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Biological assay challenges from compound solubility: strategies for bioassay optimization

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Compound solubility in buffers and dimethyl sulfoxide (DMSO) has emerged as an important issue. Many discovery compounds have low solubility but are potentially valuable as leads. Unfortunately, low solubility affects bioassays by causing underestimated activity, reduced HTS-hit rates, variable data, inaccurate SAR, discrepancies between enzyme and cell assays and inaccurate *in vitro* ADME–Tox testing. Strategies for optimizing bioassays include: considering solubility in HTS-library design; early screening for solubility; improving storage and handling of DMSO stocks; optimizing dilution protocols; and ensuring that low-solubility compounds are fully solubilized in bioassays. These approaches allow for adequate assessments of valuable pharmacophores for which solubility can be chemically optimized at a later date.

Growing awareness of solubility limitations in drug discovery

In recent years there has been a growing awareness of solubility-related limitations on many aspects of drug discovery. Initially, the focus was on the role of compound solubility in drug absorption and pharmacokinetics. Recent concerns have extended this focus to the effect of compound solubility in biological assays.

Awareness of aqueous solubility has developed in the field of drug discovery because of the high rate of candidate attrition caused by biopharmaceutical properties [1]. The work of Lipinski [2] raised awareness that low solubility will limit compound absorption after oral dosing. As a result, minimum solubility levels were recommended for candidates, based on their permeability and therapeutic dose, and fundamental properties, such as solubility, were regularly measured.

Despite this awareness, many compounds under investigation in drug discovery have low solubility. This happens because achieving good activity against a biological target is of paramount importance and structural features that produce good activity (e.g. lipophilic substructures) can reduce solubility. The common procedure of dissolving compounds for bioassays in dimethyl sulfoxide (DMSO) does not mean that compounds will necessarily have good aqueous solubility [2]. In addition, the success of pharmaceutics groups in formulating low-solubility compounds to achieve acceptable gastrointestinal absorption has encouraged discovery scientists to defer solubility issues for development groups.

The prevalence of low-solubility compounds and the availability of solubility data in drug discovery have prompted some investigators to consider the impact solubility has on other aspects of drug discovery. It has become apparent that low solubility can affect biological assays by reducing the concentration of the compound at the target protein, resulting in erroneous SAR.

Because there is a strong relationship between solubility and IC_{50} values, bioassay development and validation must ensure that low-solubility compounds are properly assessed. Active pharmacophores are very precious, so no discovery group can afford to miss out on (or underestimate) the activity of a compound series just because the bioassay protocols did not adequately assess their activity. Once recognized for their activity, low-solubility compounds can be improved by structural modification, maintaining this valuable activity. Our article reviews the effects reduced compound solubility has on bioassays and provides strategies that have been devised to improve bioassays.

The effects of low solubility on bioassays

It is useful to consider the effects of solubility on bioassays, as well as strategies to improve bioassays, by dividing the assay process into four stages (Figure 1). First, we consider the effects at each stage.

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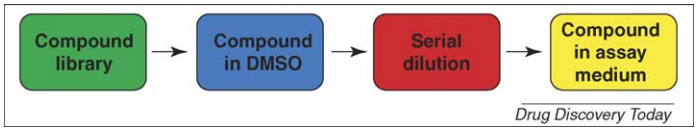

FIGURE 1

Discovery bioassay process with regard to solubility.

Compound library

Low-solubility libraries have lower HTS hit rates

Screening libraries that contain low-solubility compounds have lower HTS hit rates than libraries that contain soluble compounds. A recent study of a 2797-compound library (screened in 52 enzyme assays) showed that the soluble compounds had a hit rate of 32% but the insoluble compounds only had a hit rate of 4% [3]. The consequentially reduced compound concentration resulted in a lower success rate for low-solubility compounds.

Impurities have greater influence in low-solubility libraries

If compound libraries are not pure to start with, impurities tend to be more soluble because most of them are synthetic precursors. Potentially, assays that do not use a mass spectrometer (MS) for detection, such as those relying on UV plate-readers, might only measure impurities – minor components of the original sample. This will lead to erroneous results.

