrinsic BC subtypes were treated with variable concentrations of clofarabine for 48 h. Apart from HT1367 and HT1197, all tested BC lines showed a concentration-dependent decrease of cell viability, with IC_{50} values ranging from 0.53 to 4.4 µM. The viability of HT1367 and HT1197 was decreased by <50% in response to the highest tested concentration of 10 µM. (B) Patient-derived cell lines originating from tumours of different stages and histologic variants were treated with variable concentrations of clofarabine for 48 h. Similar to the commercially available cell lines, clofarabine caused a concentration-dependent decrease of viability in all patient-derived cultures. BC = bladder cancer; IC_{50} = half-maximal inhibitory concentration; n.d. = not defined.

Table 2 – Origins of patient-derived cell lines included in the study

Name	Sex	Stage	Grade	Intervention	Histologic type
VUC14	Male	pT2a	HG	TURB	UC with sarcomatoid differentiation
VUC38	Female	pT2a	HG	RC	Pure SaC
VUC40	Male	pT3a	HG	RC	Pure UC
VUC41	Male	pT3a	HG	RC	Pure UC
VUC48	Male	pT3b	HG	RC	Pure UC
VUC71	Male	pTa	HG	TURB	Pure UC
VUC85	Male	pT1a	HG	TURB	Pure UC
VUC99	Male	pT1a	HG	TURB	UC with squamous differentiation

HG = high grade; PDC = patient-derived culture; RC = radical cystectomy; SaC = sarcomatoid carcinoma; TURB = transurethral resection of bladder tumour; UC = urothelial carcinoma.
Patient-derived cell lines were established from tumour samples obtained during radical cystectomies or transurethral resections of bladder tumours. Seven male and one female patients suffering from bladder tumours of different tumour stages (pT1a-pT3b) were included in the study. While most PDCs derived from pure urothelial carcinoma, VUC38 originated from a pure sarcomatoid carcinoma. VUC14 and VUC99 derived from urothelial carcinoma with sarcomatoid and squamous differentiation, respectively.

xenografts to develop for 16 d before starting the treatment. Similarly to our previous approach, all animals receiving therapy ($n = 4$) underwent complete remission (Fig. 5B, left). No tumour recurrences were observed, and all animals were alive and well throughout an observation period of >10 wk (Fig. 5A and 5B, right).

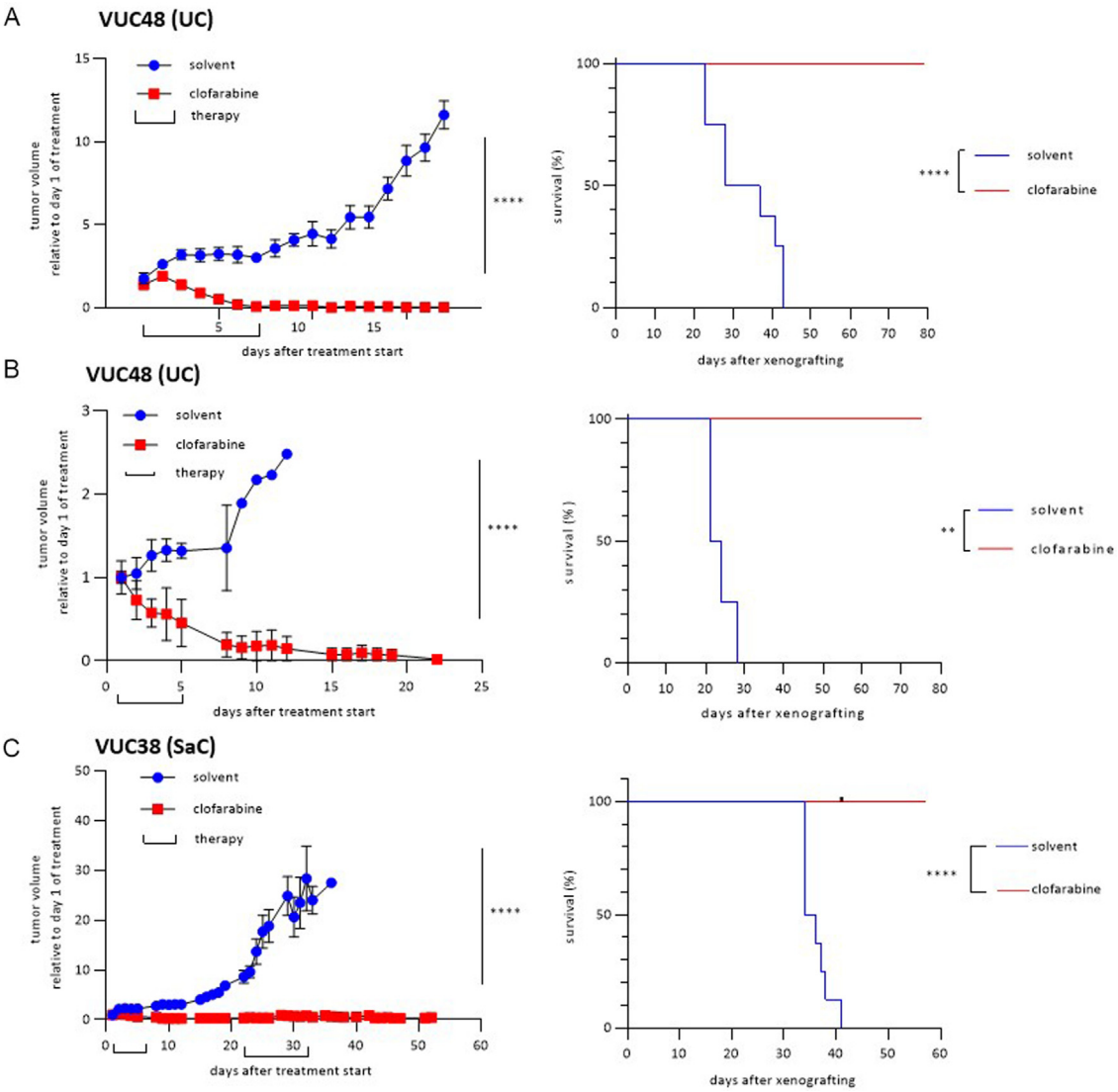

Fig. 5 – Response of mouse xenograft models to clofarabine. (A) Left: The patient-derived cell line VUC48 representing a UC was xenografted in CB17/SCID mice and clofarabine treatment was started when all animals (n = 16) had palpable tumours. After receiving seven dosages of clofarabine, all animals (n = 8) were in complete remission, lacking any palpable tumour remnants. Right: All control animals (n = 8) had to be sacrificed within 43 d after tumour implantation, while all animals of the clofarabine group (n = 8) were alive and well throughout an observation period of >10 wk. (B) Left: The patient-derived cell line VUC48 was xenografted in CB17/SCID mice, and clofarabine treatment was started when the animals (n = 8) were in advanced disease stages. Three animals of the clofarabine group (n = 4) showed complete response by day 10 and the fourth mouse by day 22. Right: All control animals (n = 4) had to be sacrificed within 28 d after tumour implantation, while all animals receiving treatment were alive and well throughout an observation period of >10 wk. (C) Left: The patient-derived cell line VUC38, representing a SaC, was xenografted in CB17/SCID mice and clofarabine treatment was started when all animals (n = 16) had palpable tumours. Similarly to UC xenograft mice, all animals of the clofarabine group (n = 8) underwent complete or subcomplete remissions with tumour remnants too small for accurate measurement. Right: One mouse of the clofarabine group (n = 8) died due to reasons unrelated to the implanted xenograft or the treatment, and was therefore excluded from the survival analysis. Control animals (n = 8) had to be sacrificed within 41 d after xenografting; the seven remaining mice of the clofarabine group were alive and well throughout the observation period of >8 wk. SaC = sarcomatoid carcinoma; UC = urothelial carcinoma. ** $p \leq 0.01$. **** $p \leq 0.0001$.

We further generated a subcutaneous SaC mouse xenograft model using the PDC VUC38 (Table 2). Clofarabine treatment was started when all animals (n = 16) had developed palpable tumours. After 5 d of treatment, all animals underwent (sub)complete remission with three animals in complete remission, while five still harboured palpable tumour remnants too small for accurate measurement (Fig. 5C, left). As after 16 d of treatment pause, tumours started to regrow in five animals, another cycle of clofarabine was applied for 10 d, leading again to remission of tumours in all treated animals (Fig. 5C, left). Except for one animal that died on day 41 after tumour implantation (in complete remission) due to reasons obviously unrelated to the implanted xenograft (spontaneous thymoma), all animals of the clofarabine group (n = 7) were alive and well throughout the observation period of 8 wk (Fig. 5C, right).