Compound in DMSO

Insolubility in DMSO causes variable data

Before use in a biological assay, each compound is dissolved in DMSO and an aliquot is used for testing. It has been shown that DMSO solubility can be limited [4]. Storage of DMSO solutions, freeze–thaw cycles and the low solubility of some compounds in DMSO (amorphous solids are generally more soluble in DMSO than crystalline solids) can result in compound precipitation. All of these effects can result in inaccurate bioassay concentrations.

For biological assays, compounds are often dissolved at a concentration of 10–30 mM in DMSO and stored at temperatures ranging from −20 to 4 °C. Approximately 10–20% of compounds in corporate collections are not soluble in DMSO at these concentrations [5,6] and lowering the storage temperature further-reduces the solubility. Inaccurate starting concentrations will lead to unknown concentrations in all subsequent dilutions. Furthermore, precipitate in DMSO stocks can also cause inaccurate dilutions. If no precipitate is carried over to the assay media, or if precipitate is carried over and does not dissolve, then the dilutions will be lower than intended. A compound might be active but it will appear to have low activity when it is not fully in solution and will be ranked as a poor inhibitor. If precipitate is carried over and dissolves then the dilutions will be deceptively high and the compound's activity will be overestimated.

Precipitates can also 'clog' low-volume pipettes used in bioassays. Inaccurate assay volumes will be delivered, especially if assays are performed unattended (i.e. by robotics), resulting in erroneous data for other compounds, not just the insoluble compound. Clogging can also cause instrument downtime [7].

Freeze–thaw cycles can reduce solubility

It is common practice to dispense compounds (from central compound stocks) in DMSO for testing. If these plates are re-used over a period of time they are often stored at cold temperatures (between −20 °C and 4 °C) between uses. The resulting freeze–thaw cycles can enhance precipitation [4,5,8–12], which is a more probable cause of compound loss than decomposition during the first six months of plate usage [13]. In this instance, precipitation is enhanced by two factors: absorption of water by DMSO and compound crystallization. DMSO is hygroscopic and will absorb water up to 10% of its original volume [14] and solubility of most compounds in wet-DMSO is lower than in dry-DMSO. Additionally, slow cooling of plates below room temperature is conducive to crystal formation. The low-energy crystals that are formed are usually less-soluble and harder to re-dissolve than the relatively high-energy amorphous material from which the DMSO solutions were initially made [4]. This is consistent with biologists' observations reporting that compounds are more active when they are tested as freshly prepared solutions than when they are tested after storage in DMSO.

Some compounds have low DMSO solubility

Typically, two types of compounds have low solubility in DMSO [15]. The first type has a strong molecular lattice for crystal packing. They tend to have relatively low molecular weights (MWs), are rigid and also hydrophilic. Organic salts, for example, tend to have lower DMSO solubility than free-bases and acids. The second type of compound is not well solvated by DMSO or water because of high MWs, high logPs, large numbers of rotatable bonds and solvent-accessible surface areas. An example is itraconazole (MW = 705 Da, ClogP^{TM} = 6, rotatable bonds = 9).

Serial dilution

IC_{50} shifts caused by compound insolubility at the highest concentrations

Most bioassays test compound activity over a concentration range – to determine the concentration of half-maximal inhibition, the IC_{50}. The range of concentrations is often produced by further-diluting a compound, from the initial DMSO stock solution, into an aqueous buffer, producing the highest concentration of compound to be tested (e.g. 100 μM), which is then subject to a serial dilution in the aqueous buffer. If the compound is not completely soluble at the highest concentration, the concentration curve will shift and the scientist will calculate the IC_{50} based on this 'apparent' concentration curve. The false, higher IC_{50} will classify the compound as less active.

Compound in assay medium

Many compounds have low solubility at screening concentrations

About 30% of discovery compounds have an aqueous solubility <10 μM [16]. This concentration is typically used for HTS and so a potential 30% of compounds could have their activity underestimated.