4. Discussion

BC is a highly heterogeneous disease comprising tumours of different intrinsic and histologic subtypes with varying

sensitivities to available drugs [3,12,17,32]. This biological and clinical variability is a major hindrance to the development of effective and safe therapies. With the goals of overcoming this challenge and identifying novel drugs for BC, we established a panel of commercially available BC cell lines (n = 23) serving as a model system. We took advantage of expression markers, most of which had originally been used to subclassify breast cancers, and assigned all cell lines to different intrinsic subtypes. Our classification was in congruence with a previous study, suggesting high robustness of the expression profiles of commercially available cell lines, independent from the batch, number of passages, and other external factors [12]. We also analysed our RNA-seq data employing the gene set predictor BASE47 and a recently established consensus classifier, obtaining congruent results [17,18]. Notably, despite being originally developed for MIBC, both systems consistently identified all luminal cell lines including those deriving from NMIBC.

We further utilised our RNA-seq data to identify mutations in protein-coding genes. This approach is certainly a limitation of our study, since it was previously reported that only 46–49% of pathogenic variants detected by whole exome sequencing were identified by RNA-Seq [7]. This bias is caused by the fact that genes expressed at low levels are hardly detectable, and that allelic imbalance and nonsense-mediated decay hinder the identification of heterozygote alterations and nonsense mutations [7]. However, despite the limited comprehensiveness of our approach, it led to the identification of >60 previously unreported alterations, all of which were verified by Sanger sequencing. Moreover, to obtain a more comprehensive characterisation of the genomic landscape of our cell lines, we complemented our own with publicly available data [10,20–24].

Taking mutation and expression data into consideration, we selected cell lines representing the widest spectrum of BC (n = 23) for drug screening, thereby identifying 471 chemical compounds with inhibitory effects. Apart from kinase inhibitors, epigenetic compounds and antimetabolites were the most strongly represented drug classes [33]. Many of these substances were approved anticancer drugs, often developed for the treatment of haematologic malignancies [33]. However, we also identified agents approved for other diseases (eg, fungicides, antipsychotics/antidepressants, and antirejection drugs), as well as dietary supplements and components isolated from medical herbs [33].

Limitations of our approach included that available substance libraries were not specifically designed for BC, but aiming to cover a wide spectrum of approved drugs/bioactive compounds. Additionally, all tested components were routinely dissolved in dimethyl sulfoxide (DMSO). Consequently, several agents approved for BC either were not included (eg, erdafitinib and gemcitabine) or gave inconclusive data due to DMSO interaction (eg, cisplatin) [34]. Moreover, the drug screen was performed in a single technical replicate, to obtain an overall impression concerning active drug classes and feasible targets. This emphasises the importance of the detailed follow-up and confirmation experiments.

Exemplarily, we selected the antimetabolite clofarabine for further investigation. Besides commercially available cell lines, we retested the drug in PDCs cultured for a limited number of passages, thus closely mimicking their tumours of origin. In accordance with the drug screen, we observed concentration-dependent effects on cell lines representing various intrinsic/histologic subtypes. The fact that most cultures with low sensitivity had exceptionally high ABCG2 expression implicates that this gene/protein may represent a biomarker for clofarabine resistance, as suggested previously [31].

These *in vitro* results were validated using a conventional UC mouse xenograft model generated by the implantation of a PDC. Independently from the disease stage, clofarabine induced complete remission in all mice without causing obvious side effects. Notably, during the observation period of >10 wk, none of the animals suffered relapse. We further observed massive antineoplastic effects of clofarabine in a xenograft model representing the rare and highly aggressive histologic BC variant SaC. This finding is of special interest, since two recent studies demonstrated that neither currently available adjuvant nor neoadjuvant chemotherapies have a significant survival benefit for this disease entity [35,36].

5. Conclusions

The potent effects of clofarabine *in vivo* suggest that our findings may be of high clinical relevance. We, thus, intend to further evaluate the effects of clofarabine on additional xenograft models. We will also assess biomarkers for the selection of BC patients potentially benefiting from clofarabine treatment. Finally, clinical trials are needed to assess the value of clofarabine in improving BC patient care sustainably and safely.