Solubility problems cause discrepancies between isolated-target and cell-based assays

DMSO helps to solubilize compounds in aqueous buffers but it can also interfere with assays [17]. Unfortunately, DMSO tolerance can

vary greatly from assay to assay. For example, some enzyme assays can tolerate 10% DMSO but, in others, 0.2% DMSO can inhibit enzyme activity. The % DMSO is one example of differences between assay-media composition that can lead to different compound concentrations. This is apparent in discrepancies between isolated-target assays (e.g. enzyme, receptor) and cell-based assays. DMSO, serum protein and buffer components are usually tolerated better by isolated targets than in cell-based assays. These additives are often used in isolated-target assays to generate more-consistent data by solubilizing low-solubility compounds. However, the compound can be less soluble in the subsequent cell assay. We have seen cases where the concentration of a compound was 10-fold higher in an enzyme assay (when 2.5% DMSO and 5% BSA were used) than it was in the respective cell-based assay, which could only tolerate 0.1% DMSO and 0% BSA.

Compounds that aggregate can cause false-positives in HTS

Some compounds are false-positive hits in diverse bioassays. A major cause of this is aggregation [18–20], especially when compounds are screened at concentrations >10 μM, and enzymes adsorb to the aggregates and are inhibited. If false-positive hits are selected for discovery optimization, medicinal chemists will be unsuccessful in achieving activity improvements and the SAR will be flat [21].

Typically, the aggregates are 30–400 nm in size and pass through standard 0.2 μm filters; they are not micelles or vesicles. Aggregation can result from compound supersaturation, as occurs when a concentrated DMSO solution is added to an aqueous phase [4] and compounds are more likely to aggregate at higher screening concentrations. A library of 1030 compounds produced 19% false-positive hits when screened at 30 μM but only 1.4% of hits were false-positives when compounds were screened at 5 μM. False-positives drain discovery resources, thus, screening at high compound concentrations can be counterproductive [21].

Low compound solubility affects drug-like property testing

Early testing for pharmaceutical properties, such as metabolic stability, cytochrome P450 (CYP) inhibition and hERG blocking, is usually performed at the discovery stage. If a compound precipitates in a microsomal stability assay it will not be in solution and, therefore, will not react with the metabolizing enzymes. Then, when the reaction is quenched by the addition of acetonitrile the compound will be re-dissolved and measured as unmetabolized compound, thus producing a deceptively high estimate of metabolic stability. Low solubility can also make CYP inhibition and hERG blockage seem to be less of a problem than they really are.

Strategies to optimize bioassays for solubility

In the following paragraphs we reveal strategies that have been devised to deal with the effects low compound solubility has on bioassays. These are described to provide alternatives for researchers.

Compound library

Include compound solubility criteria in screening-library design

Some companies routinely identify compounds that have low solubility in DMSO and water and remove them from the main screening collection. This is done to improve data quality, minimize time and resources spent on insoluble compounds and to generate more-reliable data [22].

Make good compound solubility an objective

Companies can establish minimum solubility criteria for compound advancement. Such criteria emphasize the importance of compound solubility and ensure that project teams continually consider and improve solubility. If a lead series has low solubility there is a risk that the assay data for series analogs might not be reliable.

Screen compounds for solubility early

An early screen for compound solubility provides project teams with advantages from the outset. Solubility in different aqueous matrices varies but it is still useful to know the compound solubility under generic solubility conditions. There are several methods available for this purpose [23–25]. Kinetic solubility is very useful in drug discovery because it mimics the initial dissolution of compounds into DMSO and the incubation time that is used in biological assays. Thermodynamic solubility is more relevant to formulation development in late-stage discovery.