***Author contributions*:** Walter Berger had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study concept and design: Ertl, Shariat, Berger.
Acquisition of data: Ertl, Lemberger, Ilijazi, Hassler, Bruchbacher, Brettner, Kronabitter, Gutmann, Vician, Zeitler, Koren, Lardeau, Clozel, Elemento, Kubicek.
Analysis and interpretation of data: Ertl, Berger.
Drafting of the manuscript: Ertl, Gutmann, Berger, Shariat.
Critical revision of the manuscript for important intellectual content: Shariat, Berger, Compérat, Kubicek, Elemento.
Statistical analysis: Mohr.
Obtaining funding: None.
Administrative, technical, or material support: Compérat, Oszwald, Wasinger, Haitel.
Supervision: Shariat, Berger.
Other: None.

Financial disclosures: Walter Berger certifies that all conflicts of interest, including specific financial interests and relationships and affiliations relevant to the subject matter or materials discussed in the manuscript (eg, employment/affiliation, grants or funding, consultancies, honoraria, stock ownership or options, expert testimony, royalties, or patents filed, received, or pending), are the following: None.

***Funding/Support and role of the sponsor*:** This work was supported by the Medical University of Vienna.

Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.eururo.2022.03.009.

References

[1] Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 2018;68:394–424.
[2] Yeung C, Dinh T, Lee J. The health economics of bladder cancer: an updated review of the published literature. Pharmacoeconomics 2014;32:1093–104.
[3] Choi W, Porten S, Kim S, et al. Identification of distinct basal and luminal subtypes of muscle-invasive bladder cancer with different sensitivities to frontline chemotherapy. Cancer Cell 2014;25:152–65.
[4] Kamat AM, Hegarty PK, Gee JR, et al. ICUD-EAU International Consultation on Bladder Cancer 2012: screening, diagnosis, and molecular markers. Eur Urol 2013;63:4–15.
[5] Barrio MJ, Spick C, Radu CG, et al. Human biodistribution and radiation dosimetry of 18F-clofarabine, a PET probe targeting the deoxyribonucleoside salvage pathway. J Nucl Med 2017;58:374–8.
[6] Langdon SP. Cancer cell culture: methods and protocols. Humana Press; 2004. p. 360.
[7] Kaya C, Dorsaint P, Mercurio S, et al. Limitations of detecting genetic variants from the RNA sequencing data in tissue and fine-needle aspiration samples. Thyroid 2021;31:589–95.
[8] Ross RL, Burns JE, Taylor CF, Mellor P, Anderson DH, Knowles MA. Identification of mutations in distinct regions of p85 alpha in urothelial cancer. PLoS One 2013;8:e84411.
[9] Zuiverloon TCM, de Jong FC, Costello JC, Theodorescu D. Systematic review: characteristics and preclinical uses of bladder cancer cell lines. Bladder Cancer 2018;4:169–83.
[10] Earl J, Rico D, Carrillo-De-Santa-Pau E, et al. The UBC-40 urothelial bladder cancer cell line index: a genomic resource for functional studies. BMC Genom 2015;16:403.
[11] Christian BJ, Loretz LJ, Oberley TD, Reznikoff CA. Characterization of human uroepithelial cells immortalized in vitro by simian virus 40. Cancer Res 1987;47:6066–73.
[12] Warrick JI, Walter V, Yamashita H, et al. FOXA1, GATA3 and PPARγ cooperate to drive luminal subtype in bladder cancer: a molecular analysis of established human cell lines. Sci Rep 2016;6:38531.
[13] Guo CC, Bondaruk J, Yao H, et al. Assessment of luminal and basal phenotypes in bladder cancer. Sci Rep 2020;10:9743.
[14] Subramanian A, Tamayo P, Mootha VK, et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc Natl Acad Sci U S A 2005;102:15545–50.
[15] Mootha VK, Lindgren CM, Eriksson K-F, et al. PGC-1α-responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes. Nat Genet 2003;34:267–73.