In the direct-UV kinetic-solubility method a small volume of 10 mg ml^{-1} compound–DMSO solution is added to aqueous buffer to give a final compound concentration of 100 μg ml^{-1}. After incubation for 1–18 h, the solution is filtered to remove precipitate and the concentration is measured with a UV plate-reader or HPLC [26]. In the nephelometric kinetic-solubility method the DMSO stock is diluted into an initial aqueous solution and then serially diluted with buffer. The plate is placed in a light-scattering plate-reader to detect precipitate. The highest compound concentration at which no precipitate forms is assumed to be the solubility [27,28]. Thermodynamic solubility studies use the equilibrium-shake-flask method, in which aqueous buffer is added to solid compound and mixed for 24–48 h. The solution is then filtered to remove undissolved material and the concentration is measured using HPLC [29].

Prepare compounds for use in bioassays as solid arrays

It is advantageous for HTS and other biological testing to have quick and easy access to compounds in DMSO; however, DMSO solutions can have solubility limitations. Several storage concepts, that do not involve DMSO, have been developed. NanoStore uses 1536-well plates of NanoCarrier™ to store compounds that have evaporated from DMSO [30], thus, avoiding issues of DMSO insolubility. DotFoil™ stores compounds as dry films [31] and they are rapidly re-dissolved for use in assay plates. ChemCards™ store compounds for microarray compound screening (μARCS). Here, compounds are spotted onto cards, dried and sealed in light-proof pouches in an inert atmosphere ready for direct use in an assay [13].

Compound in DMSO

Store DMSO solutions in individual-use tubes

The negative effects of water absorption by DMSO, and problems relating to repeated freeze–thaw cycles, have caused some companies to store DMSO solutions as individual-use units. These are kept in automated storage systems as single-use mini-tubes [5].

Store multi-use plates at ambient temperature

It is often assumed that storage of DMSO solutions at low temperatures will reduce compound degradation but, as we have already discussed, reduced solubility can occur. Lipinski recommends storing DMSO stock solutions at ambient temperature for short-term use (e.g. up to two weeks), minimizing precipitation induced by freeze–thaw cycles [4].

In-well sonication

A low-energy sonicator has been developed that agitates each individual well in a plate. It is effective for re-dissolving precipitated samples in seconds [5] and, in some cases, drives the solution to supersaturation. It does not cause compound decomposition and can improve the accuracy of HTS screening and benchtop bioassays.

Storage at lower compound concentrations

Storing compounds at lower concentrations reduces precipitation and storage concentrations of 2–5 mM compound have been reported to be optimal [3,32]. Unfortunately, this limits the upper compound concentration for assays that can only tolerate a limited DMSO concentration. There is a fixed ratio between compound concentration and the volume of DMSO stock that is added. For example, if DMSO >1% interferes with the cell-based assay [17] then only 1 μl of DMSO stock can be added to a final assay volume of 100 μl. Therefore, with a 2 mM compound stock in 100% DMSO the highest available assay concentration of compound will be 20 μM.

Dissolve salts in 1:1 DMSO:water

Use of a 1:1 mixed DMSO:water solvent can increase the solubility of salts. Also, other water-miscible organic solvents can be tried, depending on the compound series; these include methanol, ethanol, acetonitrile, tetrahydrofuran (THF), pyridine and dimethylformamide (DMF) [33]. In such cases, tolerance to the relevant solvent should be evaluated during assay development.

Predict compound solubility in DMSO for early alerts

Compound solubility in DMSO is not readily estimated by chemists [6]. Software has been developed for compound–DMSO solubility predictions and has a good success rate [6,15,34–36]. Use of such tools can alert discovery scientists to compounds that might have low solubility in DMSO, or these tools can be used when there is insufficient material for testing.

Determine stock concentrations

The actual compound concentration can be accurately determined without a standard by using a chemiluminescent nitrogen detector (CLND) with HPLC. The detector's response is proportional to the number of moles of compound and the measured concentration has been used to calculate accurate IC_{50} values [3,37–40].

Store compounds in 9:1 DMSO:water

Some companies dilute DMSO with 10% water for compound storage [32]. This decreases the freezing point – below 4 °C. Stocks stored at 4 °C remain in the liquid phase and, thus, compound plates do not require thawing before sampling. Hence, variability is reduced because there is no increase in volume from water absorption and no ensuing variable precipitation.

Serial dilution

Optimize dilution protocols

Probably the best improvement that can be made to an assay protocol, specifically in relation to low-solubility compounds, is to optimize the dilution protocol so that precipitation of low-solubility compounds becomes less likely [41]. We recommend performing serial dilutions in DMSO and then adding an aliquot of each DMSO solution directly into the assay media. In this way, precipitation of the high-concentration solutions does not affect the accuracy of the lower-concentration dilutions – although precipitation will still affect the dose–response curve.

Biologists have found that assay results are more reproducible when many mixing strokes (up and down) of the pipette are used when transferring solutions for serial dilution. This can happen because larger precipitates are broken up into smaller particles, forming a more homogeneous suspension. Such an assumption is, however, dangerous because one assumes that particulates are being transferred homogeneously. It is preferable to use conditions that fully dissolve the compound at the higher concentrations before serial dilution.

It is often useful to reduce the highest concentration (starting point) in the dose–response curve because there is less likelihood that the compound will precipitate and subsequent dilutions will, as a result, be more reliable.

Some dilution schemes prepare an aqueous solution from the DMSO stock and then add this aqueous solution to the assay media. This approach lengthens the time that the compound is at a high concentration in aqueous solution and increases the potential for precipitation. It is preferable to mix DMSO stock dilutions directly with each assay media solution, maximizing the interaction of the compound with the biological target. The assay media often contains proteins, cellular material, lipid membranes or microsomes, additives that all help to keep low-solubility compounds in solution. Also, direct addition of DMSO stock solution to the assay media can achieve supersaturation and slow precipitation over a certain period of time, during which, the molecules can interact with the target enzyme or receptor. Direct addition of DMSO dilutions to assay media requires the pipette or robot to deliver small volumes (typically 0.5–1.0 μl) to keep the % DMSO low. The volumes must be pipetted with high-level accuracy, which is achievable with modern robots.

Dilute into buffer, not into water

If aqueous dilution is necessary, it is preferable to dilute with an aqueous buffer at pH 7.4 rather than with pure water. The buffer maintains the pH at a constant value, whereas water has no buffering capacity and the compound will not be ionized. Compounds added from DMSO will be in the neutral state and have only limited intrinsic solubility.

Compound in assay medium

In-well sonication

In-well sonication can help to re-dissolve compounds in aqueous media. This can rescue a precipitated solution or insure that a sample is fully in solution. It is important to note that it is not always possible to observe precipitation by eye.

Screen at lower compound concentrations in assay medium

Screening compounds at a concentration of 3 μM will help to minimize precipitation compared with screening at 10 μM. Furthermore, at lower concentrations it is less likely that false-positives caused by aggregate formation will occur.

Predict aqueous solubility for early alert

Commercial software can be used to predict aqueous solubility using the molecular structure of a compound [35,42] and an accuracy of prediction within 1 log unit is common. Some programs provide a predicted solubility–pH profile and software is beneficial for alerting researchers to potential low-solubility compounds [43,44].

Determine compound solubility in biological assay media

Solubility screening in generic buffer provides a useful alert to potential problems for bioassays. However, solubility is very sensitive to its environment and changes to buffer composition, % DMSO, dilution procedure, incubation time and temperature. Determination of compound concentration under the conditions and procedure of the bioassay protocol can diagnose solubility issues and assist in optimization of the protocol. Solvent systems and dilution protocols can then be optimized to maximize solubility in bioassays. Co-solvents (e.g. DMSO, methanol, ethanol, acetonitrile, DMF, dioxane) and excipients (e.g. cyclodextrin) can improve solubility in bioassays [13,45,46]. We recommend using a set of structurally and solubly diverse compounds during assay development, providing assurance that the protocol keeps diverse compounds in solution.