[16] Charafe-Jauffret E, Ginestier C, Monville F, et al. Gene expression profiling of breast cell lines identifies potential new basal markers. Oncogene 2006;25:2273–84.
[17] Damrauer JS, Hoadley KA, Chism DD, et al. Intrinsic subtypes of high-grade bladder cancer reflect the hallmarks of breast cancer biology. Proc Natl Acad Sci U S A 2014;111:3110–5.
[18] Kamoun A, de Reyniès A, Allory Y, et al. A consensus molecular classification of muscle-invasive bladder cancer. Eur Urol 2020;77:420–33.
[19] Sondka Z, Bamford S, Cole CG, Ward SA, Dunham I, Forbes SA. The COSMIC Cancer Gene Census: describing genetic dysfunction across all human cancers. Nat Rev Cancer 2018;18:696–705.
[20] Nickerson ML, Witte N, Im KM, et al. Molecular analysis of urothelial cancer cell lines for modeling tumor biology and drug response. Oncogene 2017;36:35–46.
[21] Tate JG, Bamford S, Jubb HC, et al. COSMIC: the catalogue of somatic mutations in cancer. Nucleic Acids Res 2019;47:D941–7.
[22] Ghandi M, Huang FW, Jané-Valbuena J, et al. Next-generation characterization of the cancer cell line encyclopedia. Nature 2019;569:503–8.
[23] Williams SV, Hurst CD, Knowles MA. Oncogenic *FGFR3* gene fusions in bladder cancer. Hum Mol Genet 2013;22:795–803.
[24] Yeager TR, DeVries S, Jarrard DF, et al. Overcoming cellular senescence in human cancer pathogenesis. Genes Dev 1998;12:163–74.
[25] Kiselyov A, Bunimovich-Mendrazitsky S, Startsev V. Key signaling pathways in the muscle-invasive bladder carcinoma: clinical markers for disease modeling and optimized treatment. Int J Cancer 2016;138:2562–9.
[26] Xylinas E, Hassler MR, Zhuang D, et al. An epigenomic approach to improving response to neoadjuvant cisplatin chemotherapy in bladder cancer. Biomolecules 2016;6:37.
[27] Corsello SM, Nagari RT, Spangler RD, et al. Non-oncology drugs are a source of previously unappreciated anti-cancer activity. bioRxiv 2019; 730119.
[28] Fong MHY, Feng M, McConkey DJ, Choi W. Update on bladder cancer molecular subtypes. Transl Androl Urol 2020;9:2881.
[29] Kischel P, Girault A, Rodat-Despoix L, et al. Ion channels: new actors playing in chemotherapeutic resistance. Cancers (Basel) 2019;11:376.
[30] Gupta S, Albertson DJ, Parnell TJ, et al. Histone deacetylase inhibition has targeted clinical benefit in ARID1A-mutated advanced urothelial carcinoma. Mol Cancer Ther 2019;18:185–95.
[31] Nagai S, Takenaka K, Nachagari D, et al. Deoxycytidine kinase modulates the impact of the ABC transporter ABCG2 on clofarabine cytotoxicity. Cancer Res 2011;71:1781–91.
[32] Moschini M, D'Andrea D, Korn S, et al. Characteristics and clinical significance of histological variants of bladder cancer. Nat Rev Urol 2017;14:651–68.
[33] Kim S, Chen J, Cheng T, et al. PubChem 2019 update: improved access to chemical data. Nucleic Acids Res 2019;47:D1102–9.
[34] Hall MD, Telma KA, Chang KE, et al. Say no to DMSO: dimethylsulfoxide inactivates cisplatin, carboplatin, and other platinum complexes. Cancer Res 2014;74:3913–22.
[35] Gu L, Ai Q, Cheng Q, et al. Sarcomatoid variant urothelial carcinoma of the bladder: a systematic review and meta-analysis of the clinicopathological features and survival outcomes. Cancer Cell Int 2020;20:550.
[36] Zamboni S, Afferi L, Soria F, et al. Adjuvant chemotherapy is ineffective in patients with bladder cancer and variant histology treated with radical cystectomy with curative intent. World J Urol 2021;39:1947–53.
[37] van Bokhoven A, Varella-Garcia M, Korch C, Miller GJ. TSU-Pr1 and JCA-1 cells are derivatives of T24 bladder carcinoma cells and are not of prostatic origin. Cancer Res 2001;61:6340–4.