Correcting biological activity using compound solubility data

Some groups measure the compound concentration in the buffer solution and correct the biological activity. Compounds are added to generic phosphate buffer solutions from a 3 mM stock in DMSO, giving a final compound concentration of 150 μM in 5% DMSO [3]. The actual concentrations of the compounds in the buffers are then measured. Although this approach gives a general idea about the aqueous solubility of the compounds, solubility in the bioassay media might be very different. Thus, correction of bioactivity using generic solubility data can introduce variability into the results.

Some groups measure compound concentration in the media after preparation of the samples according to the assay protocol. Then they correct assay results using these concentration data. However, this is not recommended because the least-soluble compounds are favored and can be considered the most potent. This 'corrected activity' might never be achievable because of low solubility. Also, the discovery project will be plagued by low solubility. It is preferable to optimize the protocol so that all test compounds are completely dissolved for accurate assessment, selecting the most-potent compounds for further work.

Optimize assay for low-solubility compounds during assay development

The assay should be optimized so that it works with low-solubility compounds. Several structurally diverse, insoluble compounds can be included during assay development and assay protocols should be optimized so that acceptable solubility of these compounds is achieved (this should be validated during assay development). This is appropriate for HTS assays that are intended for large, diverse libraries and for benchtop assays intended for examining a few structural compound series. Even within a structural series, compound solubility can vary greatly.

Reduce aggregates

Promiscuous hits should be eliminated early or excluded from the screening library. False-positive hits can be recognized by one of the following methods: actives can be re-screened in the presence and absence of detergent (for example 0.1% Triton® X-100 [21,47], which will break up aggregates); a dynamic light scattering (DLS) plate reader can be used to detect the particles; IC_{50} curves can be examined for a steeper slope than would be seen with normal hits [20] (the steep slope occurs because of the strong dependence that concentration has on aggregation); screening at lower concentrations can minimize aggregation; finally, there are computational models that identify frequent hits [21,48,49].

Conclusions

In recent years, the effects of low compound solubility on bioassays, and data reliability, have been recognized (Box 1). Many

BOX 1

Discovery bioassay process with regard to solubility

Issues for low-solubility compounds in bioassays

- Low HTS hit rates
- Precipitation from dimethyl sulfoxide (DMSO) stocks
- IC_{50} appears (falsely) lower or higher than the correct value
- Discrepancies between enzyme and cell assays
- False-positive and false-negative HTS hits
- Erratic assay results
- Erroneous SAR

BOX 2

Strategies to enhance bioassay performance for low-solubility compounds

- Serial dilution in dimethyl sulfoxide (DMSO), not buffer
- Mix DMSO stock directly with assay media; avoid aqueous dilution
- Screen at lower concentrations
- Validate bioassay for low-solubility compounds during development
- In-well sonication
- Minimize freeze–thaw cycles of DMSO stock solution
- Store short-term DMSO plates at room temperature
- Dissolve salts in 1:1 DMSO:water
- Store stocks in 9:1 DMSO:water
- Use solid arrays to store compounds
- Raise organization's awareness and criteria for solubility
- Screen solubility early using generic methods
- Software prediction of DMSO and aqueous solubility
- Retest HTS hits using 0.1% Triton® X-100 to breakdown aggregates
- Correct activity with concentrations in assay media
- Measure DMSO stock concentrations using chemiluminescent nitrogen detectors (CLND)

BOX 3

Scheme for solubility optimization of bioassays

- Prepare dimethyl sulfoxide (DMSO) stocks at lowest practical concentration
- Perform serial dilutions in DMSO
- Mix small volumes of diluted DMSO stocks directly with assay media
- Run assays at the lowest useful concentrations
- Develop and validate assays to function with low-solubility compounds
- Use in-well sonication to insure dissolution

useful strategies have been developed to improve the performance of bioassays for low-solubility compounds (Box 2) and we recommend developing and validating bioassays for reliable performance for compounds across a wide solubility range. Serial dilutions should be made in DMSO and these dilutions should be added, in low volumes, directly to the assay media. Some optimal practices are recommended in Box 3.

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